

# Transcriptional regulation and influence on replication of the human cytomegalovirus *UL138* 1.4 kb transcript

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**Abstract.** Human cytomegalovirus (HCMV) exists in a latent form in hematopoietic progenitors and undifferentiated cells of myeloid lineage. Protein *UL138*, encoded by the *UL/b'* region of the viral genome, serves an important role in the establishment and/or persistence of HCMV latency. However, little information about transcriptional regulation of the *UL138* gene has been reported thus far. In the present study, the transcriptional regulation element (TRE) of the 1.4 kb *UL138* region was identified using a series of dual-luciferase constructs that contain 5' truncated deletion fragments located upstream of the transcription start site of the gene. The results demonstrated that the region from nucleotide 188995-188962 of the Han strain genome exhibits promoter activity and harbors the functional binding motif for transcription factor AP-1 (Ap-1). Using electrophoretic mobility shift assays the physical interaction of the transcription factor to a minimal essential core sequence was demonstrated. Northern blotting revealed that deletion of the TRE in a HCMV bacterial artificial chromosome or inhibition of Ap-1 using RNA interference eliminated or reduced the production of the *UL138* 1.4 kb mRNA transcript in infected human embryonic lung fibroblast cells (HELFL). Deletion of the *UL138* 1.4 kb transcript resulted in acceleration of HCMV replication in HELFL cells. To the best of the authors' knowledge, the present study is the first to analyze the transcriptional regulation of the *UL138* 1.4 kb transcript. Knowledge of the transcriptional regulation of the *UL138* gene will enhance understanding of its mechanism in HCMV latency.

## Introduction

Human cytomegalovirus (HCMV) is a highly prevalent pathogen that induces life-long infections notably through the establishment of latency in hematopoietic stem cells (1). HCMV represents a major cause of disease in transplant patients, acquired immunodeficiency syndrome sufferers and newborns (2). Primary HCMV infection and reactivation of latent HCMV infection during pregnancy is a leading cause of congenital malformations. In immunocompromised patients, HCMV reactivation carries a risk of severe morbidity and mortality (3,4). Over the past few years, several studies have demonstrated that latent infection has profound effects on host cell transcription, cell signaling, host immune surveillance, cell stress and cell death to latent genome carriage (5-8). Therefore, knowledge about the mechanism of formation, maintenance and reactivation of latent HCMV infection, and development of methods for inhibiting HCMV reactivation are key steps for the prevention of HCMV infection.

*UL138*, which is located in the *UL/b'* region of the clinical strain, has been identified to be required for HCMV to establish and/or maintain a latent infection in hematopoietic progenitor cells infected *in vitro* (9). At present, it has been confirmed that the *UL138* gene is highly expressed during HCMV latency, and the expression is associated with the formation and maintenance of HCMV latent infections (10,11). Studies also demonstrate that the protein *UL138* (p*UL138*) can increase the cell surface levels of tumor necrosis factor receptor 1 on HCMV-infected cells and may sensitize latently infected cells to TNF- $\alpha$ -mediated reactivation of HCMV (12,13). A previous study demonstrated that p*UL138* can mediate the loss of cell surface multidrug resistance-associated protein-1 (MRP1) and reduce the function of this transporter. The p*UL138*-mediated loss of MRP1 provides a therapeutic target for eliminating latently-infected cells prior to transplantation (14). In addition, it was reported that downregulation of *UL138* gene expression by hcmv-microRNA-*UL36* could promote HCMV replication (15). These previous studies indicate that *UL138* is associated with HCMV latency and reactivation.

The *UL138* transcripts are polycistronic. A total of three *UL138* transcripts of 3.6, 2.7 and 1.4 kb were identified to be transcribed from the *UL133-UL138* gene region (10,16). It is reported that p*UL138* is encoded by all the three transcripts, with the highest level of expression resulting from the 1.4 kb

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transcript. pUL138 translation from the 3.6 and 2.7 kb transcripts was induced by serum stress (10). However, the regulatory transcriptional mechanisms of *UL138* remain unclear.

The present study was undertaken to identify the transcriptional regulation element (TRE) of the 1.4 kb transcript of *UL138*, analyze transcription factors that are responsible for the regulation of its transcription, and evaluate their impact on transcription and viral replication. In the present study, the region from nucleotide (nt) 188995-188962 of the Han genome, which is located 166-133 bp from the transcription start site (TSS) of the *UL138* 1.4 kb transcript, was demonstrated to have promoter activity and harbor the functional motif for binding of transcription factor AP-1 (Ap-1). The TRE and its corresponding transcription factor, Ap-1, were involved in regulating the transcription of the *UL138* 1.4 kb transcript and HCMV replication.

## Materials and methods

**Cell lines and virus.** Human embryonic kidney cells (HEK293) were acquired from the Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (Shanghai, China). Human monocyte, THP-1 cells, and human embryonic lung fibroblasts cells (HELFL) were obtained from Professor Minhua Luo (Wuhan Institute of Virology, Wuhan, China). HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin sulfate (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). HELFL cells were maintained in modified Eagle's medium (MEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 15% FBS, 100 U/ml penicillin and 100 U/ml streptomycin sulfate. The THP-1 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin sulfate. All cell cultures were maintained at 37°C in a 5% CO<sub>2</sub> incubator.

A bacterial artificial chromosome of the HCMV Han strain (Han-BAC) was constructed with a green fluorescent protein (GFP) tag (17). To propagate the virus, HELFL cells were inoculated with 2X multiplicity of infection (MOI) of Han-BAC and maintained in MEM supplemented with 2% FBS and penicillin-streptomycin. Then the cells were lysed in TRIzol<sup>®</sup> lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 2 min, and the cell lysate was harvested and stored at -80°C.

**Virus titration.** To titrate the infectious virus a 50% tissue culture infective dose assay (TCID<sub>50</sub>) was used. HELFL cells (2x10<sup>5</sup>) in 96-well plates were inoculated with a series of dilutions of the virus stock (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup> and 10<sup>-8</sup>) and maintained at 37°C in a 5% CO<sub>2</sub> incubator. Following ~14 days, virus replication in each well was assessed by visualization of GFP under a fluorescent microscope (magnification, x40). Using the highest dilution that gave a positive result in > the half wells and was positive in the following two dilutions, the titer of the stock was calculated using the TCID<sub>50</sub> chart and presented in plaque-forming units/ml (18).

