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Use of Suppressor Analysis To Identify DNA Polymerase Mutations in Herpes Simplex Virus Which Affect Deoxynucleoside Triphosphate Substrate Specificity

YISHENG WANG,† SUANN WOODWARD, AND JENNIFER D. HALL*

Department of Molecular and Cellular Biology, University of Arizona, Tucson, Arizona 85721

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Herpes simplex virus DNA polymerase mutations which map in the N-terminal part of the protein and appear to alter deoxynucleoside triphosphate (dNTP) substrate specificity are described. These mutations suppress a drug hypersensitivity associated with the downstream mutation, Aph^{r}10. We suggest that the mutant residues form part of the dNTP-binding site, a site previously thought to be confined to the C terminus.

A genetic method for identifying interactions between functional domains of the DNA polymerase from herpes simplex virus type 1 is described. This enzyme is thought to be composed of three functional units: a C-terminal polymerization domain, a central 3′-5′ exonuclease domain, and an N-terminal RNase H domain (Fig. 1). The method described involves a suppressor analysis of upstream mutations which suppress the phenotype of a mutation within the C-terminal polymerization domain.

The location of the polymerization domain is suggested by previously described mutations which affect deoxynucleoside triphosphate (dNTP) substrate recognition. These mutations alter the ability of the enzyme to incorporate dNTP analogs (e.g., arabinosylthymine triphosphate), to respond to other inhibitors which bind at the substrate- or PP_i-binding sites (aphidicolin and phosphonoacetic acid, respectively), and to bind to normal dNTPs (5, 7, 8, 11, 12). This domain is further defined by mutations which inactivate the polymerase activity but not the 3′-5′ exonuclease in a related polymerase from bacteriophage ϕ29 (15). The location of the 3′-5′ exonuclease domain is suggested by sequence alignments between the 3′-5′ exonuclease domain from DNA polymerase I (*Escherichia coli*) and several herpesvirus-related polymerases (1) and by mutations in the ϕ29 polymerase and DNA polymerase III (*Saccharomyces cerevisiae*) which specifically inactivate this nuclease (1, 16).

The three-domain model is similar to that proposed for DNA polymerase I from *E. coli* (13). Structural and biochemical analyses suggest that the three domains of DNA polymerase I function relatively independently. The RNase H domain can be removed, producing the Klenow fragment, which encompasses a polymerization and a 3′-5′ exonuclease domain. The peptide backbone of the Klenow fragment is folded so that the N-terminal portion is entirely within the exonuclease domain and the C-terminal portion is entirely within the polymerase domain (13).

In contrast, the domains of the herpesvirus polymerase may be more interconnected. Its polymerase and 3′-5′ exonuclease activities are coinactivated by inhibitors, such as phosophonoacetic acid, which appear to bind within the polymerase domain (14). Certain antibodies made against the polymerase domain also inactivate the exonuclease activity (10). These observations could be explained if the two activities are closely linked, perhaps in one integrated domain. This paper describes two mutations which support this model.

Isolation and mapping of mutations which suppress the DNA polymerase mutation, Aph^{r}10. The above-described mutations were isolated as second-site changes which suppress the aphidicolin resistance mutation, Aph^{r}10 (Fig. 1) (8). This aphidicolin mutation causes both resistance to aphidicolin and hypersensitivity to phosphonoacetic acid. To suppress its hypersensitive phenotype while retaining aphidicolin resistance, we selected viral derivatives which grew in both aphidicolin and phosphonoacetic acid (8). Plaques from two independent selections were isolated and named Aph^{r}10-Su1 and Aph^{r}10-Su2. The Aph^{r}10-Su1 mutant has been described previously (8). It retains the original Aph^{r}10 mutation and has acquired a second polymerase mutation, Su1 (Fig. 1) (8).

The Aph^{r}10-Su2 mutant also carries the original Aph^{r}10 mutation and has acquired a second, suppressor mutation. The presence of the Aph^{r}10 mutation was shown by marker rescue (Table 1). Cells were mixedly transfected with wild-type viral DNA and mutant polymerase fragments (see reference 8 for the procedure), and the frequency of aphidicolin-resistant progeny was measured. We found that 0.22% of the progeny (>8.5-fold above the spontaneous level) were drug resistant. Hence, the Aph^{r}10-Su2-derived fragment carries a resistance mutation. DNA sequencing (data not shown) confirmed that the original Aph^{r}10 mutation was still present.

To map the second mutation in Aph^{r}10-Su2, we predicted that, since the Su mutations suppress the Aph^{r}10 hypersensitivity to phosphonoacetic acid, they would cause resistance to this drug when present as a single mutation. Therefore, we carried out marker rescue experiments by using cloned DNA fragments from the Aph^{r}10-Su2 polymerase gene and measured the frequency of progeny resistant to phosphonoacetic acid. As shown in Table 1, fragments which carry mutant-derived sequences located between the first *Bam*HI site and the *Sfi*I site (fragments A, B, and C1) confer resistance. Clones carrying Su1 also produced phosphonoacetic acid-resistant progeny (Table 1).

