Aphidicolin resistance in Herpes simplex virus type I reveals features of the DNA polymerase dNTP binding site

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ABSTRACT

We describe the mapping and sequencing of mutations within the DNA polymerase gene of herpes simplex virus type 1 which confer resistance to aphidicolin, a DNA polymerase inhibitor. The mutations occur near two regions which are highly conserved among DNA polymerases related to the herpes simplex enzyme. They also occur near other herpes simplex mutations which affect the interactions between the polymerase and deoxyribonucleoside triphosphate substrates. Consequently, we argue in favor of the idea that the aphidicolin binding site overlaps the substrate binding site and that the near-by conserved regions are functionally required for substrate binding. Our mutants also exhibit abnormal sensitivity to another DNA polymerase inhibitor, phosphonoacetic acid. This drug is thought to bind as an analogue of pyrophosphate. A second-site mutation which suppresses the hypersensitivity of one mutant to phosphonoacetic acid (but not its aphidicolin resistance) is described. This second mutation may represent a new class of mutations, which specifically affects pyrophosphate, but not substrate, binding.

INTRODUCTION

A low, but significant, level of replication errors is normally associated with DNA synthesis, thus allowing both continuity and evolution of genetic information. DNA polymerases play an essential role in controlling this error frequency by selecting correctly base-paired deoxyribonucleoside triphosphates (dNTPs) for polymerization and by removing existing errors with an associated 3'–5' proofreading exonuclease. To understand the role of the polymerase in substrate selection, it is important to identify the molecular interactions which occur in the dNTP-enzyme complex. We have attempted to identify the protein residues which participate in these interactions by characterizing mutations within the DNA polymerase of herpes simplex virus type 1 (HSV-1) which alter the substrate specificity of this enzyme.

The herpes simplex polymerase is uniquely suited for these studies because of a class of enzyme mutants which polymerize normally but are resistant to certain substrate analogue inhibitors (1–3). These analogues carry abnormal ribose rings and include the triphosphate derivatives of acycloguanosine (9-[(2-hydroxyethoxy)-methyl]guanine), arabinosyladenine (9-beta-D-arabinofuranosyladenine), arabinosylthymine (9-beta-D-arabinofuranosylthymine) and DHPG (9-(1,3-dihydroxy-2-propoxymethyl)guanine). They are utilized as polymerase substrates in herpes-infected cells and cause subsequent deterioration of chain elongation (4–6). *In vitro*, the analogues compete with normal dNTP substrates for incorporation (7,8). Hence, the resistant mutants appear to carry changes which affect binding of the DNA polymerase to the ribose moieties of the analogues. In addition, the mutations affect binding to normal dNTP substrates. Some mutants exhibit abnormal

affinities for dNTPs *in vitro* (9,10), and some influence substrate selection, as indicated by abnormal viral mutation frequencies (11) and by a polymerase with enhanced replication fidelity (9).

The HSV-1 DNA polymerase is related to a large group of polymerases, including human alpha DNA polymerase and alpha-like activities from other eukaryotic cells. These polymerases share a common sensitivity to the inhibitor aphidicolin (12–16). Where the genetic sequences of these enzymes are known, several sites of strong similarity at the protein and nucleic acid levels are found (1,17,18). Consequently, the catalytic sites in these proteins appear to have been conserved during evolution. Sequence changes associated with several of the HSV-1 drug-resistant mutants described above cluster near two of the most highly conserved regions (1–3). This coincidence suggests that the conserved regions constitute part of the dNTP binding site.

In this report, we describe our studies of HSV-1 mutants resistant to aphidicolin. We chose this drug because we suspected that aphidicolin resistant mutants would carry mutations which affect substrate binding. Our reasoning was based on kinetic studies indicating that aphidicolin inhibition of DNA polymerases is competitive with respect to one or more dNTP species (12,19,20). In addition, we noted that previously characterized aphidicolin mutants from several sources exhibit aberrant substrate interactions. Resistant mutants from the related virus, herpes simplex virus type 2 (HSV-2), and Chinese hamster cells encode DNA polymerases with enhanced affinities for pyrimidine dNTPs (21,22). Also, several of the substrate analogue-resistant mutants from HSV-1 are hypersensitive to aphidicolin (23). However, only two aphidicolin-resistant mutants from HSV-1 have been described. One has not been mapped (24); the second is only moderately resistant (25).