**Construction of luciferase plasmids.** A series of different 5'-truncated fragments located upstream of the TSS of the

*UL138* 1.4 kb transcript (Fig. 1A) were obtained from HCMV Han genomic DNA (GenBank no. KJ426589.1) by polymerase chain reaction (PCR) using the primers listed in Table I. PCR was performed using Master Mix (Thermo Fisher Scientific, Inc.) with an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was included. The PCR product was ethanol precipitated, digested and cloned into the *KpnI* and *XhoI* restriction sites of the pGL3-basic vector (Promega Corporation, Madison, WI, USA), which is a firefly luciferase (FFL) expression vector lacking eukaryotic promoter and enhancer sequences. The constructs included pGL3-B/190128, pGL3-B/189728, pGL3-B/189328, pGL3-B/189228, pGL3-B/189028, pGL3-B/188995, pGL3-B/188962 and pGL3-B/188896.

To construct the luciferase plasmids containing the fragments from nt 189028-188995, 188995-188962 and 188962-188896, sense and antisense oligonucleotides of these fragments were synthesized with *KpnI* or *XhoI* recognition sites at 5' ends, respectively. Following annealing and digestion, the fragments were inserted into the pGL3-enhancer (E) and pGL3-promoter (P) vectors (Promega Corporation), that lack promoter and enhancer sequences respectively, resulting in pGL3-E/189028-188995, pGL3-E/188995-188962 and pGL3-E/188962-188896, as well as pGL3-P/189028-188995, pGL3-P/188995-188962 and pGL3-P/188962-188896. All the constructs were confirmed by sequencing for the presence of the correct inserts (Thermo Fisher Scientific, Inc.).

**Dual-luciferase assay.** A dual-luciferase assay was used to measure the transcriptional regulatory activity of the sequences located at upstream of TSS of the *UL138* 1.4 kb transcript. A total of 1.5x10<sup>5</sup> HEK293 cells cultured in 24-well plates were transfected with 400 ng plasmids, constructed as described above, or pGL3 empty vector along with 100 ng *Renilla* luciferase vector (Promega Corporation) using 500 ng Lipofectamine<sup>®</sup> 3000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The pGL3-control vector (Promega Corporation), which contains the SV40 promoter and enhancer sequences, and exhibits strong expression of luciferase in numerous types of mammalian cells, was used as a positive control of transcription. In the 48 h following transfection, luciferase activity was measured according to the manufacturer's protocol using a Dual Luciferase Reporter assay kit (Promega Corporation). The FFL signals of the constructs were normalized against that of the *Renilla* luciferase control. All measurements were carried out in triplicate. Data from three independent experiments were collected and used for statistical analysis.

Putative transcription factors binding to the TRE of the *UL138* 1.4 kb transcript were predicted online using two different programs, Jaspas Core database ([jasper.genereg.net](http://jasper.genereg.net)) and Web Signal Scan ([www.bimas.cit.nih.gov/molbio/signal](http://www.bimas.cit.nih.gov/molbio/signal)).

**Electrophoretic mobility shift assay (EMSA).** EMSA was performed using the LightShift<sup>™</sup> Chemiluminescent EMSA kit (Thermo Fisher Scientific, Inc.), as described by the manufacturer's protocol. HELFL or THP-1 cells were seeded onto a 6 cm culture dish at a density of 2x10<sup>6</sup> cells/well. After 24 h, the cells were inoculated with Han-BAC at 2X MOI. Following

