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Short communication

Conservation of HHV-6 DNA polymerase processivity factor sequence and predicted structure suggests it as a target for antiviral development

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ABSTRACT

The replication of human herpesvirus-6 (HHV-6) DNA is catalyzed by the viral DNA polymerase pU38 and the processivity factor pU27 which stabilizes the enzyme on the DNA template. The genetic polymorphism of pU27 among 46 clinical strains of HHV-6 variant A or B and four strains resistant to antivirals was investigated. Overall, 28 amino acid changes (7.6%) and a two-amino acid deletion were identified among the 368 residues of pU27, when using the U1102 (variant A) sequence as the reference. Eleven amino acid changes (3.0%) specifically differentiated both variants. The median intravariant amino acid variability was 1.2% and 0.3% for A and B, respectively. Except for a single change, the pU27 sequence of multi-drug resistant HHV-6 strains was also conserved. Structural models of pU27 for variants A and B were derived from that of the human cytomegalovirus homologue pUL44, and showed either identical or very similar residues in the regions interacting with viral DNA polymerase and viral DNA molecule. As pU27 is both highly conserved and essential for viral replication, it might constitute an interesting target for antiviral chemotherapy.

Human herpesvirus-6 (HHV-6), which exists as two variants A and B (HHV-6A and HHV-6B), is a betaherpesvirus closely related to human cytomegalovirus (HCMV). It is lympho- and neurotropic and has been recognized as a major pathogen, especially in immunocompromised patients (De Bolle et al., 2005). Moreover, HHV-6 has been implicated in various central nervous system diseases (Komaroff et al., 2006). No consensual specific therapeutic intervention has been established for the treatment of HHV-6 infections, but drugs also used against HCMV, i.e. ganciclovir (GCV), cidofovir (CDV) and foscarnet (PFA), have proven their efficiency in various HHV-6 related disorders (Dewhurst, 2004; Ljungman and Singh, 2006). All these drugs target the viral DNA polymerase, consequently inhibiting viral replication. As for all herpesviruses, the DNA polymerase complex of HHV-6 consists of two subunits, the DNA polymerase which is the catalytic subunit encoded by U38 gene and a processivity factor, the product of the U27 gene (Agulnick et al., 1993; Chang and Balachandran, 1991). Initially named p41, this 41-kDa processivity factor contains 368 residues in the U1102 strain of HHV-6 variant A. It binds to both DNA template and DNA polymerase, allowing synthesis of extended stretches of DNA without dissociation of the catalytic subunit from the template (Lin and Ricciardi, 1998). The binding between pU27 and pU38 is specific of the viral species, and has been demonstrated both in vitro, by protein coimmunoprecipitation assays, and in infected cells (Lin and Ricciardi, 1998). It has also been shown, by means of mutational analysis, that both the 77 N-terminal and the 235 C-terminal residues of pU38 contribute to its binding to pU27 and DNA synthesis. In the case of pU27, the 130 N-terminal amino acids were required for binding to pU38 and then increasing DNA synthesis, of which the first 30 residues were apparently essential for increasing DNA synthesis, whereas the last 31 C-terminal residues were totally dispensable. Similar interactions between the processivity factors and DNA polymerases from herpes simplex virus (HSV) or HCMV have been demonstrated and shown to be essential, since disruption of the protein-protein interaction inhibits virus replication (Loregian and Palù, 2005). Furthermore, crystallization of the processivity factors of these two herpesviruses, pUL42 and pUL44, respectively, has shown that they share the same structure with a connector loop binding to the DNA polymerase by hydrophobic interactions or hydrogen bonds (Appleton et al., 2004; Zuccola

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Table 1
Primers used to amplify and sequence the HHV-6 U27 gene.

