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A Comparison between Low- and High-Passage Strains of Human Cytomegalovirus S

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To understand how human cytomegalovirus (HCMV) might change and evolve after reactivation, it is very important to understand how the nucleotide sequence of cultured HCMV changes after in vitro passaging in cell culture, and how these changes affect the genome of HCMV and the consequent variation in amino acid sequence. Strain JHC of HCMV was propagated in vitro for more than 40 passages and its biological and genetic changes were monitored. For each passage, real-time PCR was performed in order to determine the genome copy number, and a plaque assay was employed to get virus infection titers. The infectious virus titers gradually increased with passaging in cell culture, whereas the number of virus genome copies remained relatively unchanged. A linear correlation was observed between the passage number and the $\log_{10}$ infectious virus titer per virus genome copy number. To understand the genetic basis underlying the increase in HCMV infectivity with increasing passage, the whole-genome DNA sequence of the high-passage strain was determined and compared with the genome sequence of the low-passage strain. Out of 100 mutations found in the high-passage strain, only two were located in an open reading frame. A G-T substitution in the RL13 gene resulted in a nonsense mutation and caused an early stop. A G-A substitution in the UL122 gene generated an S-F nonsynonymous mutation. The mutations in the RL13 and UL122 genes might be related to the increase in virus infectivity, although the role of the mutations found in noncoding regions could not be excluded.

Keywords: Human cytomegalovirus, mutations, RL13, UL122

Introduction

Human cytomegalovirus (HCMV), also known as human herpesvirus type 5 or HHV-5, is a human beta-herpesvirus. Of all viruses infecting humans, HCMV has the largest genome [4]; about 230 kilobase pairs (kbp). It has a double-stranded DNA genome with a molecular mass of 150×10^6 daltons [7]. HCMV infection in immune-competent adults generally does not lead to any specific symptoms and establishes a latency. However, in patients with reduced or ameliorated immune function, such as AIDS patients or transplant recipients, it can lead to disease conditions that reflect virus reactivation, such as pneumonia, hepatitis, and retinitis. Moreover, HCMV is the most frequent source virus in congenital infections of infants, and about 10% of congenital HCMV infections result in fetal central nervous system damage. Although latent HCMV infection is usually maintained in myeloid-derived mononuclear cells, the specific reasons for this are not known [19]. Whole-genome nucleotide sequence analysis of HCMV was first performed in 1990 [4], and other studies have been conducted since then [1].

HCMV strain JHC was isolated from a Korean bone marrow transplant patient in 2003 and cultured by inoculating the patient's blood into cultured human foreskin fibroblast (HFF) cells [12]. The JHC genome is 235,476 bp in length, with 167 open reading frames (ORFs) [9]. These ORFs represent genes from 12 multigene families. One of these is the RL11 gene family, which includes RL5A, RL6, RL11, RL12, RL13, UL1, UL4, UL5, UL6, UL7, UL8, UL9,

UL10, and UL11. Most of the genes in the RL11 gene family play important roles in cell entry through membrane proteins. The RL13 gene is relatively conserved in all beta-herpesviruses [13]. Mutations have been seen in four HCMV strains that were cultured after their initial clinical isolation. Comparing the genome sequences from the final and initial subcultures, both the RL13 gene and the UL128 locus, which includes the UL128, UL130, and UL131 genes, showed mutations in all four of these strains. These two loci showed somewhat different mutation patterns in different cell lines, however. Whereas the RL13 gene underwent mutation in three types of cells (HFFs, epithelial cells, and endothelial cells), the UL128-locus genes underwent mutation only when cultured in HFFs [6].

HCMV DNA replication is a very complex process that engages various virus proteins and genes. During the infection, HCMV genes are expressed in a cascade, designated immediate-early (IE), early (E), and late (L) genes [18]. IE genes are expressed just after HCMV infection, reaching maximum levels after about 6 h. The HCMV IE genes include the major IE (MIE) UL122 (IE2) and UL123 (IE1) and other genes [2, 3, 24]. The mRNAs of the IE1 and IE2 genes are transcribed by alterative splicing of a single precursor RNA [23, 26]. The products encoded by IE1 and IE2 genes function in the regulation of subsequent viral gene expression. The IE2 gene is expressed according to type of host cell, and can be controlled by enhancers and silencers. The IE2 gene is essential for viral gene expression and viral replication [17]. The IE2 protein acts as an autorepressor, repressing transcription of the IE1 and IE2 genes by binding the *cis*-repressive sequence (crs), located between the TATA box and the transcription start point [5, 8, 10, 14, 21].

Viruses have evolved defense systems for evading host immune systems. The study of how HCMV might change and evolve after reactivation is an important issue. As the first step, this study addresses the issue of how the nucleotide sequence of cultured HCMV changes after in vitro passaging in cell culture, and how these changes affect the genome of HCMV and the consequent variation in amino acid sequence.

