

Herpes Simplex Virus Mutants with Multiple Substitutions Affecting DNA Binding of UL42 Are Impaired for Viral Replication and DNA Synthesis[∇]

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Herpes simplex virus mutants with single substitutions that decrease DNA binding by the DNA polymerase processivity subunit UL42 are only modestly impaired for viral replication. In this study, recombinant viruses harboring two or four of these mutations were constructed. The more substitutions, the more severe the defects in viral replication and DNA synthesis, suggesting that DNA binding by UL42 is important for these processes.

Herpes simplex virus DNA polymerase (Pol) consists of a heterodimer with a catalytic-subunit Pol and an accessory subunit, UL42. UL42 is a processivity factor that stimulates long-chain DNA synthesis *in vitro* and is essential for viral replication (3, 7, 9, 10). Unlike the sliding clamp processivity factors that interact only topologically with DNA (reviewed in reference 4), UL42 interacts with DNA directly with high affinity as a monomer (11) and increases the binding affinity of the Pol to the primer/template (2, 15) by decreasing the dissociation rate (2). Several results indicate that the DNA binding activity of UL42 is crucial for processive DNA synthesis by the viral Pol. Insertion mutations of UL42 that result in undetectable DNA binding impair long-chain DNA synthesis and prevent complementation of a *UL42* null mutant (1). UL42 mutants containing alanine substitutions for any of four conserved arginine residues (at positions 113, 182, 279, and 280), which reside on the positively charged surface of UL42, decrease DNA binding without affecting binding to the Pol C terminus and decrease the stimulation of long-chain DNA synthesis (12). However, when incorporated into herpes simplex virus, these single substitutions have only modest effects on viral replication and DNA synthesis (5). Because combining two or four substitutions resulted in more-severe effects on DNA binding *in vitro* and the quadruple substitution impaired complementation of a *UL42* null mutant (12), we wished to investigate the effects of such multiple substitutions on viral replication and DNA replication when they were incorporated into the virus.

We constructed two plasmids, pHC-R113/182A and pHC-R279/280A, each of which contains two arginine-to-alanine substitutions, as previously described using pHC700 (5) and plasmids based on pMBP-ppΔ340 containing the correspond-

ing mutations (12). Each plasmid was sequenced to confirm the presence of the desired mutations and no others. These two plasmids were tested for their ability to complement the replication of *CgalΔ42*, a *UL42* null mutant, in Vero cells as described previously (5, 12). As expected from the previous result that a plasmid containing all four substitutions was able to complement *CgalΔ42* replication, albeit with reduced efficiency, the two new plasmids were able to complement the null mutant. The complementation efficiencies of the two double-mutation plasmids were intermediate between those of the quadruple-substitution mutant and a wild-type control plasmid (data not shown).

Recombinant viruses containing *UL42* double and quadruple mutations were then constructed and purified as described previously (5). Two independent isolates of each mutant were derived from separate transfections, and the presence of the *UL42* mutations was confirmed by sequencing. Plaque assays were performed on Vero cells and on V9 cells, which express wild-type UL42 protein (6), and images of at least 20 plaques were recorded using a SPOT camera on a model TE300 inverted microscope (Nikon). Plaque sizes were measured using Photoshop software (Adobe). The data presented in Fig. 1 were from a single experiment using equal densities of cells. While all recombinants formed plaques of similar sizes on V9 cells, mutants with double and quadruple mutations formed significantly smaller plaques on Vero cells than did two single-substitution mutants that were tested or a control virus constructed in the same manner as the mutants but encoding wild-type UL42 (Fig. 1). The quadruple mutant was particularly impaired in plaque size, with a mean area more than 20-fold less than that of the control virus.

The double- and quadruple-substitution mutants along with two single-substitution mutants and the control virus were then tested in single-cycle growth assays on Vero cells, performed as previously described (5). The titers of all viruses peaked at 36 h postinfection, with the exception of that of the quadruple mutant, which peaked at 48 h postinfection. Burst sizes were then determined as the total yield of virus at the time of peak titer

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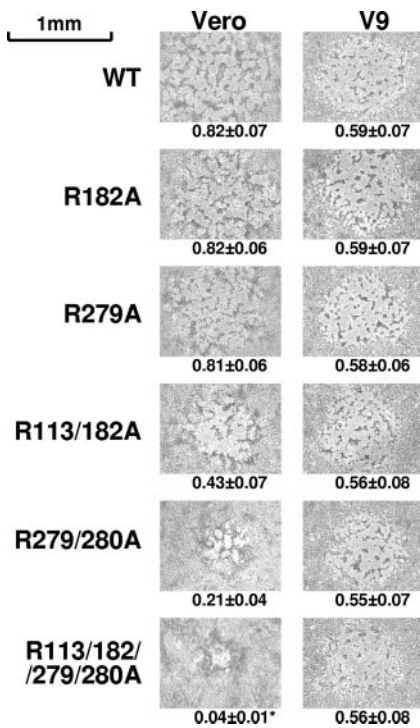