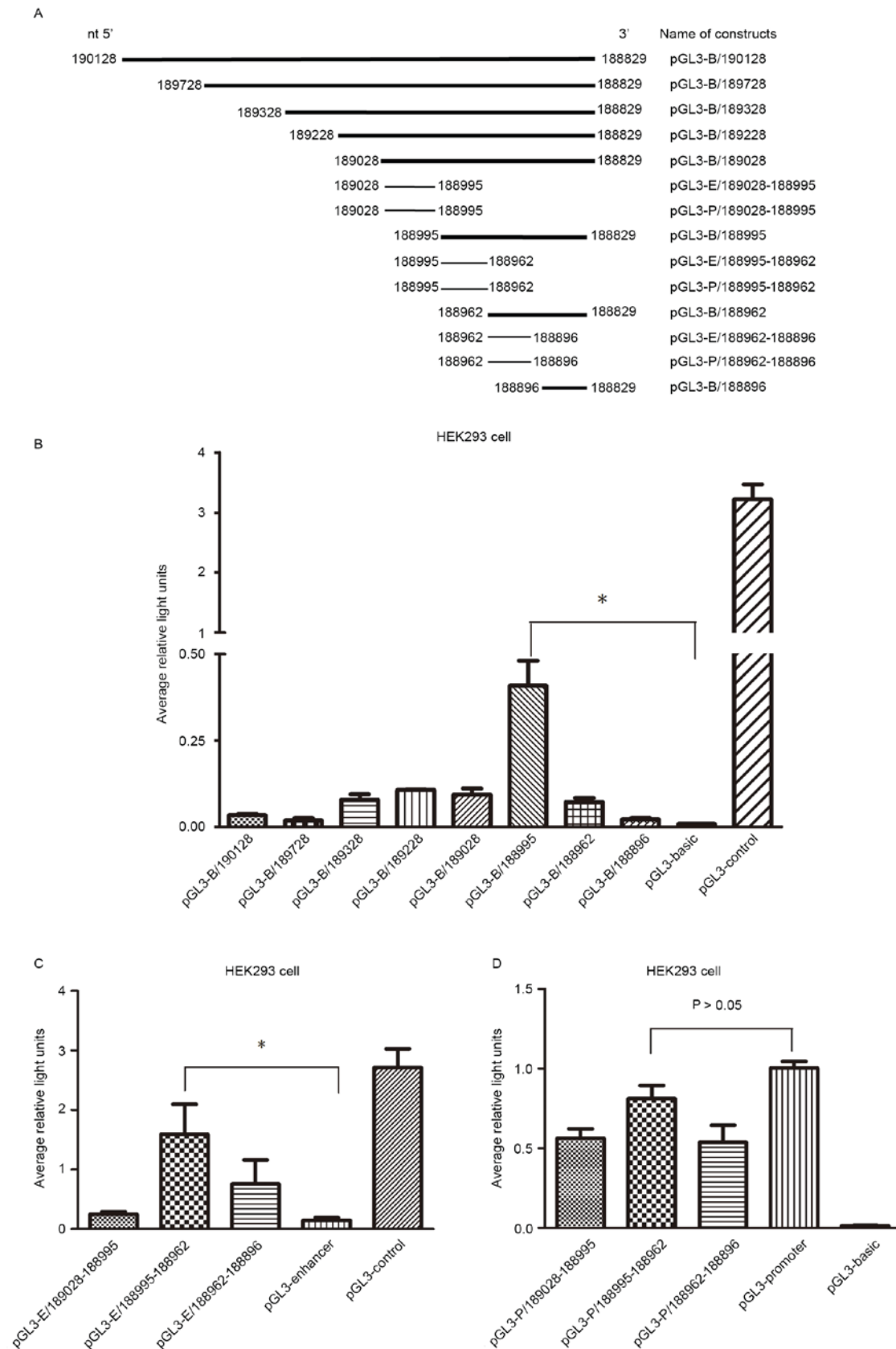


Figure 1. Identification of the promoter of *UL138* 1.4 kb transcript by dual luciferase reporter assay. (A) To find the transcriptional regulatory region of the *UL138* 1.4 kb transcript, sequences with different initiation sites and that all ended at nt 188829, which is located just upstream of the transcription start site of the *UL138* 1.4 kb transcript, were cloned into pGL3-B vector. Fragments from nt 189028-188995, nt 188995-188962 and nt 188962-188896 were cloned into pGL3-E and pGL3-P vectors. (B) The luciferase activity of each construct was measured, 48 h following transfection into HEK293 cells. The relative luciferase activity of the pGL3-B/188995 construct to that of the *Renilla* luciferase control was significantly increased compared with the pGL3-B vector. (C) The relative luciferase activity of the pGL3-E/188995-188962 construct was significantly increased compared with the pGL3-E vector. (D) However, no significant difference was observed between the relative luciferase activities of the pGL3-P/188995-188962 and pGL3-P vector alone. Data are presented as the mean  $\pm$  standard error. \* $P < 0.05$ . nt, nucleotide; HEK, human embryonic kidney cells; B, basic; P, promoter; E, enhancer.

Table I. Primers for generating the sequences at upstream of the TSS of the *UL138* 1.4 kb transcript.

| Primer name | 5' location (nt) | Sequence (5'-3')                    |
|-------------|------------------|-------------------------------------|
| UL138-F1    | 190128           | <i>CCGGGTACCCGGACTGGCTTCCCTG</i>    |
| UL138-F2    | 189728           | <i>CGGGGTACCCGAGCTCTTTGTCGTCCT</i>  |
| UL138-F3    | 189328           | <i>CGGGGTACCCGCCTATCGTGTCGGTTTC</i> |
| UL138-F4    | 189228           | <i>CGGGGTACCTCGATGCAGATGACCTGAG</i> |
| UL138-F5    | 189028           | <i>CGGGGTACCAACGATTCTGGGAATGTC</i>  |
| UL138-F6    | 188995           | <i>CGGGGTACCCGGCGTAAGAGAAACCGA</i>  |
| UL138-F7    | 188962           | <i>CGGGGTACCAACGCGTCCCCGACGCCG</i>  |
| UL138-F8    | 188896           | <i>CGGGGTACCTACGCTTATGGTTATGGC</i>  |
| UL138-R     | 188829           | <i>CCGCTCGAGGCCAACTGTCTCTGGTGGT</i> |

The TSS of the *UL138* 1.4 kb transcript is at nt 188829 of the Han genome. Positions of nt are presented according to the Han genome. The underlined nt are enzyme recognition sites, and the nts in italics are those that protect the enzyme recognition site. F, forward; R, reverse; TSS, transcription start site; nt; nucleotide.

Table II. Oligonucleotide sequences used for electrophoretic mobility shift assay.

| Name     | Sequence (biotin-5'-3')              |  |
|----------|--------------------------------------|--|
|          | Forward                              | Reverse                                |
| Ap-1-BS  | GCTGAGTGACGCCGGCGTA<br>AGAGAACCGACCC | GGGTCGGTTTCTCTTACGCCGGC<br>GTCACCTCAGC |
| Ap-1-BSM | GCTGAGCATCGCCGGCGTAG<br>AGAAACCGACCC | GGGTCGGTTTCTCTTACGCCGGC<br>GATGCTCAGC  |

The underlined bases represent the mutation points. Ap-1, transcription factor AP-1; BS, oligonucleotides containing the putative binding site of AP-1; BSM, oligonucleotide with mutated nucleotides in the putative binding site of AP-1.

a 48 h incubation, nuclear extracts were prepared using the NE-PER<sup>TM</sup> nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. An oligonucleotide containing the putative binding site of the predicted transcriptional factor Ap-1 was synthesized and labeled with biotin (Thermo Fisher Scientific, Inc.; Table II). Following annealing, the double stranded fragment of the biotin-labeled oligonucleotide was incubated with 20  $\mu$ g nuclear extract for 30 min at room temperature. For the supershift assay, a specific antibody of c-jun (Abcam, Cambridge, UK), which is the key functional component of Ap-1, was incubated with the nuclear extract for 30 min on ice, prior to the addition of the labeled oligonucleotide. In addition, 200X unlabeled oligonucleotide was used in the competition experiment. And the labeled mutant oligonucleotide (Table II) was used as a control. The reactions were separated on a 6% polyacrylamide gel for 1 h at 100 V. Following electrophoretic transference onto a nylon membrane (Thermo Fisher Scientific, Inc.), the transferred DNA was cross-linked to the membrane by incubating the membrane face down on a UV transilluminator (254 nm) for 15 min. The biotin-labeled DNA was detected by chemiluminescence using the LightShift<sup>TM</sup> Chemiluminescent EMSA kit, which was performed according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). The results were recorded using ChemiDoc<sup>TM</sup> XRSt System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*Western blot and RNA interference.* To detect whether Ap-1 protein expression is affected by HCMV infection, HELF cells cultured in 60-mm plates, were inoculated with Han-BAC at 2X MOI. At 0 (non-infected cells), 24, 48, 72 and 96 h post infection (hpi), proteins of the infected cells were extracted using NE-PER reagent. The quantity of the proteins was determined using a Nanodrop 2000 spectrophotometer. Then, 45 mg protein/well was loaded and separated via SDS-PAGE on a 10% gel. Separated proteins were transferred onto polyvinylidene fluoride membranes, which were blocked in 5% non-fat milk (BD Biosciences, Franklin Lakes, NJ, USA) and TBST solution (TBS containing 0.05% Tween-20) for 2 h at room temperature on a shaker. The protein levels of Ap-1 and GAPDH were detected using the specific antibodies, anti-c-Jun (dilution, 1:1,000; cat. no. ab428; Abcam) and anti-GAPDH (dilution, 1:2,000; cat. no. ab8245; Abcam) in 10 ml TBST solution, containing 5% non-fat milk, on a plate shaker overnight at 4°C. They were then incubated with a horseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000; cat. no. AB501; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) on a plate shaker at room temperature for 2 h. Visualization was performed using the Enhanced Chemiluminescence Detection Reagent (EMD Millipore, Billerica, MA, USA) and the ChemiDoc<sup>TM</sup> XRS + imaging system with Image Lab<sup>TM</sup> software version 5.0 (Bio-Rad Laboratories, Inc.).