Materials and Methods

Cell Culture

HFF cells were used to culture HCMV. The cells were cultivated in Dulbecco's minimum essential medium (DMEM; Sigma-Aldrich, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technology, USA). DMEM with 2% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin was used as a maintenance medium for the cell monolayers. Cultivation was carried out in an incubator at 37°C in an atmosphere of 5% CO_2 in order to maintain a proper acidity level.

Virus Culture

The virus used in this experiment was a sixth generation subculture of the HCMV JHC strain, which was isolated from a Korean patient in 2003. The whole-genome nucleotide sequence of this strain has been reported [9]. To obtain infectious HCMV stocks, viruses were inoculated into the HFF cell layer to present a multiplicity of infection of 0.01–0.05 plaque forming units (pfu)/cell. The infected cell layer was then inoculated at 37°C for 1 h to allow adsorption. The flask was gently rocked every 15 min in order to prevent cell drying and to evenly spread the viruses. Maintenance medium was applied after the 1-h adsorption period. After 5–6 days, the medium was changed to a fresh one as some cell lesions had appeared. Ten days after infection, the cells were cultivated by scraping, using a cell scraper. Then, the cells were centrifuged at 3,000 rpm for 5 min in order to collect the supernatant, and the pellet was resuspended in the one-tenth volume of the supernatant. A cycle of freezing and thawing of the cells was repeated three times in liquid nitrogen and a 42°C water bath, and the cells were centrifuged at 3,000 rpm for 5 min in order to collect the supernatant. This supernatant was then mixed with the initially obtained supernatant. The combined supernatant was filtered using a 0.45 μm filter and the virus stock was preserved in a –70°C chamber in 1 ml aliquots.

Titration of Infectious Viruses

A plaque titration assay was used to quantify the infectious viruses. The virus sample was diluted 10-fold at room temperature, and 0.2 ml of this diluted virus sample, from 10^{-2} to 10^{-5}, was inoculated into the confluent monolayer of HFF cells cultured in a 35-mm-diameter cell-cultivation dish. The cultivation dish was smoothly shaken every 15 min during a 1-h adsorption period at 37°C. Then, the virus inoculum was removed and overlay medium (DMEM with 2% FBS, 0.37% sodium bicarbonate, 0.25% agarose, 100 U/ml penicillin, and 100 μg/ml streptomycin) was added. At 14 days post infection, the cells were fixed over 24 h using 10% formalin. After removing the formalin and overlay medium, the number of plaques was counted using a dissecting microscope after staining with 0.03% methylene blue.

Quantitative Real-Time PCR

DNA was extracted using a Qiagen DNA Mini Kit (Qiagen, Germany), according to the manufacturer's instructions, and the extracted DNA was used as the template for PCR using the $UL141_{b2}$ primer set ($UL141_{b2}$F-18mer, 5′-TCG GCT GAT GAA CGG ACT-3′; $UL141_{b2}$R-20mer, 5′-CCA AGT GGT AAC GAT AGG AT-3′) [15]. Amplification of the target was confirmed by gel electrophoresis, and the PCR products were purified using the GeneAll Expin Gel SV kit (GeneAll, Korea). The purified amplicon was ligated into

the pGEM T Easy Vector (Promega, USA) and the vector was used to transform *E. coli* DH10B cells, which were cultivated in LB broth with ampicillin (50 μg/ml) for 12 h after verification of insertion using IPTG and X-gal plates. The DNA-spin Plasmid DNA Purification Kit (iNtRON Biotechnology, Korea) was used to extract plasmid DNA. The DNA concentration of the extracted plasmid DNA was measured using the NanoDrop 2000 spectrometer (Thermo Fisher Scientific, USA), with 260 nm representing about 256.34 ng/μl. The number of DNA copies was calculated using the following equation: copies/μl = (concentration of DNA)/(660 × size of dsDNA/Avogadro's number). Avogdro's number = 6.023e23 molecules/mole; average MW of a double-stranded DNA molecule = 660 g/mol. This method indicated 6.036×10^9 copies/μl. Ct values for each concentration were calculated using two methods: (i) real-time PCR, using the primers UL141 F-22mer (5′-CCG CTG TTG GAC AGT GAT ACA G-3′) and UL141 R-20mer (5′-TGG CGT CAC CGG TAA RAA TT-3′) [25, 27]; (ii) real-time PCR with SYBR Green, in which the number of DNA copies had already been determined as 10^{-7} after 10-fold dilution. Then, a standard curve was produced according to the Ct values based on the number of DNA copies. Real-time PCR was conducted as follows. The final volume was brought to 20 μl by adding sufficient sterile water to the reaction mix of 1 μl of extracted virus DNA, 10 μl of iQ SYBR Green Supermix, and 0.6 μl each of 10 pmol UL141F and UL141R primers [27]. After denaturation at 94°C for 5 min, amplification was conducted, using the iCycler iQ real-time PCR detection system (Bio-Rad, USA), for 40 cycles of denaturation at 94°C for 5 sec, annealing at 55°C for 10 sec, and extension at 72°C for 10 sec. After the 40 cycles, a final extension reaction was applied at 72°C for 15 sec.

