AN INTRASTRAIN VARIATION OF A 189-BP REPEAT REGION IN THE HUMAN CYTOMEGALOVIRUS REPLICATION ORIGIN

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Summary. – A 189-bp repeat has been reported previously to be present in the replication origin (ORI) of Human cytomegalovirus (HCMV) Towne strain and to contain essential and supporting sequences (Chen *et al*., *Arch. Virol.* **141,** 13–301 (1996)). In this study, clones of HCMV strain Towne were isolated and analyzed for the copy number of the 189-bp repeat within its ORI (oriLyt). Southern blot analysis revealed that out of 14 clones, two contained a dimer, two contained a tetramer, and the remaining contained a trimer of the 189-bp repeat. Nucleotide sequence analysis disclosed that, within the dimer and the tetramer, the 189-bp repeat was present in a directly repeated manner. In this way we demonstrated an HCMV intrastrain variation in the copy number of the 189-bp repeat. Dot blot hybridization and growth curve analysis showed that the clones containing the dimer or the tetramer replicated in infected cells with similar efficiencies. We conclude that the HCMV oriLyt with spatial separation of the essential flanking regions by various copy number of the 189-bp repeat acts as ORI in the viral genome.

Key words: Human cytomegalovirus; DNA replication; replication origin

Introduction

HCMV (the species *Human herpesvirus 5*, the genus *Cytomegalovirus*, the subfamily *Betaherpesvirinae*) is a serious, life-threatening opportunistic pathogen in newborn and immuno-compromised individuals (Deayton *et al*., 2004; Lurain *et al*., 2004; Tamarit *et al*., 2004; Whitley, 2004). Researchers are seriously concerned about the reactivation of HCMV from a latent state in immuno-suppressed or immuno-compromised individuals. HCMV possesses a large DNA genomes of about 230 kbp with more than 200 ORF (Britt and Alford, 1996). Viral DNA synthesis is a critical regulatory event in the branch point between lytic and latent pathways. In the lytic infection, replication of viral genome starts at oriLyt, which has been identified by a ganciclovir-induced chain termination method (Hamzeh *et al*., 1990) and transient transfection assay (Anders *et al*., 1992; Anders and Punturieri, 1991; Masse *et al*., 1992; Pari and Anders, 1993; Watanabe and Yamaguchi, 1993). The oriLyt is localized around the 0.4 map unit of the genome. We have previously determined the nucleotide sequence of the oriLyt of HCMV Towne strain and demonstrated the differences between the oriLyts of strains Towne and AD169 (Chen *et al*., 1996). The oriLyt of Towne strain contained a trimer of the 189-bp repeat, whereas, that of AD169 strain only a monomer. Although we have confirmed the presence of the trimer in the Towne strain as a major band in Southern blot analysis of viral DNA, we could not rule out a possibility of the presence of the monomer or other multimers as minor bands.

We have also shown previously that the 189-bp repeat contained essential sequences at either or both the 5'- and 3'-ends and a supporting inner sequence (Chen *et al*., 1996, 1999). The inner sequence could be deleted or inverted but

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Abbreviations: HCMV = Human cytomegalovirus; MOI = multiplicity of infection; ORI = replication origin; oriLyt = replication origin of lytic phase; p.i. = post infection

not replaced with other sequence without loss of replication ability. Meanwhile, we have generated oriLyt-derivative-containing plasmids with the monomer or dimer of the 189-bp repeat and examined their replication ability with a result that these plasmids had similar replication efficiencies. Since in this study we employed a modified transient transfection assay of DNA replication in virus-infected cells, we could not address a question whether or not the variation of the 189-bp repeat in the HCMV genome affects the efficiency of virus replication in infected cells.

In this study, we attempted to isolate clones of HCMV Towne strain with different copy number of the 189-bp repeat and to examine their replication efficiency in infected cells.

Materials and Methods

Cells and viruses. Human embryonic lung (HEL) cells were cultured as previously described (Chen *et al*., 1996). Clones of HCMV Towne strain were isolated by limiting dilution in HEL cell cultures in 96-well microplates. Culture medium from the well containing a single focus of CPE was inoculated into a HEL cell culture in a 6-cm plate. Titers ($TCID_{50}$/ml) of isolated clones were determined in HEL cell cultures on the basis of CPE.