Table III. Primer sequences for the production of specific RNA probes in northern blot analysis.

| Primer | 5' location (nt) | Sequence (5'-3')                              |
|--------|------------------|---|
| UL138F | 188273           | GTGATGCTCGTGCTGA                              |
| UL138R | 187834           | <u>TAATACGACTCACTATAGGG</u> GATGGCTACGGTGGTGA |
| UL83F  | 122168           | GTCAGCAGAACCAGTG                              |
| UL83R  | 121781           | <u>TAATACGACTCACTATAGGG</u> TGTGAGGTAAAAGCCA  |

Positions of nt are presented according to the Han genome. The underlined nts are the promoter sequence of T7 RNA polymerase added to the 5' ends of the reverse primers to synthesize the RNA probes. nt, nucleotide; F, forward; R, reverse.

Pre-designed individual small interfering RNA (siRNA) targeting c-Jun (the major functional unit of Ap-1) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) with the following sequences: Sense, 5'-GACUGUAGAUUGCUCUGUdTdT-3' and antisense, 5'-ACAGAAAGCAAUCUACAGUCdTdT-3'. Scrambled siRNA from Sigma-Aldrich (Merck KGaA) was used as negative control, with the following sequences: Sense, 5'-UUCUCCGAACGUGUCACGUCdTdT-3' and antisense, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. To evaluate the inhibition efficiency of the siRNA on Ap-1 expression,  $2.5 \times 10^5$  HELF cells were seeded onto 6-well plates. Following 24 h, the cells were transfected with 50 nM siRNA for Ap-1 using Lipofectamine 3000 according to the manufacturer's protocol. Proteins were extracted at 48 and 72 h following transfection using the NE-PER reagent. Expression of Ap-1 in the transfected cells was detected by western blotting, as described above.

**Construction of the mutant virus.** To evaluate the effect of the identified TRE on the transcription of the *UL138* 1.4 kb transcript in HCMV infected cells, a virus with a deleted TRE region, Han-BAC/ $\Delta$ UL138p, was constructed. Fragments containing the kanamycin resistance protein (*KanR*) gene with 50 bp homologous flanking sequences to the *UL138* gene at the two ends of the TRE were obtained from a *KanR* gene containing plasmid, pGEM, which was provided by Professor Minhua Luo at the Wuhan Institute of Virology (Wuhan, China), using the following primers: 5'-GCCCTGAGTCGCATTCACCGATTCTGGGAATGTCGGCTACGGGTGTGGTGTCTGTCTCTTATACACATCTCAACCATC-3' (sense) and 5'-ATAACGTGAAACACCGCGGTTCATCCAAGTCGGGCGGCGTCGCGGACGCGGCTGTCTCTTATACACATCTCAACCTG-3' (antisense), in which the nts underlined are *UL138* specific sequence. The amplicons were identified by PCR (PCR Reagent kit; Takara Bio, Inc., Otsu, Japan) using the following *KanR* gene specific primers: 5'-GTATGTCAGTCAAGGG-3' (sense) and 5'-GCCATAACCATAAGCG-3' (antisense). PCR was performed with an initial denaturation at 98°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min 30 sec; a final extension step at 72°C for 5 min was included. Following fractionation on a 1% agarose gel and visualization using an automatic gel imaging analyzer (GIS-2020; Tianneng Corp., Shanghai, China), the fragments containing the *KanR* gene with the *UL138* sequences were purified from the original amplicons using the Wizard Genomic DNA Purification

kit (Promega Corporation). The purified fragments (200 ng) were transformed into the DY380 cells containing Han-BAC plasmid, which were provided by Professor Minhua Luo at the Wuhan Institute of Virology. The mutant Han-BAC plasmid with the deletion of the putative TRE sequence of the *UL138* 1.4 kb transcript was selected on chloramphenicol and kanamycin containing LB agar plates. Monoclones with *KanR* positive recombinants were selected and identified by PCR (PCR Reagent kit; Takara Bio, Inc.) using the following primers: 5'-GCTGAGTGACGCCGGCGT-3' (sense) and 5'-GCCAACTGTCCTGGTGGT-3' (antisense). PCR was performed with an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min 30 sec; a final extension step at 72°C for 10 min was included. Plasmid of the Han-BAC/ $\Delta$ UL138p was isolated using the Nucleiobond kit, according to the manufacturer's protocol (Machery-Nagel GmbH, Düren, Germany). The mutant sequence in the Han-BAC/ $\Delta$ UL138p was further confirmed by sequencing analysis. To produce the virus stock, HELF cells ( $1 \times 10^7$  cells) were electroporated with 3  $\mu$ g Han-BAC/ $\Delta$ UL138p plasmid in the presence of 1  $\mu$ g pSVpp71 plasmid, which was provided by Professor Minhua Luo (Wuhan Institute of Virology). The cells were maintained in MEM supplemented with 2% FBS and penicillin-streptomycin. Between 7 and 10 days following 100% cytopathic effect, the extracellular fluid and the monolayer of cells were collected.

**Northern blot analysis.** To evaluate the effect of the identified TRE and its corresponding transcription factor, Ap-1, on the transcription of the *UL138* 1.4 kb transcript in HCMV-infected cells, transcription of *UL138* was detected by northern blotting. HELF cells were transfected with Ap-1 siRNA or blank sequences using Lipofectamine 3000. In the following 6 h, the mock-transfected cells were inoculated with Han-BAC and Han-BAC/ $\Delta$ UL138p, and the cells treated with Ap-1 siRNA were inoculated with Han-BAC. The cells were harvested at 72 hpi. Total RNA was isolated from the HELF cells and treated with TURBO DNA-free™ kit (Thermo Fisher Scientific, Inc.).