Whole-Genome Sequencing of HCMV JHC p37

The DNA of JHC p37 was extracted using the Qiagen DNA mini kit (Qiagen). The DNA concentration was 26.1 ng/μl and the total amount was about 6.525 μg. The extracted DNA was sequenced using the 454 GS Junior sequencing system serviced by ChunLab (Korea). A total of 126,997 nucleotide sequence reads with an average length of 476 bp were used to construct the whole-genome nucleotide sequence. The obtained nucleotide sequence reads were arranged based on the whole-genome sequence of another JHC strain (HQ380895). From these reads, 21 contigs were assembled. The completed contigs represented a coverage of about 256.32×, which can be considered reliable. There were 22 gaps in the interpretation of the sequence using the 454 GS Junior method and these gaps were filled in by PCR sequencing. PCR gap-filling was implemented by designing primers on either side of the gap between contig sequences and sequencing the resulting PCR products. The sequencing of the PCR products was performed by SolGent Co. (Korea).

Comparison and Analysis of the Nucleotide Sequence

To investigate changes in the JHC sequence, the whole-genome nucleotide sequences of the high-passaged JHC p37 and the low-passaged JHC p6 were aligned using ClustalW (ver. 2.0.1, http://www.clustal.org/clustal2/#Download). Using the ORF locations in the JHC strains registered in NCBI, the ORFs of JHC p37 were found in the p37 whole-genome nucleotide sequence. For the single nucleotide polymorphism (SNP) analysis, the whole-genome nucleotide sequences of JHC p6 and p37 were compared and analyzed using the BioEdit Sequence Alignment Editor (ver. 7.0.9.0, http://www.mbio.ncsu.edu/bioedit/) and the CLC Sequence Viewer (ver. 6.6.1; CLC bio, USA). The nature of the SNPs (substitutions, insertions, and deletions) was noted, as was whether the substitutions were transitions or transversions (purine-purine/pyrimidine-pyrimidine or purine-pyrimidine/pyrimidine-purine substitutions, respectively). Finally, the effects of the SNPs on proteins via changes in the deduced amino acid sequences were analyzed. To analyze the SNPs, 16 total genome sequences, including AD169-varUK (NC_001347.6), AD169-varUC (FJ527563.1), Towne (FJ616285.1), HAN38 (GQ396662.1), HAN20 (GQ396663.1), HAN13 (GQ221973.1), 3157 (GQ221974.1), 3301 (GQ466044.1), JP (GQ221975.1), Toledo (GU937742.1), Merlin (NC_006273.2), and JHC (HQ380895.1), registered in GenBank, were used.

Results

Changes in Infectious Virus Titer after Serial Passage

The whole-genome nucleotide sequence of HCMV JHC p6 has already been reported and analyzed [9]. In the present study, the infectious viruses were investigated using a plaque assay, continuously subculturing the JHC strains in HFF cells for up to 43 passages. The experiments were performed in an overlapping manner, and each result represents the mean and standard deviation of four repeated experiments. The quantity of infectious virus was presented in pfu/ml, for each passage stock. Virus titers gradually increased along with increasing passage number (Fig. 1). More specifically, increases in virus titer were small until p25 but showed relatively large increases after p25.

Changes in the Number of Viral Genome Copies after Serial Passage

To investigate the relationship between changes in the infectious virus titer and the number of genome copies with each JHC passage, the HCMV UL141 gene, which includes the target section for the real-time PCR, was amplified by PCR and cloned. Quantification was performed using the cloned plasmid as a positive control. The number of plasmids per unit volume was calculated as 6.036×10^9 plasmids/μl. That is, the number of positive control plasmids containing the HCMV UL141 gene was 6.036×10^9/μl. Real-time PCR was carried out with a 10-fold dilution of this plasmid DNA. The number of genome copies and value of Ct were calculated using the standard equation, $Y = -0.291X + 9.757$,

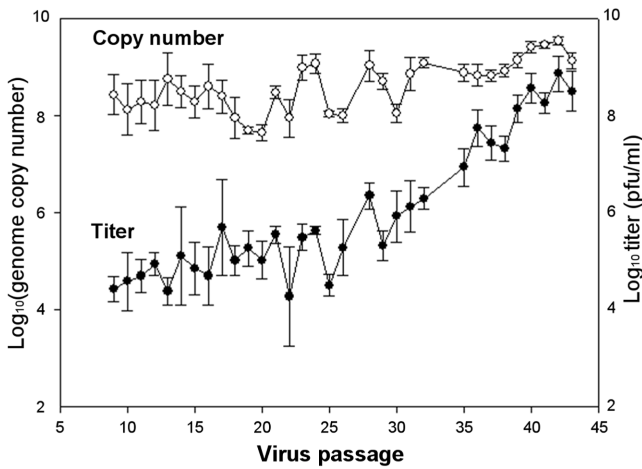

Fig. 1. Changes in the infectious virus titer and the total number of viral genome copies.