Primer	Sequence 5′→3′ (orientation)	Location[a]	Use
U27A	AAG GCG CGA GTT GGT GCT AGG (+)	39897-39877	PCR, sequencing
U27Abis	AAA GCA CAT AAG GCG CGA GTT GGT (+)	39906-39883	PCR, sequencing
U27A1	CGA ATC CTG AGG TTA CGA AG (+)	39530-39511	Sequencing
U27A2	CTG CCA AAA ATT TAC AGC AGG C (+)	39161-39140	Sequencing
U27B	GGA ATA ACG GCA CCC TTA GCA AG (−)	38737-38715	PCR, sequencing
U27Bbis	GAA TAA CGG CAC CCT TAG CAA GAC C (−)	38740-38716	PCR, sequencing
U27B1	CCG TCA ACA ACG TGC TTT TGG AAG (−)	39062-39039	Sequencing
U27B2	ACC GAC GCC TGT GTA CAT GTA TC (−)	39456-39434	Sequencing

[a] Refers to the nucleotide sequence of the HST strain (accession number AB021506) (Isegawa et al., 1999).

et al., 2000). As far as HHV-6 pU27 is concerned, little is known about its interstrain variability, its interactions with pU38 and viral DNA, and its putative role in the resistance of HHV-6 to antivirals. In this study, the U27 gene of 46 strains of HHV-6A or HHV-6B was sequenced to determine its natural polymorphism. Four mutant strains of HHV-6B selected in vitro and resistant to GCV, PFA and/or CDV were also examined. Finally, using the available crystal structure of pUL44 from HCMV, a structural model of pU27 was built in order to better understand the interactions of the HHV-6 processivity factor with the DNA polymerase and the DNA molecule.

1. Intervariant and intravariant polymorphism of pU27

In this study, 46 HHV-6 strains (16 HHV-6A and 30 variants HHV-6B) were examined. The DNA from strains previously isolated and propagated in cell culture, i.e. U1102, GS, SIE, TAN, HST, BLA, BLE, BOU, TRA, BOB, MAR, MBE strains (Manichanh et al., 2001), and the DNA from HHV-6 positive clinical blood samples from distinct patients (12 HHV-6A and 21 HHV-6B) were extracted using either the rapid lysis method as previously described (Bonnafous et al., 2007) or the QIAamp DNA Blood kit (Quiagen, Courtaboeuf, France). The full-length U27 gene was then amplified by a one-step PCR for isolated strains and clinical samples with a viral load higher than 2.25×10^5 copies of viral DNA per mL of blood using the TaKaRA LA Taq™ kit (Lonza, Verviers, Belgium) and the U27A and U27B primers (Table 1). The thermal cycling reaction consisted of 94 °C for 5 min, followed by 38 cycles at 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min 50 s and finally 72 °C for 10 min. A nested PCR was performed for the other extracts with a lower DNA load, using the U27Abis and U27B primers for the first step, and the U27A and U27Bbis primers for the second step, under the same PCR conditions, except that the annealing temperature was 56.8 °C. PCR products were sequenced with the BigDye v3.1 Cycle Sequencing kit (Applied Biosystems, Courtaboeuf, France) using the primers specified in Table 1. The nucleotide and derived amino acid sequences were analyzed with Seqscape 2.5 software and then aligned using Genedoc software. The U27 sequence of strain Z29 (HHV-6B) was also included in this alignment (GenBank accession number AF157706). For both HHV-6A and HHV-6B, the obtained sequences were compared with each other and an amino acid consensus sequence was established (Fig. 1). Among all strains sequenced, 74 point mutations on 1107 nucleotides (6.7%), leading to 28 changes on 368 amino acids (7.6%) were identified. In addition, a deletion of six nucleotides at the C-terminal end (1071–1076) causing a two-amino acid deletion (N358-P359) was found in eight sequences out of 16 HHV-6A strains. The same deletion was also