Southern blot analysis. Total DNA was extracted from cell cultures in 6-cm plates infected with the clones using a lysis buffer (0.6% SDS, 50 mol/l EDTA, and 50 mmol/l Tris-HCl pH 8.1), treated with RNase A and proteinase K, and purified by phenol extraction and ethanol precipitation (Chen *et al.*, 1996). The purified DNA was digested with *Rsr*II, electrophoresed in a 1.2% agarose gel, blotted onto a nylon membrane. The latter was hybridized with a probe generated by random priming of a 152-bp *Bam*HI-*Bam*HI fragment of the 189-bp repeat in oriLyt. After threefold washing with 0.2 x SSC and 0.1% SDS at 65°C, the blot was exposed to an Image Plate and analyzed in a Bio Image Analyzer BAS 2000 (BAS 2000, Fuji Film, Japan).

DNA sequencing of viral clones. Viral DNA was extracted from virions collected from culture medium by ultracentrifugation, purified with proteinase K as described above, and subjected to sequencing in a 377 DNA Sequencer (Applied Biosystems, USA). To verify the obtained sequence the *Xho*I-*Xho*I fragment containing the 189-bp repeat was cloned into vector pME18S and subjected to sequencing.

Dot blot hybridization. Total DNA was extracted every 24 hrs post infection (p.i.) from HEL cell cultures in 3.5-cm plates infected with the clones at a multiplicity of infection (MOI) of 0.1 using the lysis buffer and purified as described above. An aliquot of the purified DNA was treated with 0.5 N NaOH in 1.5 mol/l NaCl and blotted onto a nylon membrane. The blot was hybridized with a probe generated from the *Xho*I-*Xho*I fragment described above by random priming. After the washing described above, the blot was analyzed in a BAS 2000 Bio Image Analyzer.

Growth curves of viral clones. HEL cell cultures in 25-cm^2 flask were infected at a MOI of 0.1. After virus adsorption for 1 hr, the inoculum was replaced with fresh culture medium. Supernatants from two separate cultures for each clone were sampled daily p.i. and stored at -80°C. Infectious virus was plaque-titrated on HEL cells.

Results

Intrastrain variation of HCMV in the replication origin

To investigate the presence of intrastrain variation in the copy number of the 189-bp repeat within the HCMV oriLyt we isolated 14 viral clones, extracted total DNA from cells infected with them, and subjected these DNAs to Southern blot analysis using a 152-bp *Rsr*II-*Rsr*II fragment from the 189-bp repeat (Fig. 1). A previous study (Chen *et al*., 1996) has shown that the oriLyt of HCMV Towne strain contains an 189-bp trimer in a 1112-bp *Rsr*II-*Rsr*II fragment. In this

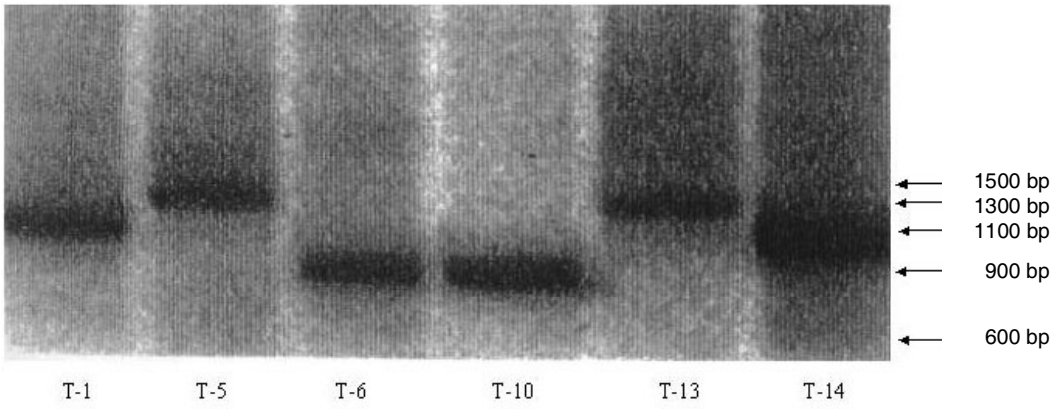

Fig. 1

Southern blot analysis of the clones

The clones T-1, T-5, T-6, T-10, T-13, and T-14 were analyzed.

study, out of 14 clones, the majority contained a trimer (a 1.1 kbp fragment), two (T-5 and T-13) contained a tetramer (a 1.3 kbp fragment), and two (T-6 and T-10) contained a dimer (a 0.9 kbp fragment). These results suggest the presence of an intrastrain variation in the copy number of the 189-bp repeat.