The infectious virus titer (lower line) steadily increased, whereas the number of genome copies (upper line) remained roughly constant (x-axis: the number of virus passages; left y-axis: the number of infectious virus genome copies; right y-axis: infectious virus titer).

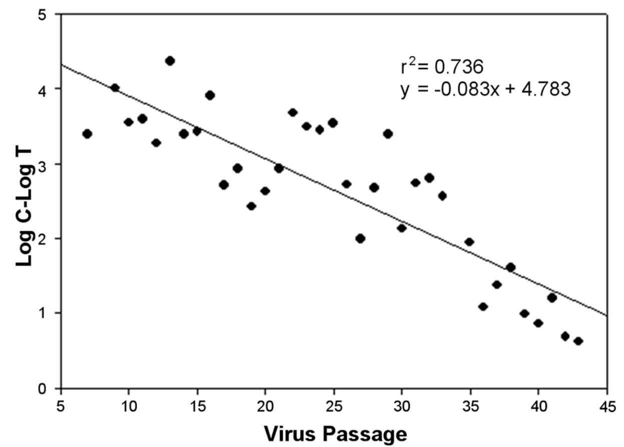

Fig. 2. Differences between the number of genome copies and the infectious virus titer, according to passage number.

The difference between the log_{10} number of viral genome copies and the log_{10} infectious virus titer was correlated with passage number, with a correlation coefficient, r^2, of 0.736. Log C, log_{10} number of viral genome copies; Log T, log_{10} number of infectious viruses.

where Y is the number of log_{10} genome copies, and X is the value of Ct. The correlation coefficient, r^2, of this equation was very high, 0.998. The result shows the whole-genome DNA copies of the virus stocks in each passage obtained by real-time PCR based on the standard equation above (Fig. 1). The number of genome copies showed no significant increases with passage number.

Relationship between Infectious Virus Titer, Viral Genome Copy Number, and Passage Number

As mentioned above, the infectious virus titer increased with increasing passage number in JHC strains, but there was no significant change in the number of genome copies (Fig. 1). This reveals a tendency towards decreasing differences between the number of genome copies and the number of infectious viruses along with increasing passage. To further illustrate this pattern, the difference between the log_{10} number of infectious viruses and the log_{10} number of genome copies is presented for each passage (Fig. 2). Although the changes in the values varied with each passage, there was a clearly observable tendency towards a decreasing difference between the values ($r^2 = 0.736$). That is, the number of infectious viruses was much smaller than the number of genome copies at low-passage number. However, the number of infectious viruses increased, relative to the number of genome copies, with increasing passage number, resulting in a steadily decreasing difference between the two.

Comparison of the Whole HCMV Genome Sequence between Low- and High-Passage Viruses

Whole-genome sequencing of the high-passage virus, JHC p37, through next-generation sequencing using JHC p6 as a reference passage, yielded 110,513 reads, incorporated into 23 contigs and 22 gaps (Table S1). The whole-genome nucleotide sequence of JHC p37 was completed by filling in the gaps through PCR using primers designed in this study, followed by sequencing of the PCR amplicons (Table S2). The whole-genome nucleotide sequence of JHC p37, as determined by a combination of next-generation sequencing and PCR gap-sequencing, was 237,480 bp in length. This represented an extension by 4 bp compared with the whole-genome length of JHC p6, which was 235,476 bp in length. However, the GC content and proportions of A, G, C, and T nucleotides showed no significant differences compared with those of the reference sequence, JHC p6 (Table 1). Comparison of the whole-genome nucleotide sequences between the low-passage JHC p6 and the high-passage JHC p37 revealed a total of 100 differences (Table 2). Nucleotide substitutions were classified as transitions (TS, non-intersectional substitutions; from purines to purines or from pyrimidines to pyrimidines) or transversions (TV, intersectional substitutions; from purines to pyrimidines or from pyrimidines to purines). Of the differences, 50 were substitutions, including two within ORFs: one TS and one TV in the RL13 and UL122 ORFs, respectively. The TS substitution in RL13 was a change

Table 1. Comparison of the nucleotide sequences of JHC p6 and p37.