For the northern blotting, the RNA was separated by electrophoresis on a 1.25% formaldehyde agarose gel and transferred onto a positively charged nylon membrane. To obtain specific RNA probes for *UL138* and *UL83* transcripts, gene specific sequences were generated by PCR amplification using the primers as described in Table III. The RNA probes were

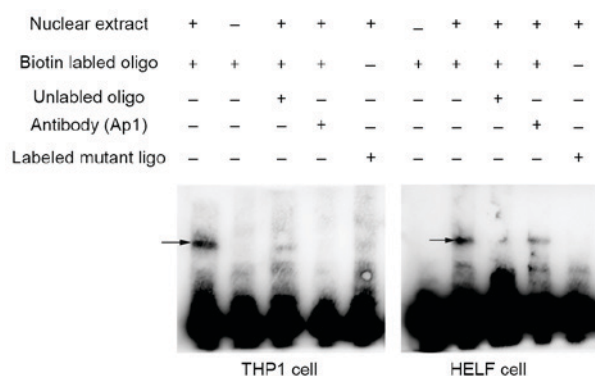


Figure 2. Confirmation of binding between Ap1 and its putative binding site in the identified TRE of the *UL138* 1.4 kb transcript by EMSA analysis. Equal amounts of nuclear extract from HCMV infected HELF and THP-1 cells were incubated with 5'-end biotin labeled oligonucleotide containing the predicted binding site of the Ap1. The biotin labeled oligonucleotide was able to form complexes with the nuclear extracts from HCMV infected HELF cells and THP-1 cells. However, the complexes disappeared upon competition with the unlabeled oligonucleotide, addition of antibody for c-Jun and replacement by the labeled oligonucleotide bearing a 3 bp mutation in the predicted binding region. HELF, human embryonic lung fibroblasts cells; HCMV, human cytomegalovirus; EMSA, electrophoretic mobility shift assay. Ap1, transcription factor AP-1.

synthesized using T7 RNA polymerase and labeled with digoxigenin (DIG), according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN, USA). The nylon membranes were prehybridized for 30 min at 65°C in the preheated hybridization solution supplied with the kit. Following denaturation by heating at 100°C for 5 min, the probes were added to the prehybridized membrane and incubated at 65°C overnight. Following incubation at room temperature for 30 min with an anti-DIG antibody conjugated to alkaline phosphatase (dilution, 1:1,000; cat. no. 12039672910; Roche Diagnostics), the blots were visualized with the CDP-Star® (Roche Diagnostics) substrate and recorded using ChemiDoc™ XRS + imaging System (Bio-Rad Laboratories, Inc.).

**Growth curve analysis.** HELF cells grown in 6-cm plates were transfected or mock transfected with Ap-1 siRNA and transfected respectively with Han-BAC and Han-BAC/ $\Delta$ UL138p strains as described above. At 24, 48, 72 and 96 hpi, the infected cells were collected with 2 ml media, and frozen overnight. Following thawing, the cells were mixed using a pipette. Infectious particles released in the supernatants were titrated to 50% TCID<sub>50</sub>.

**Statistics.** All calculations and statistical analyses were performed with GraphPad Prism (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance was applied for comparing the treated groups with the control group, followed by pairwise multiple comparisons using the Bonferroni post hoc test. Data are presented as the mean  $\pm$  standard error.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*The minimal essential promoter of the UL138 1.4 kb transcript is located within a 33 bp sequence upstream of*

*the transcription start site.* TSS of the *UL138* 1.4 kb transcript has already been identified by Grainger *et al* (10) using 5' rapid amplification of cDNA ends experiment in 2010. To identify the minimal essential TRE of the *UL138* 1.4 kb transcript, a set of constructs containing sequences starting at different points but ending at nt 188829, which is located one nt prior to the TSS of the *UL138* 1.4 kb transcript, were integrated into the pGL3-basic vector (Fig. 1A). The transcriptional regulatory activities of the constructs were measured in HEK293 cells at 48 h following transfection using a dual-luciferase assay. The relative FFL activity of the pGL3-B/188995 to that of the *Renilla* luciferase control was significantly increased compared with that of pGL3-basic vector alone ( $P < 0.05$ ; Fig. 1B). Compared with that of the pGL3-B/188995, the relative FFL activity of the pGL3-B/188962, which lacks the 33 bp sequence from nt 188995-188962, decreased by 82%.

To identify the transcriptional regulatory activity of the 33 bp fragment located between nt 188995 and 188962, the fragment and its neighboring fragments were inserted into pGL3-E and pGL3-P vectors. The relative FFL activity of the pGL3-E/188995-188962 was significantly increased compared with the pGL3-enhancer vector alone ( $P < 0.05$ ; Fig. 1C). However, no significant difference was observed between the pGL3-P/188995-188962 and pGL3-P vector alone ( $P > 0.05$ ; Fig. 1D). These results suggest that the 33 bp sequence located within nt 188995-188962, which is -166 to -133 bp from the TSS of the *UL138* 1.4 kb transcript, possesses promoter activity in HEK293 cells.

*Ap-1 has the ability to bind to the specific TRE of the UL138 1.4 kb transcript.* To predict the putative transcription factors binding to the TRE of the *UL138* 1.4 kb transcript, which is located between nt 188995 and 188962, two different programs of Jaspar Core database and Web Signal Scan were used. By comparing the results of the two systems, it was demonstrated that the TRE possesses a binding site of 5'-TGAGTGA-3' for Ap-1, which agrees with the sequence of 5'-TGAC/GTGA-3' reported by Shaulian *et al* (19). In the EMSA experiment, the biotin labeled oligonucleotide was able to form complexes with the nuclear extracts from HCMV-infected HELF cells and THP-1 cells. However, the complexes disappeared upon competition with the unlabeled oligonucleotide, addition of antibody for c-Jun or replacement with the labeled oligonucleotide with a 3 bp mutation in the predicted binding region (Fig. 2). This result indicates that the predicted binding site of Ap-1 in the TRE of the *UL138* 1.4 kb transcript has the ability to bind specifically to the transcription factor Ap-1.