To confirm the presence of the intrastrain variation in the copy number of the 189-bp repeat, we determined the nucleotide sequence of an *Xho*I-*Xho*I fragment within the oriLyt of two clones, T-5 and T-6, which contained the tetramer and the dimer of the 189-bp repeat, respectively (Fig. 2). The sequencing confirmed that the clones T-5 and T-6 actually contained the tetramer and the dimer of the 189-bp repeat in a direct manner. The nucleotide sequences of the 189-bp repeat within the dimer and the tetramer was identical to that previously reported for the trimer (Chen *et al*., 1996).

Replication ability of the clones

To test the relationship between the copy number of the 189-bp repeat in the HCMV oriLyt and the replication efficiency we first determined viral DNA in HEL cells infected with the clones T-5 and T-6 at a MOI of 0.1 by dot blot hybridization on days 1–5 p.i.(Fig. 3). During this period, the amount of viral DNA in both clones was similar and increased in similar manner. As T-6 and T-5 clones contained a dimer and a tetramer of the 189-bp repeat, respectively, the copy number of the repeat does not seem to play any role in replication ability of the virus.

Next, we analyzed growth curves of these clones in HEL cells after infection at an MOI of 0.1. Virus titers of the supernatants of cell cultures were determined by plaque assay. The results showed (Fig. 4) that the two clones exhibited similar growth kinetics and confirmed the conclusion drawn from the dot blot hybridization experiments.

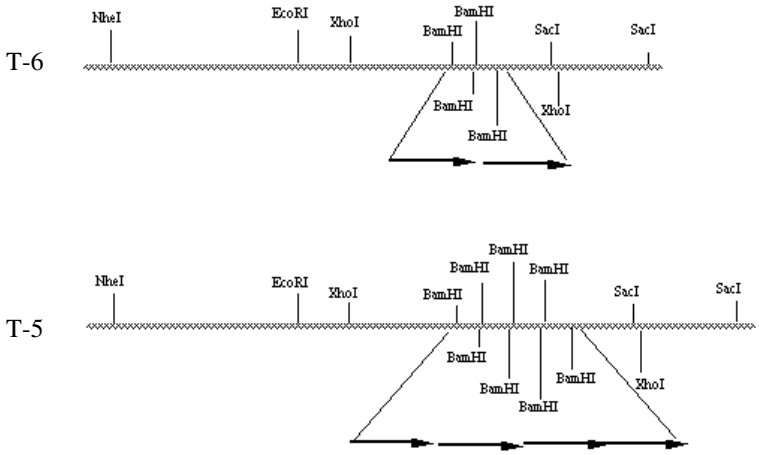

Fig. 2

Schematic representations of the oriLyt of the clones T-5 and T-6

The 189-bp repeats are marked with arrows.

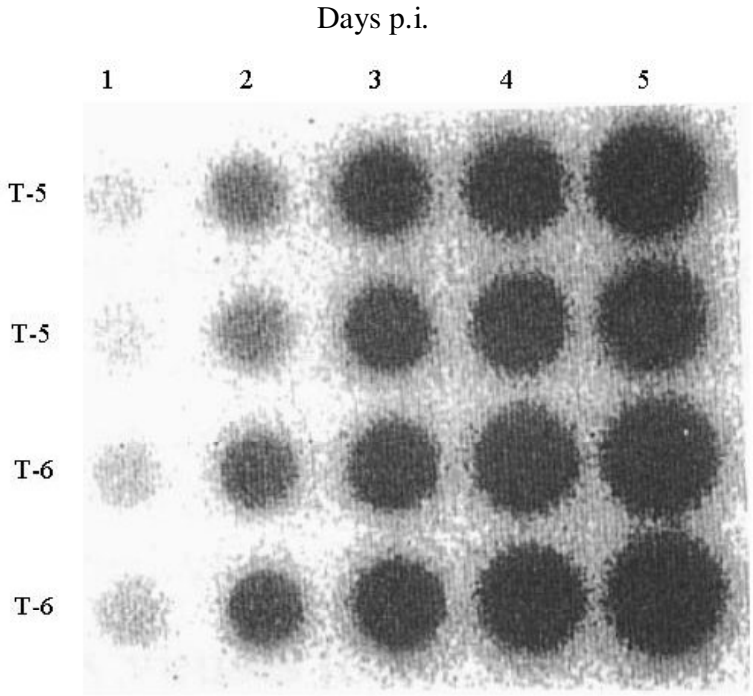

Fig. 3

Dot blot hybridization analysis of the clones T-5 and T-6 for viral DNA

Viral DNA was assayed on days 1–5 p.i.