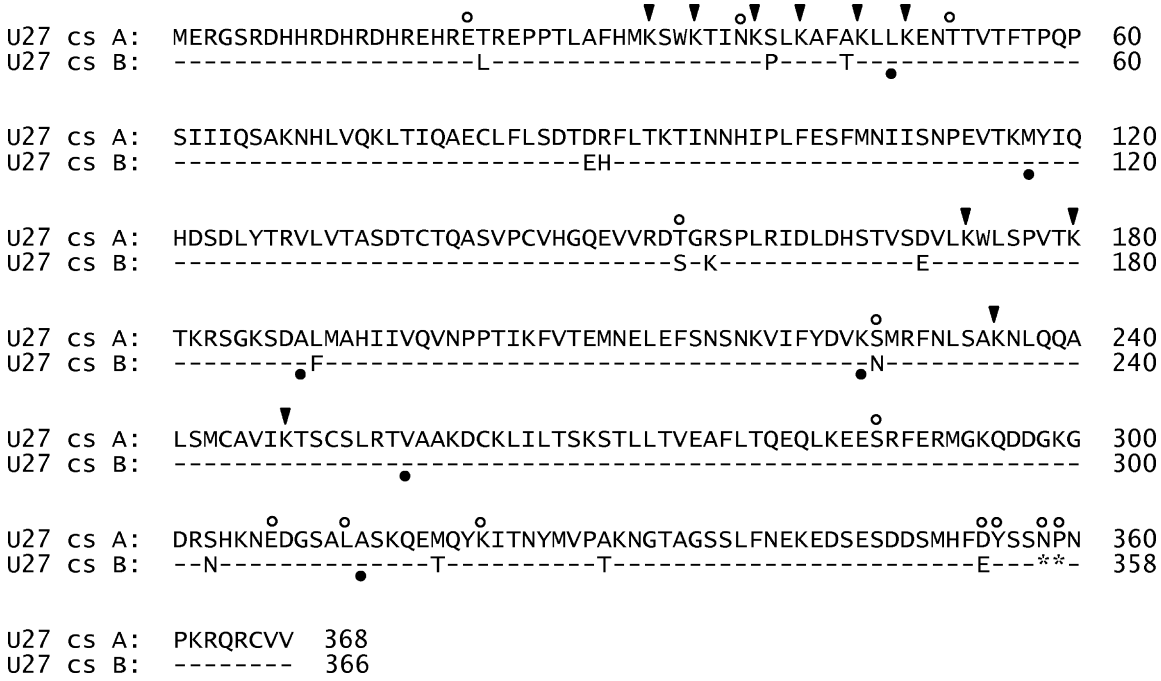

Fig. 1. Consensus protein sequence of HHV-6A and HHV-6B. Nucleotide sequencing was performed on DNA amplified from 46 HHV-6 strains. The consensus HHV-6A U27 sequence (U27 cs A) was established from the sequences of U1102, SIE, TAN, GS strains and 12 HHV-6A strains from patient blood samples. The consensus HHV-6B U27 sequence (U27 cs B) was established from the sequences of HST, BLA, BLE, BOU, TRA, BOB, MAR, MBE, Z29 (GenBank accession number AF157706) strains and 21 HHV-6B strains from patient blood samples. HHV-6B amino acids identical to HHV-6A are represented by a dash; the asterisk indicates the absence of an amino acid residue. The open and closed circles indicate an intravariant A and intravariant B variability, respectively. The black triangles indicate the lysine residues located in the four helices of the structural model.

present in all HHV-6B strains. Of all mutations, 37 nucleotide positions (3.3%) and 11 amino acids (3.0%) were variant-specific and allowed discrimination between the two variants of HHV-6. Of note, the present study confirmed the divergence at position 328, between variants A (serine) and B (asparagine), that previously permitted to define a monoclonal antibody specific for HHV-6A (Xu et al., 2001). The median intravariant HHV-6A nucleotide variability was 1.1%, corresponding to a median amino acid variability of 1.2%. The intravariant HHV-6B variability was lower, with a median value of 0.2% in nucleotides and 0.3% in amino acids. This low variability was within the same range as the divergence of homologous UL44 gene among HCMV strains, with an interstrain variability ranging from 0.5% to 1.5% at the nucleotide level and being less than 0.5% for amino acid residues (Boutolleau et al., 2009). These genes are part of the most conserved genes in human herpesviruses, as expected given their essential role in replication.

In addition, four HST-derived strains, selected in vitro under increasing concentrations of GCV, CDV and/or PFA, were also investigated (Bonnafous et al., 2007, 2008). U27 gene sequences were identical to that of HST, except for one strain (GPFAR1 resistant to GCV, CDV and PFA) that exhibited an a976g mutation leading to an M326V change. It is assumed that this change either would not impact DNA synthesis or, at best, provide a benefit in terms of fitness. Indeed, the growth of this resistant strain was found similar to that of wild-type HST despite the observed changes in the viral DNA polymerase and phosphotransferase which are believed to explain resistance pattern (Bonnafous et al., 2007, 2008). It is unlikely that the M326V change would play a role in the resistance to GCV, CDV and PFA since these drugs all target the catalytic site of DNA polymerase and are not expected to interfere with the processivity factor.