*Deletion of the UL138 TRE and inhibition of Ap-1 expression affects transcription of the UL138 1.4 kb transcript.* Ap-1 expression during HCMV infection at different time points was investigated and the inhibitory effects of Ap-1 specific siRNA were evaluated with Ap-1 expression levels being detected by western blotting. The result demonstrated that expression of Ap-1 was highest at 24 hpi and then decreased with increasing duration of infection. A significant difference in Ap-1 expression was observed between cells at 24 hpi and mock infection (Fig. 3A). The expression of Ap-1 was inhibited by its siRNA in HELF cells at 48 and 72 h post transfection (Fig. 3B).

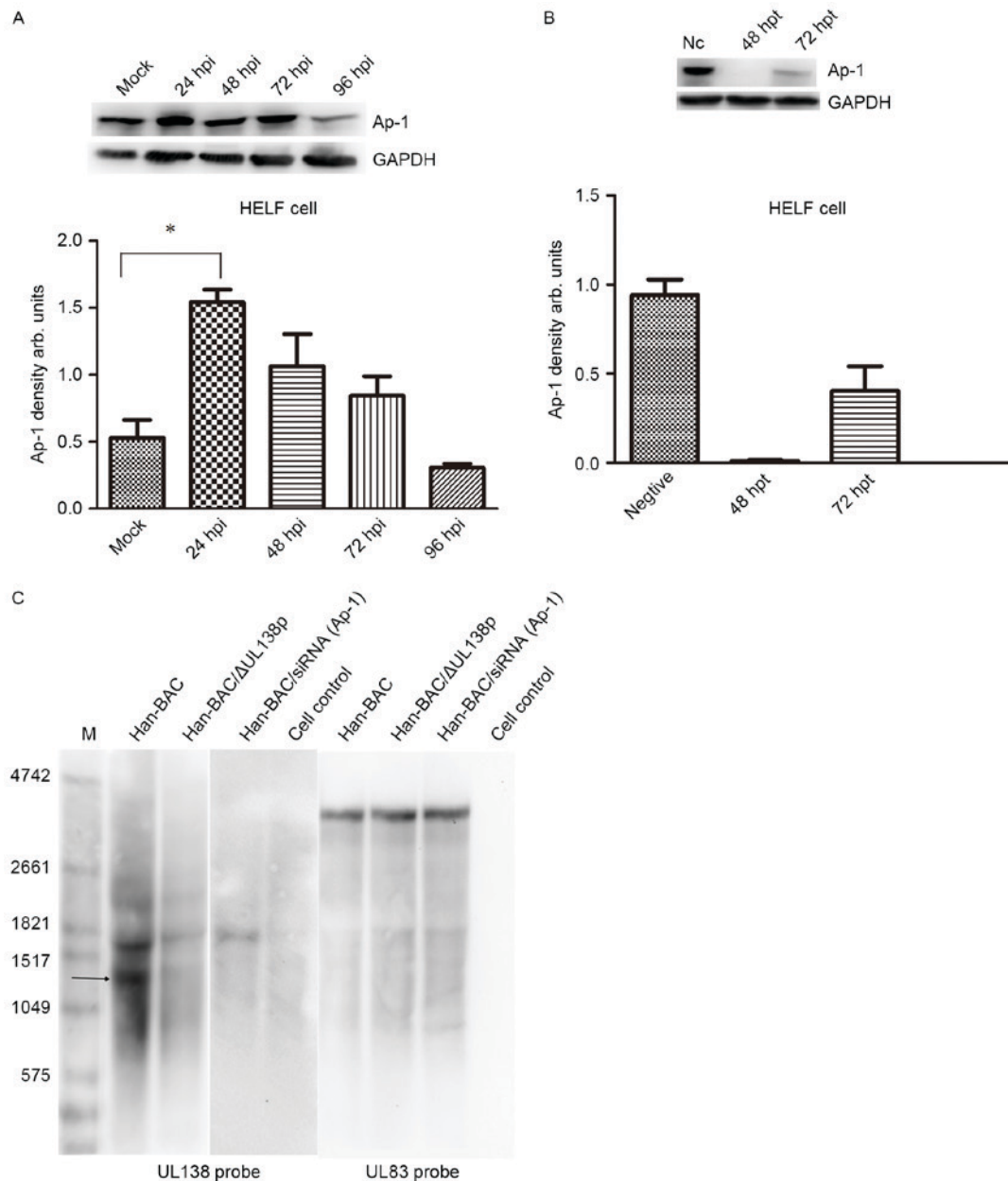


Figure 3. Effects of the promoter and Ap-1 on *UL138* transcription. (A) To understand expression of Ap-1 in HCMV-infected cells, HELF cells were infected with the Han-BAC strain at 2X MOI, and the cells were collected at different time points. Expression of Ap-1 in the infected cells was detected by western blotting. The result demonstrated that expression of Ap-1 began to increase at 24 hpi and decrease keep 24 hpi with the duration of infection. (B) Inhibition efficiency of the predesigned siRNAs for the Ap-1 was detected by western blotting in the transfected HELF cells. At 48 and 72 hpi, expression of Ap-1 was significantly inhibited in the cells compared with the negative control. (C) Transcriptions of the *UL138* 1.4 kb transcript in HCMV infected HELF cells were detected by northern blotting at 72 hpi. Compared with the Han-BAC infected cells, the *UL138* 1.4 kb transcript was undetected in the Han-BAC/ΔUL138p infected cells and was decreased in the Han-BAC infected cells treated with Ap-1 siRNA. Data are presented as the mean  $\pm$  standard error. \* $P < 0.05$ . HCMV, human cytomegalovirus; MOI, multiplicity of infection; HELF, human embryonic lung fibroblasts; siRNA, small interfering RNAs; hpi, h post infection; BAC, bacterial artificial chromosome. Ap-1, transcription factor AP-1.

To understand the regulatory effects of the TRE and the transcription factor Ap-1 on transcription of the *UL138* 1.4 kb transcript, a mutant Han-BAC, Han-BAC/ΔUL138p, with the identified TRE deleted, was successfully constructed and rescued (data not shown). Transcription of the *UL138* 1.4 kb transcript in HCMV infected HELF cells was detected by northern blotting at 72 hpi. Compared with the Han-BAC-infected cells, the *UL138* 1.4 kb transcript was not detected in the Han-BAC/ΔUL138p-infected cells and was decreased in the Han-BAC-infected cells treated with

siRNA for Ap-1 (Fig. 3C). The results support the theory that transcription of the *UL138* 1.4 kb transcript in fibroblasts is dependent on the TRE and the transcription factor of Ap-1.