FIG. 1. *UL42* mutants form smaller plaques on Vero cells than the wild type (wt). Plaques formed on Vero and V9 cells were analyzed as described in the text. Numbers shown below the plaques are average sizes (mm^2) of at least 20 plaques \pm standard deviations. *, the mutant with quadruple mutations formed only tiny plaques, which could be observed clearly only under a high magnification ($\times 200$). The plaques formed by other viruses were observed under a magnification of $\times 100$ at 3 days postinfection.

divided by the number of infected cells (Table 1). Consistently with a previous report (5), the single-substitution R182A and R279A mutants exhibited two- and fourfold-reduced virus yields, respectively, relative to the yield of the control virus. The double-substitution R113/182A and R279/280A mutants had 10- and 30-fold-decreased virus yields, respectively, while the quadruple mutant with the R113/182/279/280A mutations yielded nearly 100-fold less virus. Thus, the double and quadruple mutants were substantially impaired for viral replication.

To examine the kinetics of viral DNA synthesis, a quantitative real-time PCR assay was applied, as described previously (5). Figure 2 shows the amounts of viral DNA synthesized at 2, 5, 8, 12, 24, 36, and 48 h postinfection by the various viruses. Similarly to what was observed previously with single-substitution mutants (5), the two double-substitution mutants replicated DNA with slower kinetics during the early phase of infection than that of the control virus with wild-type *UL42*. Interestingly, the R113/182A mutant synthesized less DNA than the single mutants during the early phase of infection ($P < 0.05$ and $P = 0.003$ at 5 and 8 h postinfection, respectively; Student's *t* test), and the R279/280A mutant replicated DNA even more slowly during this phase ($P < 0.05$ and $P < 0.001$ at 5 and 8 h postinfection, respectively). These double mutants, however, synthesized amounts of DNA similar to that of the wild-type *UL42* control virus by 36 h after infection. The

quadruple mutant, on the other hand, was substantially impaired for viral DNA synthesis at all time points (P was ≤ 0.001 except at 48 h postinfection, at which time, P was 0.003); it synthesized fourfold-less DNA than the control virus with wild-type *UL42* even at late times. Even so, the double and quadruple mutants exhibited significantly higher ratios of DNA copy numbers to numbers of PFU relative to those of viruses with wild-type *UL42* or a single-substitution mutation (Table 1).

The increasing severity of replication and DNA synthesis phenotypes with an increased number of substitutions and their increasingly severe effects on DNA binding (11) are consistent with the interpretation that reduced DNA binding by *UL42* results in a reduction in viral DNA synthesis and thus viral replication. It must be cautioned that in the study of the effects of the double and quadruple mutations on DNA binding, we could not rule out an effect on Pol binding. Nevertheless, no single substitution affected the ability of *UL42* to bind to Pol in vitro (12). In addition, we cannot exclude the possibility that these substitutions might also influence other properties of *UL42*, such as interactions with other proteins. Regardless, the simplest interpretation of the results is that DNA binding by *UL42* is important for viral replication and DNA synthesis.

Given this interpretation, one could ask why these mutants synthesize any viral DNA since the mutant proteins were so highly impaired in DNA binding (12). It should be noted, however, that not every arginine and lysine on the positively charged surface of *UL42* is replaced by alanine in the quadruple mutant, so the mutant likely retains some affinity for DNA. Moreover, *UL42* may interact not only with Pol but also with *UL9* (8, 14), the origin binding protein, and *UL29* (13), the single-stranded DNA binding protein. The interactions of these other proteins with DNA in infected cells may permit the mutant *UL42*s to function, albeit less efficiently, in supporting processive DNA synthesis, which may result in slower kinetics of viral DNA replication and the synthesis of less viral DNA during the early phase of infection.