	Total length (bp)	Nucleotide base				GC%
		A	G	C	T	
p6	235,476	50,565 (21.50%)	67,111 (28.50%)	68,253 (29.00%)	49,546 (21.00%)	135,364 (57.50%)
p37	235,480	50,580 (21.50%)	67,100 (28.50%)	68,243 (29.00%)	49,577 (21.00%)	135,343 (57.50%)

from G to T at nt 11,642 of the whole-genome sequence (nt 631 from the start of the ORF), leading to a change from glutamic acid (E) to a stop codon at position 211 of the deduced amino acid sequence. The TV substitution in UL122 was a change from G to A at nt 171,040 of the whole-genome sequence (nt 1,127 from the start of the ORF), leading to a change from serine (S) to phenylalanine (F) at position 376 of the deduced amino acid sequence. In addition to the substitutions, 50 insertions and deletions were found in non-coding regions (Table 2).

The substitution in the RL13 gene caused early termination of the amino acid sequence. Specifically, the G-T substitution, at position 11,642 on the JHC p37 whole-genome nucleotide sequence, changed a glutamic acid (E) residue, encoded by GAA, to a TAA stop codon. Five other HCMV strains (AD169-UK, AD169-UC, TOWNE, TOLEDO, and 3157) also include early termination of the RL13 gene. The introduced stop codons in these strains are at different positions, so their ORF lengths are different (Table 3).

UL122, the other ORF with a substitution mutation, was stably preserved through an evolutionarily stable strategy. Comparing the UL122 gene in JHC p37 with those in other HCMV strains, a G to A substitution at nt position 171,040 resulted in a change from AGA (S, serine, a polar R group) to AAA (F, phenylalanine, a nonpolar aromatic R group) (Fig. 3). Characteristic changes in amino acids would be expected to affect the secondary structure of the UL122 protein. The UL122 ORF has four exons: 1, 2, 3, and 5. It is

Table 2. Number and types of mutations.

		ORF	NCR	Total
Substitutions	TS	1	19	20
	TV	1	29	30
In/Dels	In	0	8	8
	Del	0	42	42
All mutations:		2	98	100

In/Dels: Insertion or deletion.

Table 3. Human cytomegalovirus strains with early termination caused by mutations in the RL13 gene.

Strains without early termination		Strains with early termination	
HCMV strain	ORF length (bp)	HCMV strain	ORF length (bp)
3301	924	AD169_UK	444
AF1	912	AD169_UC	444
HAN20	909	TOWNE	129
JP	909	TOLEDO	630
U8	909	JHC_P37	633
HAN38	906		
VR1814	897		
U11	897		
HAN13	885		
MERLIN	882		
3157	883		
JHC	921		

located in a region that plays a role in the function of an IE2 transactivator and creates IE86 and IE56 proteins. The IE86 protein has 425 amino acids and becomes IE56 after deletion of 155 amino acids between aa 365 and aa 519, due to removal of exon 5 of IE86 [2]. The UL122 variation in JHC p37 was caused by a mutation affecting aa 376. This variation is expected to only affect the IE86 protein, which includes several different functional domain structures [2]. It is possible that this one variation, S → F at aa 376, could affect six functions of the IE86 protein (Fig. 4).

Discussion

HCMV is an important pathogen as it can lead to serious disease conditions in immune-compromised individuals. To better understand how HCMV might change and evolve after reactivation, we used a cell culture model that would allow us to examine changes after high passage. We examined the DNA sequence of the viral genome before and after it had reproduced in human foreskin fibroblast cells for at least 30 passages. Although the number of viruses themselves remained constant, the proportion that were infectious steadily increased. This suggests that while the viruses were reproducing at the same rate, they were becoming more infectious with each passage.

Next, we searched for the specific mutations that might be responsible for this effect. The PCR-based whole-genome sequence comparison between JHC p6 and p37