**Deletion of the *UL138* TRE is able to accelerate HCMV replication.** In order to ascertain the role of the *UL138* TRE in HCMV replication, mock transfected HELF cells were infected with Han-BAC and Han-BAC/ΔUL138p at 2X MOI. The HELF cells transfected with Ap-1 specific siRNA were infected with 2X MOI Han-BAC. Infectious virus particles in



the cells collected at 24, 48, 72 and 96 hpi were titrated by TCID<sub>50</sub> (Fig. 4A). At 96 hpi, the virus titer in cells infected with Han-BAC/ $\Delta$ UL138p was increased 2.5 fold compared with the cells infected with Han-BAC. However, no difference in viral titer was observed in Han-BAC infected cells transfected with or without Ap-1 siRNA (Fig. 4B). The result indicates that HCMV replication can be accelerated by deletion of the *UL138* 1.4 kb transcript specific TRE.

## Discussion

HCMV *UL138* is a 510 nt gene within the UL/b' region of the genome, which is unique to clinical or low-passage strains (20). *UL138* is expressed during active and latent infections in a variety of cell types, and is the first viral gene demonstrated to function in promoting HCMV latency (9,21). It has been reported that pUL138 retards HCMV replication in human CD34<sup>+</sup> macrophage progenitors and therefore promotes latency (21,22). High expression in the primary hematopoietic progenitor cells (HPCs) indicates that *UL138* serves an important role in viral persistence (8). Studies have indicated that pUL138 is associated with formation, maintenance (10,11) and reactivation (12,13) of latent HCMV infections.

The *UL138* transcripts are polycistronic. The two transcripts of 3.6 and 2.7 kb were identified to contain the *UL133-UL138* gene sequences by northern blotting and rapid amplification of cDNA ends in 2009 (16), and a third transcript of *UL138* corresponding to 1.4 kb with 5' end at 292 nt downstream of the predicted start of *UL136* was reported in 2010 (10). Mechanisms of pUL138 translation have been well studied (10). However, knowledge about the transcriptional regulation of this important gene remains unclear.

In the present study, a 33 bp fragment from nt 188995-188962, which is located from -166 to -133 bp upstream of the TSS of the *UL138* 1.4 kb transcript, was identified to possess promoter activity. In addition to the 33 bp fragment that demonstrated positive transcriptional regulatory function, another fragment between nt 189028 and 188995, which is located upstream of the 33 bp fragment, should not be ignored for the region may serve a negative regulatory role in the expression of the *UL138* 1.4 kb transcript. The relative luciferase activity of the pGL3-P/189028-995 decreased ~45% compared with that of the pGL3-P vector alone which supports the preceding judgment. Further study of the functions of the negative regulatory region may be required in the future.

The binding of Ap-1 to the TRE in the 33 bp fragment was demonstrated by EMSA in THP-1 and HELF cells. A possible explanation for the disappearance of the complex in the supershift lane in EMSA is that the addition of an antibody for c-Jun reduced the formation of the complex. The TRE and its corresponding transcription factor Ap-1 were confirmed to be responsible for initiating transcription of the *UL138* 1.4 kb transcript during active infection in HELF cell. Deletion of the *UL138* 1.4 kb transcript specific TRE in virus can accelerate HCMV replication in HELF cells. These results indicate that AP-1 is a key transcription factor for transcription of the *UL138* 1.4 kb transcript, which is involved in suppressing viral lytic gene expression and virus production in actively infected cells.

Petrucelli *et al* (16) argued that *UL138* expression in fibroblasts is dependent on the synthesis of the immediate-early (IE)

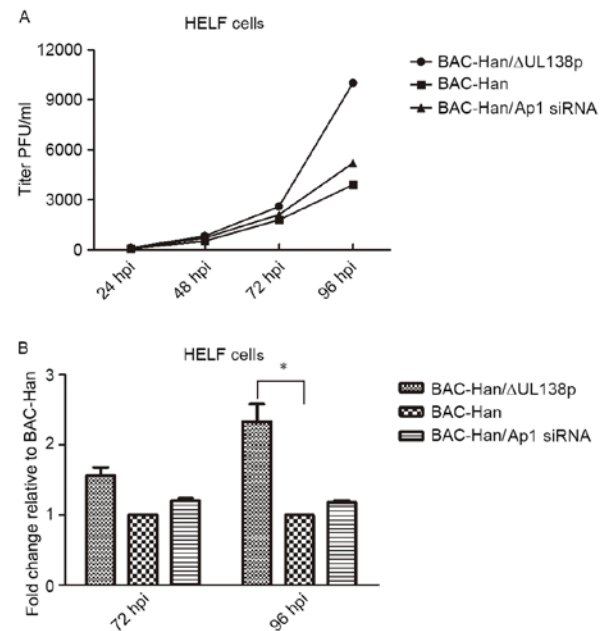


Figure 4. Effect of *UL138* expression on viral replication. (A) HELF cells grown in 6-cm plates were transfected with blank siRNA and infected with Han-BAC and Han-BAC/ $\Delta$ UL138p strains at 2X MOI. Following 6 h transfection with the Ap-1 siRNA, the HELF cells were infected with Han-BAC at 2X MOI. At 24, 48, 72 and 96 hpi, the cells were collected with 2 ml media and thawed. Infectious particles in the infected cells were titrated by TCID<sub>50</sub> assay. Average numbers from two separate analyses were used for drawing the growth curve. (B) Data collected at 72 and 96 hpi were used in the statistical analysis. Results demonstrated that the titer of infectious particles in Han-BAC/ $\Delta$ UL138p-infected cells was significantly increased compared with in Han-BAC-infected cells. Data are presented as the mean  $\pm$  standard error. \* $P < 0.05$ . HCMV, human cytomegalovirus; MOI, multiplicity of infection; HELF, human embryonic lung fibroblasts; siRNA, small interfering RNAs; TCID<sub>50</sub>, 50% tissue culture infective dose; hpi, h post infection; BAC, bacterial artificial chromosome; PFU, plaque forming units; Ap-1, transcription factor AP-1.

proteins. In addition, the study also reported that *UL138* may be expressed independently of IE genes as viral gene expression is dictated by the unique milieu of the particular cellular environment during an active infection. In the present study, Ap-1 was demonstrated to be involved in the transcriptional regulation of the *UL138* 1.4 kb transcript, indicating that Ap-1 may be a key factor in the unique milieu of infected cells. Whether transcription of the *UL138* 1.4 kb transcript is mediated by Ap-1 alone or together with IE proteins need to be studied in the future.