TABLE 1. Burst sizes and DNA copy numbers per PFU of *UL42* mutants

Virus (recombinant) ^c	Burst size ^a (PFU/cell)	No. of DNA copies/PFU ^b
Wild-type virus (A)	30 \pm 6.1	190 \pm 10
R182A mutant (A)	18 \pm 1.8	490 \pm 10
R279A mutant (A)	7.9 \pm 1.1	1,200 \pm 160
R113/182A mutant (A)	3.6 \pm 0.1	2,000 \pm 70
R113/182A mutant (B)	2.6 \pm 0.2	2,900 \pm 40
R279/280A mutant (A)	1.1 \pm 0.0	4,800 \pm 1,400
R279/280A mutant (B)	1.1 \pm 0.2	5,200 \pm 1,900
R113/182/279/280A mutant (A)	0.4 \pm 0.0	4,800 \pm 80
R113/182/279/280A mutant (B)	0.4 \pm 0.0	3,200 \pm 1,000

^a Burst size was calculated as the ratio of the peak titer (at 48 h postinfection for the quadruple mutants and at 36 h postinfection for all other recombinants) to the number of cells. Data are averages \pm standard errors of the means from the same two experiments of single-cycle growth curves, and the amounts of viral DNA were synthesized as indicated for Fig. 2.

^b Numbers of DNA copies per PFU were calculated by determining the ratios of total DNA copy numbers synthesized to peak titers (at 48 h for the quadruple mutant and at 36 h postinfection for other recombinants).

^c Wild-type virus refers to the control virus with wild-type *UL42*. The A and B recombinants were two independent constructs. All recombinations were performed with the control virus and the indicated mutant.

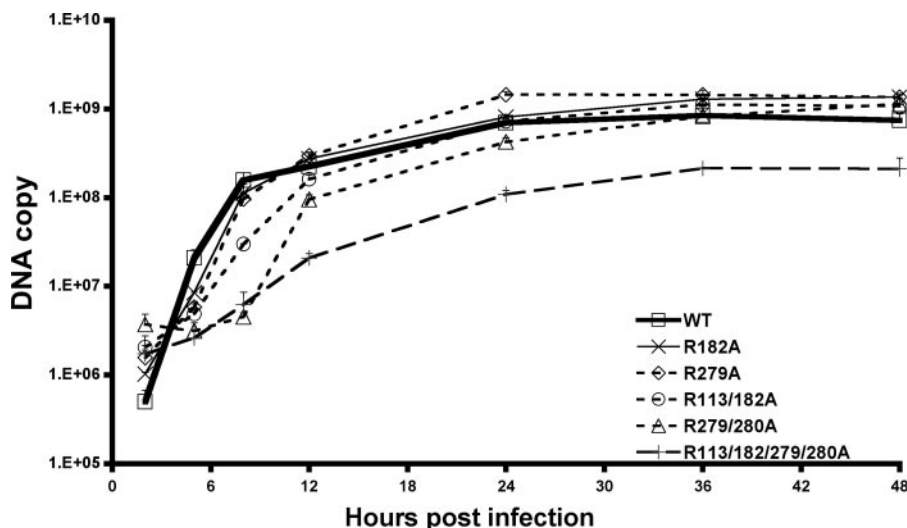


FIG. 2. Viral DNA synthesized by *UL42* recombinants. Vero cells (1×10^5) were inoculated with *UL42* recombinants at a multiplicity of infection of 3. Infected cells were harvested at different times postinfection. DNA was isolated from aliquots of both infected cells and medium containing cell-free virus. Purified DNA was serially diluted and quantified by real-time PCR as described in reference 5 to determine the relative copy numbers of viral DNA. Data shown are averages from two independent experiments. Since the independent isolates of each virus exhibited similar kinetics, only the results for one recombinant (recombinants A in Table 1) are shown for clarity. WT, wild type.

An interesting property of all of the viral *UL42* mutants containing arginine-to-alanine substitutions is that they synthesize relatively large amounts of DNA per PFU. This was true even for the quadruple-substitution mutant, which is substantially defective in viral DNA synthesis, even at late times postinfection. This mutant is 25-fold-more defective in viral yield than it is in viral DNA synthesis. It is possible that the high DNA copy number/PFU ratio is due in part to the mutagenic effect of the *UL42* mutation (5; unpublished results). Indeed, examination of the copy numbers of cell-free virion DNA by real-time PCR, which mimicked the numbers of cell-free virus particles, and of the relative virus titers demonstrated that the quadruple-substitution mutant exhibited a 10-fold-higher ratio of particle numbers to PFU than the control virus with wild-type *UL42*. This suggests that the mutant synthesized more virions that contain lethal mutations and thus do not form plaques. Another possible explanation for the elevated DNA copy number/PFU ratio stems from the delayed kinetics of DNA replication exhibited by the mutants. It may be that much of the DNA made later in infection is not productively packaged into virions. An electron microscopy study of the distributions of different nucleocapsids formed in cells infected with viruses with mutant and wild-type *UL42* should clarify this possibility. A third possibility is that *UL42* has a role in viral replication other than DNA synthesis and that the mutations affect this function. Further studies of these and other *UL42* mutants should resolve the possibility.

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