2. Three-dimensional structural model of the HHV-6 processivity factor

In order to identify the regions of pU27 important for binding to pU38 and viral DNA, models of the three-dimensional structure of HHV-6A and HHV-6B pU27 were generated with the homology-modelling server SWISS-MODEL (Arnold et al., 2006; Guex and Peitsch, 1997; Kopp and Schwede, 2004), starting from consensus amino acid sequences and using the crystallographic protein structure of processivity factor pUL44 of HCMV as the template [Protein Data Bank (PDB) accession number 1yyp] (Appleton et al., 2006). The two structures obtained were energy-minimized in 100 steps using the program CNS and visualized using PyMOL (Brünger et al., 1998; DeLano, 2002). As the entire pUL44 protein has not been crystallized, the structure of pU27 was derived from residue 23 to residue 281 (Fig. 2). Furthermore, the structure of a short part of pUL44 (residues 163–174) has not been determined and the corresponding part of pU27 (residues 182–192) consequently formed a randomized loop. The pU27 models of both variants were extremely similar. It was also shown using PyMOL software that the very modest intravariant changes did not induce any dramatic local conformational change that could modify the interactions with DNA or DNA polymerase. As a whole, the processivity factor of both HHV-6A and HHV-6B shares the same structure as HCMV pUL44 and HSV pUL42, i.e. two topologically similar domains linked by a connector loop and four alpha helices located at the back side (Appleton et al., 2004; Zuccola et al., 2000). The sequence of the connector loop of pU27 was compared to that of pUL44 (Fig. 3). Most amino acids were either identical or similar between both betaherpesvirus species, suggesting that the connector loop of pU27 interacts with the polymerase pU38 in the same manner as pUL44 with pUL54. In contrast, the sequence of the connector loop of HSV pUL42 was very different (data not shown). In addition, several lysine residues, which were found in the four helices of pU27 at