It is known that a number of factors may be involved in gene transcription. Although mutation of the TRE in the 33 bp fragment or inhibition of Ap-1 expression in infected cells can block or decrease the transcription of the *UL138* 1.4 kb transcript, it does not mean the binding of the TRE and Ap-1 is the only mechanism to change transcription of the *UL138* 1.4 kb transcript. Other mechanisms and factors involved in the transcription of the *UL138* 1.4 kb transcript cannot be precluded.

It has been reported that human monocytic leukemia cells (THP-1) are not only widely used as a model of HCMV latent infection (23,24), but also have been employed as a lytic infection model (25). Although evidence remains lacking in a reliable cell model (for example CD34<sup>+</sup> cells), the binding of Ap-1 to the TRE in the 33 bp fragment of *UL138* gene region in



HCMV-infected THP-1 cells may imply a similar role for Ap-1 in regulating *UL138* expression in quiescent/lytic THP-1 cells.

The Ap-1 family including members of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families participate in the regulation of multiple genes involved in control of cell differentiation, apoptosis, immune responses and in response to a wide array of stimuli, including stress, growth factors, and proinflammatory cytokines (26). An early study demonstrated that HCMV early infection could result in a rapid and substantial increase of Ap-1 mRNA. Furthermore, the transcriptional activation of cellular Ap-1 could provide the means for the rapid activation of the HCMV IE genes (27). Other reports demonstrated that HCMV infection could induce Ap-1 in a biphasic mode in which the initial interaction of viral particles with the cell caused a rapid induction of the major components of Ap-1, c-Jun, and later increase of the c-Fos RNA expression, when the viral IE1 and IE2 proteins were involved (28,29). To determine the appropriate time for the evaluation of Ap-1-siRNA inhibitory effect and for the detection of *UL138* transcripts affected by the inhibition of Ap-1 expression in infected HELF cells, expression kinetics of Ap-1 were detected by western-blotting in the present study. The result illustrated that expression of the major functional member of Ap-1, c-Jun, increased at 24 hpi, but decreased with increased duration of HCMV infection in HELF cell line.

The *UL138* encodes a 21 kDa type-1 transmembrane protein that localizes to the Golgi apparatus (16). pUL138 was expressed from each of the three transcripts of *UL138*, with the greatest expression resulting from the 1.4 kb transcript, and pUL138 translated from the 3.6 and 2.7 kb transcripts was induced by serum stress (10). Viral infection or cell stress is able to activate an alternative mechanism of translation initiation when canonical cap-dependent mechanisms of translation were inhibited (30,31). The function of the *UL133-138* gene locus in viral replication is controversial. It has been reported that the genes of *UL133-138* locus in clinical strains can slow down the viral replication process in human fibroblast cells by affecting viral egress as well as reducing the viral major IE RNA and protein (32). Furthermore, recent research reports that a long form of *UL138* initiates translation from codon 1 which appears more potent at silencing IE1 transcription shortly following infection, while the short form initiating from codon 16 appears more potent at restricting progeny virion production at later time points, promoting HCMV latency (33). However, *UL133/8* null mutants exhibit a delay in replication at lower MOIs during viral replication in fibroblasts (34). As a member of the *UL133-138* gene locus, contribution of *UL138* to viral replication is different in various cells. It has been proved *in vitro* that *UL138* could retard viral replication in human CD34<sup>+</sup> macrophage progenitors to promote latency (35). However in CD34<sup>+</sup> hematopoietic progenitor cells, it has been demonstrated that the *UL133-138* gene locus promotes viral replication in endothelial cells in culture by mediating formation of the virion tegument and secondary envelope (36). To understand the effects of the *UL138* 1.4 kb transcript on viral replication, a growth curve of virus with a deletion of the *UL138* 1.4 kb transcript specific TRE was assayed in HELF cells. The result demonstrated that infectious particles in cells infected with the deleted virus Han-BAC/ $\Delta$ UL138p at 96 hpi increased by 2.5 fold compared with the cells infected with

Han-BAC. These results imply that the role of *UL138* on viral replication may be different in various cells and infection states. An explanation for these paradoxical results is that *UL138* may encode multifunctional proteins such as other viral genes and may modulate the cell-type specific outcome of infection.

A recent article reported that the 23-/19-kDa isoforms of pUL136 act to suppress, while the 33- and 26-kDa isoforms function to promote HCMV replication in CD34<sup>+</sup> HPCs and endothelial cells (ECs) (37). However, the pUL136 isoforms are dispensable for viral replication in fibroblasts (38). *UL136* is transcribed together with two transcripts of 3.6 and 2.7 kb from the *UL133* to *UL138* gene locus (16). Although mutation of the deleted virus Han-BAC/ $\Delta$ UL138p may affect the transcription of *UL136* in theory, the result of northern blotting demonstrated that the two transcripts, which contain the *UL136* sequence, had not been affected, indicating that the relatively high replication efficiency of the mutant virus in HELF cells is caused by deletion of the *UL138* 1.4 kb transcript only.

Unlike deletion of the *UL138* 1.4 kb transcript specific TRE, HCMV infectious particles from HELF cells treated with Ap-1 specific siRNA were similar to those from non treated cells at different infection points. It has been reported that *UL135*, another member of the *UL133/8* locus, comprises of a molecular switch with *UL138* whereby *UL135* is required for viral replication, and able to overcome *UL138*-mediated suppression of virus replication to balance states of latency and reactivation (11). In addition, different isoforms of pUL136 exhibit different functions in HCMV replication in CD34<sup>+</sup> HPCs and ECs (37) and are dispensable for virus replication in fibroblasts (38). Inhibition of Ap-1 by its siRNA may result in the loss of transcriptional activity on other genes that may promote HCMV replication. Anyway, the results of the present study confirmed that inhibiting the transcription of the *UL138* 1.4 kb transcript could slightly accelerate virus replication in HELF cells.

In conclusion the present study identified that the TRE and its corresponding transcription factor Ap-1 contribute to transcription of the *UL138* 1.4 kb transcript in HCMV infected HELF cells. Transcriptional suppression of the *UL138* 1.4 kb transcript benefits HCMV replication in HELF cells. It can be proposed that high-level expression of *UL138* may be helpful for developing and maintaining virus latency, and inhibition of *UL138* expression during latency may be helpful for virus reactivation. Therefore, the present study provides a novel insight about transcriptional regulation of *UL138* gene, and interactions between virus and the host cells during natural infection.

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