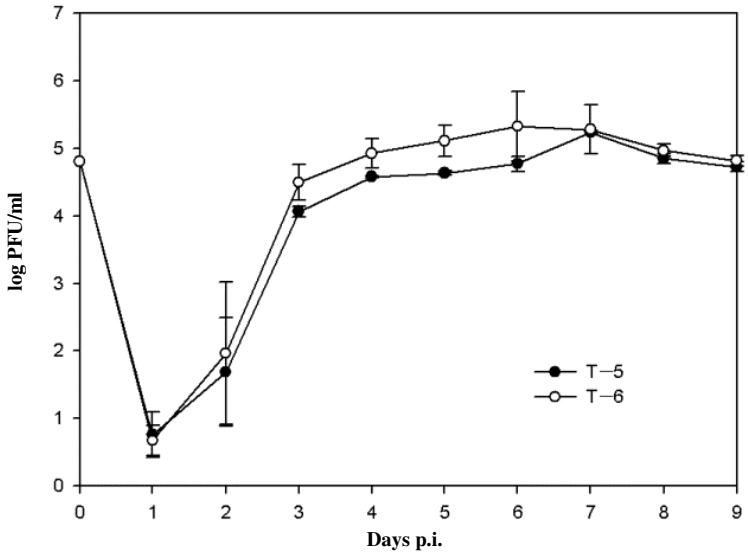

Fig. 4

Growth curves of the clones T-5 and T-6

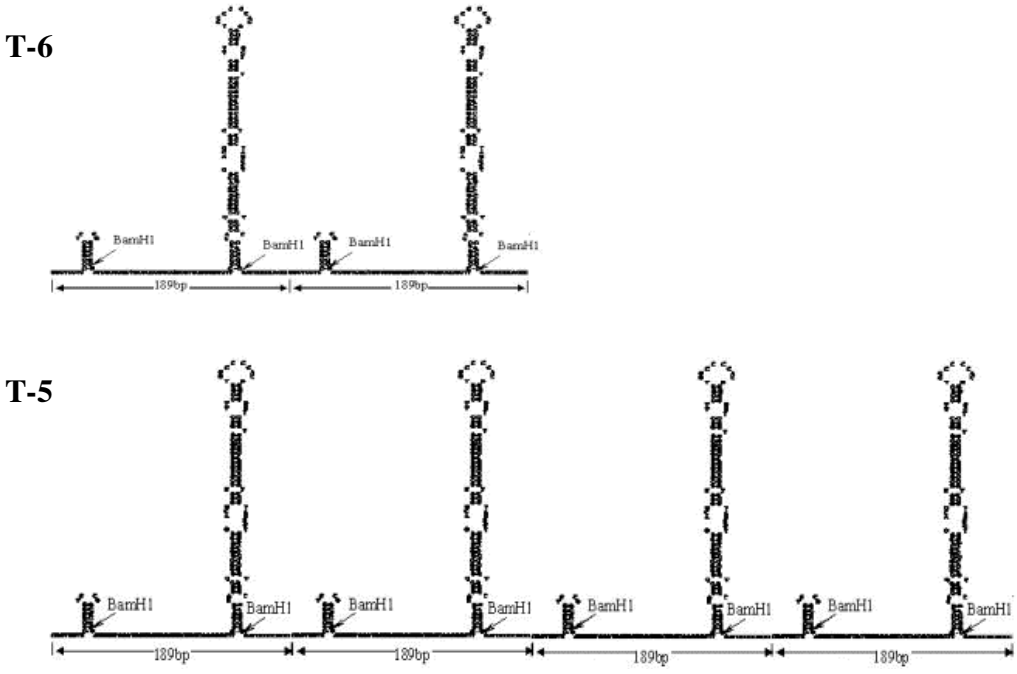

Fig. 5

Putative imperfect palindromes present within the 189-bp repeat region of the oriLyt of the clones T-5 and T-6

The 189-bp repeats contain a small (18-bp) and a large (117-bp) palindrome. The *Bam*HI sites are marked by arrows.