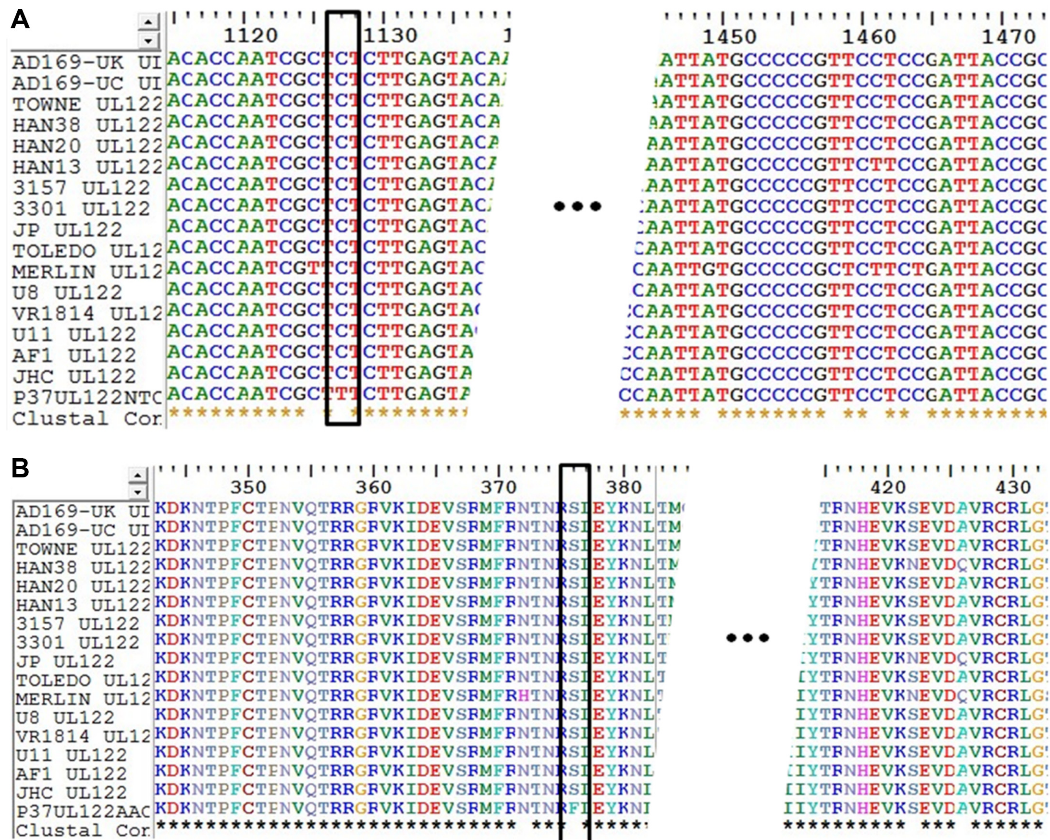

Fig. 3. Comparison of the UL122 gene between different human cytomegalovirus strains.

(**A**) Among all of the analyzed HCMV strains, the change from a cytosine to thymine at nucleotide position 1,127 of the UL122 gene, causing the codon to change from TCT (serine) to TTT (phenylalanine), was observed only in the JHC p37 strain. (**B**) Amino acid alignment, showing a change from serine to phenylalanine in the JHC p37 strain only.

revealed 100 mutations in the later passage strain. Of these 100, two were located in open reading frames, in locations that could result in changes in the infectiousness of the virus. The first was a G → T substitution that introduced a stop codon in the RL13 gene, causing early termination and truncation of the RL13 protein, which is involved in entry of the virus into a host cell. Five other HCMV strains have been found with early terminations in RL13: AD169-UK, AD169-UC, TOWNE, TOLEDO, and 3157. Some clues to the functional consequences of early termination of the RL13 gene were found in a previous study of the TOLEDO strain [22]. That study found that RL13 functions as a potent inhibitor of replication, and that truncation of the protein potentially enhances the efficiency of TOLEDO replication, dissemination, and pathogenesis during an acute infection. JHC may suffer from similar functional consequences of RL13 mutation. RL13 encodes a membrane glycoprotein, and mutations may affect its production. Although further investigation will be required to elucidate the specific effects of this mutation, it can be expected that truncation should affect functions related to replication and infection.

The second mutation was in the UL122 gene, which encodes the protein IE86, also known as IE2, expressed immediately after infection. The resulting amino acid substitution, S→F, in IE86 could be expected to have strong effects not only because of the nature of the change (small, polar amino acid to bulky, non-polar amino acid) but because of the large number of functions affected. UL122 is part of the major immediate-early gene, considered the important gene in HCMV, as it regulates subsequent viral gene expression, and its expression can cause a virus in its latent form (no symptoms) to change into its active form. IE72 and IE86 can act synergistically to activate HCMV early gene promoters, as well as heterologous promoters in a variety of cell types. IE72 can upregulate the major IE promoter, and IE86 also acts during the infection to negatively regulate its own promoter via direct binding to