Our studies have identified two mutations within the HSV-1 DNA polymerase gene which cause resistance to aphidicolin. One mutation is identical to a mutation previously reported from HSV-2 (26,27). However, its associated phenotype differs from that of the HSV-2 mutant. In addition, we describe a third polymerase mutation which suppresses one of the phenotypes (hypersensitivity to phosphonoacetic acid) of one of our aphidicolin-resistant mutants. These three mutations help define the site of inhibitor and substrate binding within the herpes polymerase.

MATERIALS AND METHODS

Cells and Viruses. Methods for cell maintenance, virus preparation and plaque assays have been described (11). Vero cells were used to prepare virus stocks and for marker rescue. TC7 cells were used to measure viral drug sensitivity. Both cell lines were originally established from African green monkey kidney cells. Viral mutants were derived from the KOS HSV-1 strain.

Anti-viral Drugs. Stock solutions of aphidicolin (National Cancer Institute and Sigma) were prepared at 100 µg/ml in Dulbecco's modified Eagle's medium supplemented with 2% calf serum and 10% dimethyl sulfoxide. Stocks of other drugs were prepared at 5 mg/ml in H₂O (phosphonoacetic acid, Abbott Laboratories), 0.04 N NaOH (arabinosylthymine, Sigma; acycloguanosine, Burroughs Wellcome) and 0.06 N NaOH (DHPG, Burroughs Wellcome and Syntex Research). Drug stocks were filtered and stored at –20°C.

Isolation of Mutants. Isolation of the aphidicolin-resistant mutants has been described (28). The suppressor mutant (Aph^r10-Su1) was isolated by growing Aph^r10 virus in aphidicolin and phosphonoacetic acid. Initially, TC7 cells were inoculated with 5 × 10⁵ plaque forming

units (pfu) of virus at 0.1 pfu/cell. The virus was grown at 37°C in the presence of aphidicolin (0.9 µg/ml) and phosphonoacetic acid (15 µg/ml) until extensive cytopathic effects were observed. Progeny virus was cycled twice more in aphidicolin and phosphonoacetic acid (20 µg/ml) at the same multiplicity and then plated in a plaque assay containing aphidicolin and phosphonoacetic acid (25 µg/ml). A resistant plaque was picked, and its progeny were plaque purified without drug to yield Aph¹⁰-Su1.

Drug Sensitivities of Mutant Viruses. 200–500 plaque forming units of virus were plated onto 60mm plates of TC7 monolayers at several drug concentrations, including duplicate plates without drug. The surviving fractions of plaques were determined at each concentration.

Plasmid Isolation. Plasmids were purified according to the second procedure in reference 29 with the following modifications. The lysate was extracted with phenol and chloroform, ethanol precipitated, resuspended in 5 ml 10 mM Tris (pH 7.5) and 1 mM EDTA, and then centrifuged in CsCl for 40 hr at 40 Krpm at 20°C in a Ti50 rotor.

Cloning of DNA Polymerase Fragments from Mutants. The cloning scheme is depicted in Figure 1. Restriction digests and ligations were performed as directed by the manufacturers. Viral DNA was prepared from each mutant (30) and digested with BamHI. The Q fragment was recovered from an agarose gel, using NA-45 paper (as directed by Schleicher and Schuell). The fragment was ligated into the tetracycline gene of pBR322, at the plasmid BamHI site, to produce a given plasmid pBRQ. These plasmids were then transferred into *Escherichia coli* strain DH1 (31) and recovered from ampicillin-resistant, tetracycline-sensitive colonies. The orientation of the insert was determined from the PstI digestion pattern, and, in all cases, was as shown in Figure 1.

Mapping of Mutations. Vero cells were co-transfected with mutant DNA fragments and wild type viral DNA and the progeny examined for acquisition of aphidicolin resistance, using a procedure modified from reference 32. First, 1.5–3 µg of wild type viral DNA was precipitated with 0.5–10 µg of mutant DNA and 4 µg salmon sperm DNA in 600 µl HEPES buffered saline supplemented with 125 µM CaCl₂. The mutant DNAs were either an appropriate pBRQ (carrying the BamHI Q fragment from a given mutant), EcoRI-generated fragments from pBRQ (called large and small) or the EcoRI M fragment from a viral DNA digestion (see Figure 1). EcoRI fragments were isolated from agarose gels using the NA-45 paper method (see above) or by electroelution (31), with the modification that the buffer was 0.1 × TBE, the elution was carried out at 100 mA for 10–15 min, and the current was reversed for 5 sec. Plasmids or fragments carrying vector sequences were first digested with BamHI to release viral DNA.