The Su2 mutation was determined by sequencing the *Bam*HI-*Sfi*I region (see reference 8 for the procedure). A single T-to-C transition at DNA residue 773, which produced

* Corresponding author.
† Present address: Lederle Laboratories, 401 N. Middletown Rd., Pearl River, NY 10965.

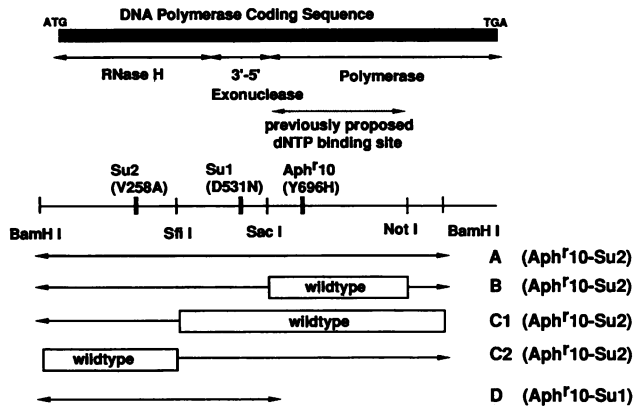

FIG. 1. The herpes simplex virus DNA polymerase gene, protein domains, and DNA sites. Previously proposed protein domains and the dNTP-binding region are shown relative to the coding sequence. Pertinent restriction sites are indicated, and mutation sites are shown with amino acid changes and residue numbers in parentheses. The region between the two *Bam*HI sites constitutes the *Bam*HI Q fragment. It was cloned from wild-type and mutant viruses into pBR322 as described previously (8). Fragments from these clones were also exchanged to produce recombinant clones carrying both wild-type and mutant-derived sequences. Mutant-derived sequences are indicated by thin lines, and the corresponding mutant virus is shown in parentheses. Fragments A, B, C1, C2, and D were used for the marker rescue experiments in Table 1.

a valine-to-alanine change at protein residue 258, was found (Fig. 1).

Characterization of the suppressor phenotypes. To isolate viruses carrying only the suppressor mutations, we plaque purified phosphonoacetic acid-resistant progeny from these marker rescue experiments. Three potential Su1 and two potential Su2 plaques were characterized, each from a separate transfection experiment. Within each group, the phenotypes were identical. Hence, we concluded that these viruses represented true recombinants, not spontaneous mutants. We therefore show the results for only one representative from each group.

These mutants exhibit altered sensitivity to several inhibitors (Fig. 2). Sensitivity was measured in plaque assays

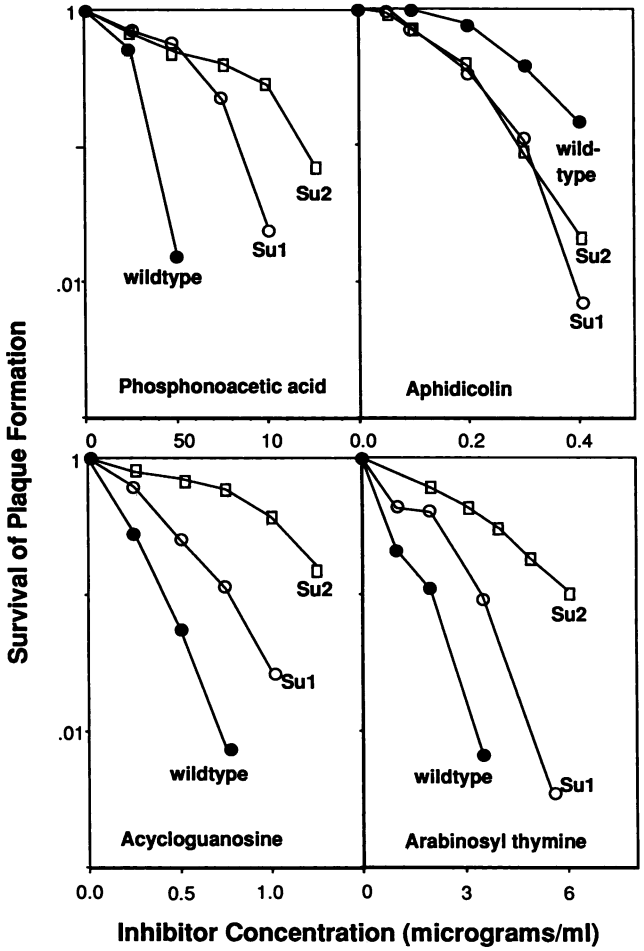

FIG. 2. Survival of plaque formation by mutant and wild-type viruses in the presence of polymerase inhibitors. Results are averages of two experiments.