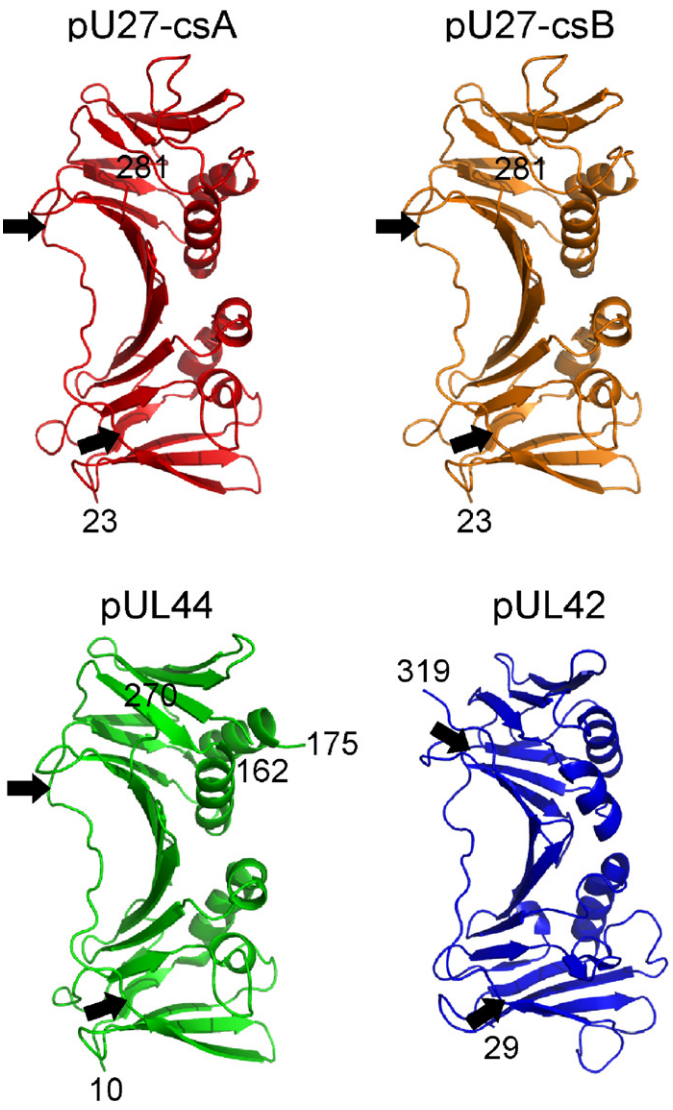

Fig. 2. Structural models of consensus HHV-6A and HHV-6B pU27 in comparison with the structures of HCMV pUL44 and HSV pUL42. Ribbon diagram representations of HHV-6A pU27 (pU27-csA), HHV-6B pU27 (pU27-csB), HCMV pUL44 template [Protein Data Bank (PDB) accession number 1yyp (Appleton et al., 2006)] and HSV pUL42 [Protein Data Bank (PDB) accession number 1dml (Zuccola et al., 2000)] are shown in red, orange, green, and blue, respectively. The models of pU27-csA and pU27-csB were generated from the consensus amino acid sequences of HHV-6A and HHV-6B, respectively, from residue 23 to residue 281, using the crystallographic protein structure of pUL44 as a template. The arrows indicate both ends of connector loop (residues 144–155, 144–155, 129–140 and 160–175 for pU27-csA, pU27-csB, pUL44 and pUL42, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the positions 32, 35, 39, 42, 46, 49, 173, 180, 235 and 248, were conserved among HHV-6A and HHV-6B strains (Fig. 1). These basic residues, as for other processivity factors, would interact with the negatively charged backbone of DNA. Interestingly, the pU27 pro-

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U27 HHV-6 cs A   :   144   CVHGQEVVRDTG   155
U27 HHV-6 cs B   :   144   ----------S-   155
UL44 HCMV        :   129   ----[-DI-]-ESE  140
                                 **  *
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Fig. 3. Connector loop sequences of HHV-6A pU27, HHV-6B pU27, and HCMV pUL44. The consensus HHV-6A and HHV-6B pU27 sequences were compared with HCMV pUL44 sequence (AD169 strain, GenBank accession number BK000394). The open circle indicates a position of intravariant A variability. The asterisks indicate the similarity of amino acids. The residues crucial for the binding to pUL44 DNA polymerase are framed.

tein deleted of the 30 N-terminal and 31 C-terminal residues was found to bind to pU38 but failed to increase DNA synthesis (Lin and Ricciardi, 1998). It is likely that this deletion in the N-terminus disorganizes the structure of nearby helices containing lysine residues, and prevents the binding to DNA. However, the active form of pU27 protein is yet unknown. Indeed, it might be a monomer like HSV pUL42, or, in contrast, a dimer forming a C clamp-shape where DNA could bind, as in HCMV pUL44 (Appleton et al., 2004; Zuccola et al., 2000). Only crystallization and analysis of the structure of pU27 in complex with DNA molecule may provide such information.

Because its sequence appears very constant and the proposed structural model suggests similar interactions to the processivity factors of other herpesviruses, pU27 could constitute an interesting target for new antiviral drugs. Indeed, in the case of HSV and HCMV, peptidic compounds mimicking the C-terminal part of the DNA polymerase, as well as diverse small molecules, were screened for their capability to interfere with the binding of processivity factor with DNA polymerase, and some were efficient in inhibiting viral replication in infected cells (Loregian and Palù, 2005). Furthermore, some of them had an antiviral activity 500-fold higher than their cytotoxicity, which reflected a high selectivity index (Loregian and Coen, 2006). In the same manner, although the precise identification of residues involved in pU27–pU38 interaction is still needed, new drugs aiming at the disruption of this interaction could be developed.

References

Agulnick, A.D., Thompson, J.R., Iyengar, S., Pearson, G., Ablashi, D., Ricciardi, R.P., 1993. Identification of a DNA-binding protein of human herpesvirus 6, a putative DNA polymerase stimulatory factor. J. Gen. Virol. 74, 1003–1009.

Appleton, B.A., Brooks, J., Loregian, A., Filman, D.J., Coen, D.M., Hogle, J.M., 2006. Crystal structure of the cytomegalovirus DNA polymerase subunit UL44 in complex with the C terminus from the catalytic subunit. Differences in structure and function relative to unliganded UL44. J. Biol. Chem. 281, 5224–5232.

Appleton, B.A., Loregian, A., Filman, D.J., Coen, D.M., Hogle, J.M., 2004. The cytomegalovirus DNA polymerase subunit UL44 forms a C clamp-shaped dimer. Mol. Cell 15, 233–244.

Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201.

Bonnafous, P., Boutolleau, D., Naesens, L., Deback, C., Gautheret-Dejean, A., Agut, H., 2008. Characterization of a cidofovir-resistant HHV-6 mutant obtained by in vitro selection. Antiviral Res. 77, 237–240.

Bonnafous, P., Naesens, L., Petrella, S., Gautheret-Dejean, A., Boutolleau, D., Sougakoff, W., Agut, H., 2007. Different mutations in the HHV-6 DNA polymerase gene accounting for resistance to foscarnet. Antivir. Ther. 12, 877–888.

Boutolleau, D., Deback, C., Bressollette-Bodin, C., Conan, F., Ait-Arkoub, Z., Imbert-Marcille, B.M., Agut, H., 2009. Genetic analysis and putative role in resistance to antivirals of the human cytomegalovirus DNA polymerase UL44 processivity factor. Antivir. Ther. 14, 847–852.

Brünger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T., Warren, G.L., 1998. Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. 54, 905–921.

Chang, C.K., Balachandran, N., 1991. Identification, characterization, and sequence analysis of a cDNA encoding a phosphoprotein of human herpesvirus 6. J. Virol. 65, 7085.

De Bolle, L., Naesens, L., De Clercq, E., 2005. Update on human herpesvirus 6 biology, clinical features, and therapy. Clin. Microbiol. Rev. 18, 217–245.

DeLano, W.L., 2002. The PyMOL Molecular Graphics System. DeLano Scientific, Palo Alto, CA, USA. Available from http://www.pymol.org.

Dewhurst, S., 2004. Human herpesvirus type 6 and human herpesvirus type 7 infections of the central nervous system. Herpes 11 (Suppl. 2), 105A–111A.

Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714–2723.

Isegawa, Y., Mukai, T., Nakano, K., Kagawa, M., Chen, J., Mori, Y., Sunagawa, T., Kawanishi, K., Sashihara, J., Hata, A., Zou, P., Kosuge, H., Yamanishi, K., 1999. Comparison of the complete DNA sequences of human herpesvirus 6 variants A and B. J. Virol. 73, 8053–8063.

Komaroff, A.L., Jacobson, S., Ablashi, D.V., Yamanishi, K., 2006. Highlights from 5th International Conference on HHV-6 and -7. Herpes 13, 81–82.

Kopp, J., Schwede, T., 2004. The SWISS-MODEL Repository of annotated three-dimensional protein structure homology models. Nucleic Acids Res. 32, D230–D234.

Lin, K., Ricciardi, R.P., 1998. The 41-kDa protein of human herpesvirus 6 specifically binds to viral DNA polymerase and greatly increases DNA synthesis. Virology 250, 210–219.

Ljungman, P., Singh, N., 2006. Human herpesvirus-6 infection in solid organ and stem cell transplant recipients. J. Clin. Virol. 37 (Suppl. 1), S87–S91.

Loregian, A., Coen, D.M., 2006. Selective anti-cytomegalovirus compounds discovered by screening for inhibitors of subunit interactions of the viral polymerase. Chem. Biol. 13, 191–200.

Loregian, A., Palù, G., 2005. Disruption of the interactions between the subunits of herpesvirus DNA polymerases as a novel antiviral strategy. Clin. Microbiol. Infect. 11, 437–446.

Manichanh, C., Olivier-Aubron, C., Lagarde, J.P., Aubin, J.T., Bossi, P., Gautheret-Dejean, A., Huraux, J.M., Agut, H., 2001. Selection of the same mutation in the U69 protein kinase gene of human herpesvirus-6 after prolonged exposure to ganciclovir in vitro and in vivo. J. Gen. Virol. 82, 2767–2776.

Xu, Y., Linde, A., Dahl, H., Winberg, G., 2001. Definition of a divergent epitope that allows differential detection of early protein p41 from human herpesvirus 6 variants A and B. J. Clin. Microbiol. 39, 1449–1455.

Zuccola, H.J., Filman, D.J., Coen, D.M., Hogle, J.M., 2000. The crystal structure of an unusual processivity factor, herpes simplex virus UL42, bound to the C terminus of its cognate polymerase. Mol. Cell 5, 267–278.