Next, an aliquot of 10⁶ cells was added to each DNA mixture and the suspensions were agitated for 45 min at 37°C. 10⁶ additional cells were added, and each transfection mixture was plated onto duplicate 35 mm plates in Dulbecco's modified Eagle's medium containing 10% calf serum. Plates were incubated at 37°C and refed at 5 and 16 hr with medium containing 2% calf serum. Incubation was continued until the cells became uniformly infected (approximately 3 days). (In parallel experiments in which plaque formation was measured instead of progeny virus production, these DNA mixtures yielded 150–300 plaques).

Finally, progeny viruses were harvested and titered in the presence and absence of aphidicolin (0.9 µg/ml) to determine the fraction of aphidicolin resistant recombinants. In experiments using fragments from Aph¹⁰-Su1, several aphidicolin resistant progeny were then plaque purified without drug and titered in the presence of phosphonoacetic acid

(25 µg/ml) to determine whether they had lost their hypersensitivity to phosphonoacetic acid.

DNA sequencing. DNA sequencing was carried out using a T7 DNA sequenase kit (US Biochemicals) on pBRQ plasmid templates. In each case, the sequence of the HSV-1 segment within the large EcoRI fragment (Figure 1) was determined. Seventeen-base primers were synthesized (Synthetic Genetics) so as to anneal every 300–350 nucleotides along the sequence. Sequencing was performed as described (33), except that the DNA was denatured and dried prior to mixing with the primer. Each reaction contained 4 µg DNA and 20 ng primer. Reaction products were resolved on 5–6% polyacrylamide gels containing 7 M urea. Gels were run at 35 mA until the xylene cyanol dye approached the bottom. Then, a second sample was loaded, and electrophoresis was continued. This procedure allowed reading of up to 450 nucleotides from the primer. Gels were fixed in 10% acetic acid and 10% methanol, dried at 80°C and auto-radiographed (20–40 hr). Because herpes simplex DNA is very GC-rich, 7-deaza-dGTP was used in some reactions to reduce GC compression of sequencing bands.

Computer Analyses. Pair-wise sequence alignments were obtained using LOCAL (34), LFASTA (35) and/or by eye. Multiple alignments were compiled manually from these results. Structural predictions were performed based on the method of references 36 and 37.

RESULTS

Isolation of Aphidicolin-Resistant Mutants: We previously reported the isolation of four aphidicolin-resistant HSV-1 mutants (28). These mutants fall into two phenotypic classes based on their sensitivities to various DNA polymerase inhibitors. Aph^r10 and Aph^r16 constitute one class and Aph^r12 and Aph^r14 the other. These mutants appear to be independently derived, because Aph^r10 and Aph^r12, although from the same viral stock, have differing phenotypes, and because Aph^r14 and Aph^r16 were isolated from different plaque-purified stocks.

Mapping of Aphidicolin Resistance Mutations: To determine whether the mutations conferring aphidicolin-resistance mapped within the viral DNA polymerase, we first cloned the BamHI Q fragment from the polymerase gene of each mutant. As shown in Figure 1, this fragment encompasses all but the 13 percent at the 3' end of the gene. The Q fragment was chosen, because it includes the mutation sites in all known HSV-1 polymerase mutants which exhibit altered substrate binding affinities (2,3). Fragments were cloned, as shown in Figure 1, into pBR322, to produce recombinant plasmids called pBRQ.

These clones were tested for the presence of the resistance mutation by mixedly transfecting cells with a given pBRQ plasmid plus intact wild type viral DNA. Any viral mutation on the plasmid fragment should be transferred to the viral DNA by recombination,

Figure 1: Locations of restriction fragments encompassing the HSV-1 DNA polymerase gene and the scheme for cloning the BamHI Q fragment from mutant viral DNA into pBR322. The HSV-1 genome contains three repeated regions (stippled bars) and two unique regions (U_L and U_S). The region encompassing the polymerase gene is expanded to show the relevant restriction sites. Cloning was conducted as described in Materials and Methods. BamHI Q fragments from mutant viruses were inserted into the BamHI site of the vector pBR322. Plasmids containing the insert (pBRQ) were transferred into *Escherichia coli* and recovered from ampicillin resistant (Amp^r), tetracycline sensitive (Tet^s) colonies. Also shown are the fragments used to map polymerase mutations by marker rescue. These are the BamHI Q fragment (heavy, stippled arrow; 3365 base pairs), the large and small EcoRI fragments (dashed lines; 5873 and 1843 base pairs respectively), and the EcoRI M fragment (heavy striped arrow; 8073 base pairs).