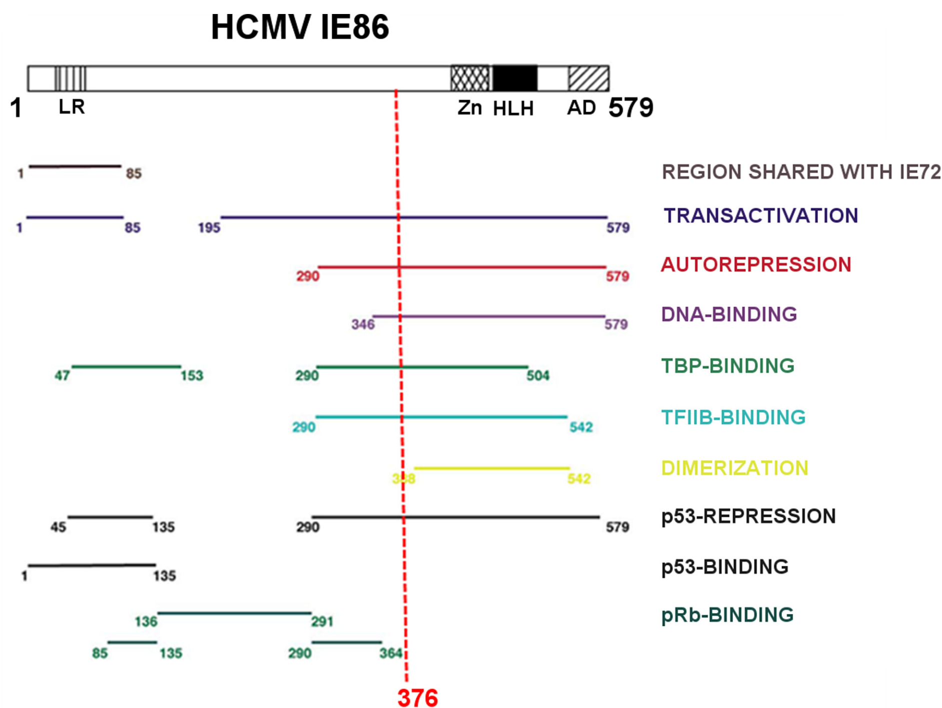

Fig. 4. Functional domain structure of IE86.

Functional domains of IE86, one of the UL122 proteins. Six domains include amino acid 376, so up to six functions may be influenced by variation in this amino acid.

a 14 bp palindromic IE86-binding site, the crs, located between the MIE promoter TATA box and the start of the transcription [11]. The up- and down-regulation functions of IE72 and IE86 are associated with domains in the N- (aa 1–81) and C-terminals (aa 195–579), respectively [16, 20]. IE86 can decrease the number of receptors on the cell surface through a crs located at aa 290–579. This region also functions in DNA-binding to the C-terminal in order to activate polymerase activity, and zinc finger domains. As aa 376 is within this region, mutations at this position are expected to affect IE86-influenced receptor expression on the cell surface [10, 16, 20, 23]. In addition, crs-containing regions are found in other domains, and appear to be involved in several other functions, including growth of virus-infected cells (aa 1–85, aa 195–579) [16, 18], inhibition of enzyme synthesis (aa 290–579), DNA-binding functions (aa 346–579), TATA-binding functions (aa 47–153 and aa 290–504), TF-B-binding functions (aa 290–542) [12, 18, 22, 23], and p53 repression (aa 45–135 and aa 290–579) [26].

Since the functions of RL13 and UL122 are related to infectivity of HCMV, and since we found that the proportion of infectious viruses increased in the high-passage viruses with these mutations, we consider that these mutations may be capable of causing increased infectivity in viruses after serial passage. The determination of gene changes correlated with increasing passage of viruses leads logically to associated analyses; for example, examination of both functional and presumed non-functional mutations (*i.e.*, all 100 mutations identified in the present study) using phylogenetic trees. Such analyses would contribute to the investigation of these changes and characteristics from the viewpoint of virus evolution.

In conclusion, to understand how the HCMV genome might evolve over several generations, we examined the DNA sequence of the viral genome before and after it had reproduced in human cells for at least 40 viral generations, known as passages. Whereas the number of viruses themselves remained constant, the proportion that were infectious steadily increased. This suggests that while the viruses were reproducing at the same rate, they were becoming more infectious with each passage. To find out what specific mutations might underlie this, we sequenced a virus that had reproduced 37 times and compared this with the full genome sequence of the same strain that had reproduced only 6 times. Two loci, RL13 and UL122, with functions related to infectiousness, were found that might be responsible for the observed increase in infectivity after serial passage.