TABLE 1. Marker rescue of phosphonoacetic acid or aphidicolin resistance[a]

Cloned fragment	Percent resistant progeny from transfections with cloned DNA polymerase sequences			
	Phosphonoacetic acid		Aphidicolin	
	No fragment	With fragment	No fragment	With fragment
From Aph^r10-Su2				
A	0.018	1.60	<0.026	0.22
B	0.080	1.62		
C1	0.011	1.63		
C2	0.011	0.0036		
From Aph^r10-Su1				
D	0.031	0.138		

[a] Marker rescue experiments were performed by cotransfection with wild-type viral DNA and the fragments described in Fig. 1. The fractions of drug-resistant progeny were then determined. Phosphonoacetic acid-resistant progency were also plaque purified from transfections with fragments D and B and used to study drug sensitivities (Fig. 2) and mutation frequencies (Fig. 3).

performed as described in reference 6 at several inhibitor concentrations, and the fraction of surviving plaques was determined. In addition to the expected resistance to phosphonoacetic acid, the Su mutants were hypersensitive to aphidicolin and resistant to acycloguanosine and arabinosyl thymine.

These abnormal sensitivities suggest that substrate binding is altered in the mutants. Aphidicolin competes with dNTPs for binding to the polymerase (3, 4, 9). Hence, altered sensitivity to this drug suggests changes at the dNTP-binding site. Arabinosylthymine and acycloguanosine are substrate analogs. Resistance to these compounds could be explained by either more discriminate incorporation or more efficient nuclease editing. We favor the former explanation, since acycloguanosine is not normally removed by the editing nuclease (2).

We also measured the spontaneous mutation frequencies of our mutants (Fig. 3). Single plaques were grown into ministocks, and the fractions of progeny resistant to iododeoxycytidine (ICdR) were determined (6). Mutants Su1, Su2, and Aph^r10-Su1 all produced fewer resistant progeny than the wild type, indicating an antimutator phenotype. Since the residues altered by the Su mutations may participate in dNTP binding, we suggest that the antimutator phenotype

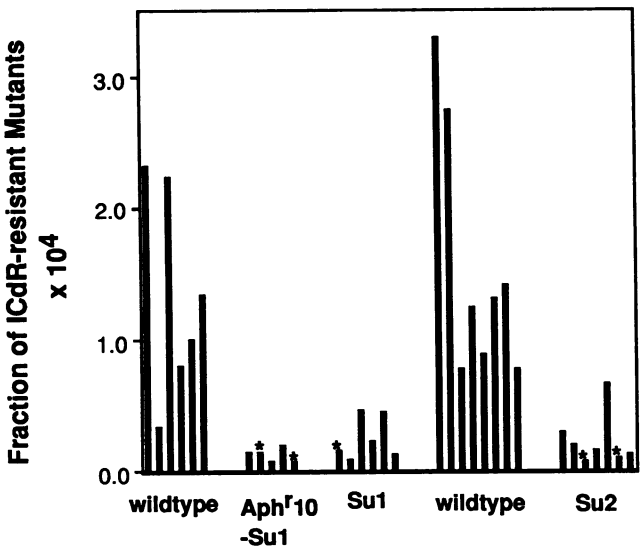

FIG. 3. Mutation frequencies of mutant and wild-type viruses. Two experiments are shown, one with wild-type, Aph^{r}10-Su1, and Su1 viruses and one with wild-type and Su2 viruses. Each bar represents the results of a separate infection. Bars marked with asterisks indicate infections in which no ICdR-resistant mutants were detected. In these cases, the frequencies shown assumed that one plaque was present, and, hence, the actual frequencies are lower than the values indicated. The average fractions of ICdR-resistant mutants for each virus are 1.34×10^{-4} (wild type), $<1.25 \times 10^{-5}$ (Aph^{r}10-Su1), and $<2.5 \times 10^{-5}$ (Su1) in the first experiment and 1.55×10^{-4} (wild type) and $<2.27 \times 10^{-5}$ (Su2) in the second. A t test indicated that the mutation frequencies for the three mutants are significantly lower than for the wild type (Aph^{r}10-Su1, $t = 2.97$, $P < 0.01$; Su1, $t = 3.05$, $P < 0.01$; and Su2, $t = 3.61$, $P < 0.005$).

results from more accurate discrimination at the insertion step of DNA synthesis.

Conclusions. We have used the pattern of inhibitor resistance and sensitivity to obtain suppressor mutations in the herpes simplex virus DNA polymerase which appear to affect substrate binding. Although the substrate-binding site has been thought to occur downstream in the C terminus (Fig. 1), our mutations map upstream in either the 3′-5′ exonuclease or RNase H regions. We therefore suggest that the polymerase domains are interconnected or form one large domain. The upstream region may participate directly in the dNTP-binding site or, alternatively, may alter protein folding, thus indirectly influencing binding.

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