Discussion

In this study, we demonstrated an intrastrain variation in the copy number of the 189-bp region of the ORI (oriLyt) of HCMV genome and that it does not affect the virus replication efficiency. Previously, we have obtained consistent findings with plasmids containing the HCMV oriLyt with various mutations in the 189-bp repeat (Chen *et al*., 1999). We proved that the mutant plasmids containing one or two copies of the 189-bp repeat had a replication efficiency similar to that of the wild-type oriLyt containing three copies of the 189-bp repeat. The study also suggested a minimum oriLyt of HCMV Towne strain consisting of three regions, the 189-bp repeat region and two flanking essential regions. The data presented in this report demonstrated that the clones containing two or even four copies of the 189-bp repeat replicated with similar efficiency. It seems that the HCMV oriLyt is able to function only provided the two essential regions are closely located or spatially separated by the 189-bp repeat, irrespective of the copy number of the latter. Furthermore, the higher structure of the 189-bp repeat might play a role in the HCMV replication. The 189-bp repeat could form two palindromes: a small one of 18 bp and a large one of 117 bp (Fig. 5). The palindrome structure can explain the above results to certain extent. It may function as a bridge linking the two essential regions, which shortens the distance between them due to its palindromic character. This distance increased proportionally with increasing copy number of the 189-bp repeat, however, the oriLyt could still function properly.

Initiation of replication of viral DNA involves the assembly of multienzyme complexes, replisomes, at unique locations. Direct or inverted repeats and palindromic structures have been found in the ORI of many DNA viruses and are believed to play important roles in viral DNA synthesis (Aslani *et al*., 2001; Cheung, 2004; Fruscalzo *et al*., 2001; Wang *et al*., 2004; Willwand *et al*., 1998). Herpes simplex virus 1 genome contains three ORIs: one oriL containing a 144-nts palindrome, and two oriS containing a 46-nts palindrome. The oriS palindrome is required for complex formation and efficient assembly of the viral replication machinery (Aslani *et al*., 2000, 2001). The oriLyt of Epstein-Barr virus consists of two essential and several auxiliary elements that are required for full activity. The two essential elements, the upstream and the downstream components constitute a minimum oriLyt (Baumann *et al*., 1999; Portes-Sentis *et al*., 1997). The upstream component plays a dual role in DNA transcription and replication. The downstream one contains a homopurine-homopyrimidine sequence that forms an H palindrome. All mutations that altered it impaired the oriLyt-dependent replication (Portes-Sentis *et al*., 1997). Interestingly, compensatory mutations that restored the H palindrome also restored th oriLyt-dependent replication (Baumann *et al*., 1999; Portes-Sentis *et al*., 1997). Subregions of ORI similar to the 189-bp repeat have not been identified yet in other herpesviruses. It appears worth of elucidating molecular mechanisms specific to HCMV replication. The 117-bp palindromic structure within the 152-bp inner sequence (the *Bam*HI-*Bam*HI fragment) is not likely the site of the assembly of replisomes, since the 152-bp *Bam*HI-*Bam*HI fragment of the 189-bp repeat could be deleted or inverted but not be replaced with other sequence without destroying the replication ability (Chen *et al*., 1996,1999). The 117-bp palindromic structure is likely the linking bridge between the two essential regions of the HCMV oriLyt, which may function even after its inversion. In addition, a mutant oriLyt completely lacking the 189-bp repeat could not function properly; this demonstrated that the 189-bp repeat contained an essential sequence at either or both the 5'- and 3'-ends. The role of the small 18-bp palindrome located at the 5'-end of the 189-bp repeat in the HCMV oriLyt is still not clear.

The HCMV oriLyt consists of more complex structure than other DNA viruses (Deb and Doelberg, 1988; Hardwicke and Schaffer, 1995; Knopf *et al*., 1986; Lockshon and Galloway, 1986, 1988; Stow and Davison, 1986; Stow and McMonagle, 1983; Stow *et al*., 1990; Weller *et al*., 1985). Some authors have suggested that the HCMV oriLyt consists of multiple components (Anders *et al*., 1992; Anders and Punturieri, 1991). Based on results from a transient replication assay, namely the sequence of oriLyt, Masse *et al*. (1992) have claimed that the minimum HCMV oriLyt consists of two domains. In this report, we showed that the HCMV oriLyt with spatial separation of the two essential regions by various copy numbers of the 189-bp repeat could act as ORI, exhibiting similar virus replication efficiencies. These results could also lead us to divide the oriLyt into three parts: two essential flanking regions and the 189-bp repeat region. We believe that the higher structure of the 189-bp repeat region might play a role in HCMV replication.

In conclusion, these findings may contribute to the understanding of functional relationships among individual regions of the HCMV oriLyt and the elucidation of initiation of viral DNA synthesis.

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