References

1. Bradley AJ, Lurain NS, Ghazal P, Trivedi U, Cunningham C, Baluchova K, *et al*. 2009. High-throughput sequence analysis of variants of human cytomegalovirus strains Towne and AD169. *J. Gen. Virol.* **90:** 2375-2380.
2. Castillo JP, Kowalik TF. 2002. Human cytomegalovirus immediate early proteins and cell growth control. *Gene* **290:** 19-34.
3. Colberg-Poley AM. 1996. Functional roles of immediate early proteins encoded by the human cytomegalovirus UL36-38, UL115-119, TRS1/IRS1 and US3 loci. *Intervirology* **39:** 350-360.
4. Chee MS, Bankier AT, Beck S, Bohni R, Brown CM, Cerny R, *et al*. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154:** 125-169.
5. Cherrington JM, Khoury EL, Mocarski ES. 1991. Human cytomegalovirus ie2 negatively regulates alpha gene expression via a short target sequence near the transcription start site. *J. Virol*. **65:** 887-896.
6. Dargan DJ, Douglas E, Cunningham C, Jamieson F, Stanton RJ, Baluchova K, *et al*. 2010. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. *J. Gen. Virol.* **91:** 1535-1546.
7. Geelen JL, Walig C, Wertheim P, van der Noordaa J. 1978. Human cytomegalovirus DNA. I. Molecular weight and infectivity. *J. Virol.* **26:** 813-816.
8. Isomura H, Stinski MF, Kudoh A, Nakayama S, Murata T, Sato Y, *et al*. 2008. A *cis* element between the TATA box and the transcription start site of the major immediate-early promoter of human cytomegalovirus determines efficiency of viral replication. *J. Virol.* **82:** 849-858.
9. Jung GS, Kim YY, Kim JI, Ji GY, Jeon JS, Yoon HW, *et al*. 2011. Full genome sequencing and analysis of human cytomegalovirus strain JHC isolated from a Korean patient. *Virus Res.* **156:** 113-120.
10. Jupp R, Hoffmann S, Stenberg RM, Nelson JA, Ghazal P. 1993. Human cytomegalovirus IE86 protein interacts with promoter-bound TATA-binding protein via a specific region distinct from the auto repression domain. *J. Virol.* **67:** 7539-7546.
11. Kerry JA, Sehgal A, Barlow SW, Cavanaugh VJ, Fish K, Nelson JA, Stenberg RM. 1995. Isolation and characterization of a low-abundance splice variant from the human cytomegalovirus major immediate-early gene region. *J. Virol.* **69:** 3868-3872.
12. Lee GC, Lee DG, Choi SM, Yoo JH, Park SH, Choi JH, *et al*. 2005. Use of time-saving flow cytometry for rapid determination of resistance of human cytomegalovirus to ganciclovir. *J. Clin. Microbiol.* **43:** 5003-5008.
13. Li M, Ma Y, Ji Y, He R, Qi Y, Sun Z, *et al*. 2011. Human cytomegalovirus RL13 gene transcripts in a clinical strain. *Virus Genes* **43:** 327-334.
14. Liu B, Hermiston TW, Stinski MF. 1991. A cis-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. *J. Virol*. **65:** 897-903.
15. Ma YP, Ruan Q, He R, Qi Y, Sun ZR, Ji YH, *et al*. 2006. Sequence variability of the human cytomegalovirus UL141 open reading frame in clinical strains. *Arch. Virol.* **151:** 827-835.
16. Malone CL, Vesole DH, Stinski MF. 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. *J. Virol.* **64:** 1498-1506.
17. Marchini A, Liu H, Zhu H. 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. *J. Virol*. **75:** 1870-1878.
18. McDonough SH, Spector DH. 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. *Virology* **125:** 31-46.
19. Mocarski ES, Shenk T, Pass RF. 2007. Cytomegaloviruses, pp. 2701-2772. *In* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds.). *Fields Virology*, 5th Ed. Lippincott Williams & Wilkins, Philadelphia, PA.
20. Pizzorno MC, Mullen MA, Chang YN, Hayward GS. 1991. The functionally active IE2 immediate-early regulatory protein of human cytomegalovirus is an 80-kilodalton polypeptide that contains two distinct activator domains and a duplicated nuclear localization signal. *J. Virol.* **65:** 3839-3852.
21. Pizzorno MC, O'Hare P, Sha L, LaFemina RL, Hayward GS. 1988. trans-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. *J. Virol*. **62:** 1167-1179.
22. Stanton RJ, Baluchova K, Dargan DJ, Cunningham C, Sheehy O, Seirafian S, *et al*. 2010. Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to be a potent inhibitor of replication. *J. Clin. Invest.* **20:** 3191-3208.
23. Stenberg RM, Thomsen DR, Stinski MF. 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. *J. Virol.* **49:** 190-199.
24. Stenberg RM. 1996. The human cytomegalovirus major immediate-early gene. *Intervirology* **39:** 343-349.
25. Tanaka N, Kimura H, Iida K, Saito Y, Tsuge I, Yoshimi A, *et al*. 2000. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J. Med. Virol.* **60:** 455-462.
26. Tsai HL, Kou GH, Chen SC, Wu CW, Lin YS. 1996. Human cytomegalovirus immediate-early protein IE2 tethers a transcriptional repression domain to p53. *J. Biol. Chem.* **271:** 3534-3540.
27. Ziemann M, Unmack A, Hennig H. 2010. A novel set of real-time PCRs for rapid differentiation between human cytomegalovirus wild-type and highly passaged laboratory strains. *J. Virol.* **170:** 155.