TABLE 1: Marker Rescue of Aphidicolin Resistance

Mutant	Percent of Aphidicolin Resistant Progeny from Experiments with Herpes Simplex DNA Polymerase Cloned Fragments:			
	No fragment	BamHI Q fragment	Large EcoRI fragment	Small EcoRI fragment
Aph ^r 10	<0.02	1.2	0.095	≤0.01
Aph ^r 12	<0.02	0.35	2.9	≤0.089
Aph ^r 14	<0.001	0.19	0.047	≤0.0007
Aph ^r 16	<0.001	0.97	0.10	0.0023

The marker rescue protocol is described in Materials and Methods. Briefly, Vero cells were co-transfected with wild type viral DNA and cloned DNA fragments containing DNA polymerase sequences from mutant viruses. Progeny virus were analyzed for drug-resistance by plating in the presence of 0.9 µg/ml aphidicolin. The locations of the BamHI Q and the large and small EcoRI fragments are indicated in Figure 1. The results are the average of two or more separate experiments.

resulting in an increased level of drug-resistant viral progeny. As shown in Table 1, experiments with pBRQ clones from all four mutants resulted in 0.19 to 1.2 percent drug-resistant progeny, while infections without plasmid or with pBR322 alone (data not shown) failed to produce significant resistant progeny. These results indicate that the resistance mutations in these viruses map within the BamHI Q fragment.

Additional mapping refinements localized these mutations to the 3' portion of the BamHI Q fragment. pBRQ plasmids from each mutant were digested with EcoRI and the two resulting fragments (see Figure 1) isolated. These fragments each contain approximately half of the BamHI Q sequence. The isolated fragments were used in separate marker rescue experiments and the fraction of aphidicolin-resistant progeny virus determined. As shown in Table 1, only the large EcoRI fragment, containing sequences near the 3' end of the herpes DNA polymerase gene, produced a significant yield of resistant progeny.

From each mapping experiment with the large EcoRI fragments, we recovered two aphidicolin-resistant recombinants. We determined their sensitivity to phosphonoacetic acid and to several substrate analogues, and we measured their mutation frequencies (28). In every case, the phenotypes of the recombinants were identical to those of the original mutants. Since (see below) only a single mutation was found in each mutant, this mutation must confer both aphidicolin resistance and the other phenotypes.

Sequencing the mutations. The region encompassing the large EcoRI fragment of each mutant gene was sequenced as described in Materials and Methods. Two mutations were found, one in each of two mutants (see Table 2). The first mutation (in Aph^r10 and Aph^r16) is identical to a mutation in an aphidicolin-resistant derivative of HSV-2 (26,27). However, our mutant fails to exhibit the mutator phenotype associated with the HSV-2 mutant (28). The mutation in Aph^r12 and Aph^r14 is new.

Isolation of the Aph^r 10-Su1 Suppressor Mutant: We previously showed that mutant Aph^r10 was both resistant to aphidicolin and hypersensitive to phosphonoacetic acid (28). Since both these phenotypes result from a single mutation, we reasoned that a second mutation in a near by residue might allow the virus to recover its normal sensitivity to phosphonoacetic acid while retaining its resistance to aphidicolin. Consequently, we grew the Aph^r10 virus in both aphidicolin and phosphonoacetic acid, as described in Materials and Methods. One plaque, called Aph^r10-Su1 was recovered for further characterization.

TABLE 2: Sequence Changes in the DNA Polymerase Genes from Drug-resistant Mutants

Mutant	Aph ^r 10, Aph ^r 16.		Aph ^r 12, Aph ^r 14	Su1
Amino Acid	H		C	N
Sequence	V G Y Q G		S V Y G F	I T D K I
	696		818	531
	C		G	A
DNA Sequence	GTGGGGTACCAAGGG		TCGGTGTACGGGTTTC	ATAACCGACAAGATC
	2086		2453	1591

Mutant sequences are shown above the wild type sequence, and residue numbers appear below the sequence. Residue numbers refer to the intact HSV-1 DNA polymerase sequence. Su1 indicates the new change found in the Aph^r10-Su1 double mutant.

Figure 2: Survival of plaque formation by mutant and wild type (wt) viruses in the presence of the DNA inhibitors: (A) aphidicolin, (B) phosphonoacetic acid, (C) arabinosyl thymine (araT), (D) DHPG. Procedures are described in Materials and Methods. The results are the average of two experiments. In the case of DHPG, although several additional drug concentrations were used to test mutant survival, both mutants failed to yield detectable plaques at any concentrations greater than 0.02 μ g/ml.

Characterization of the Suppressor Mutant. The response of this mutant to various DNA polymerase inhibitors is shown in Figure 2. The mutant exhibits the same sensitivity as its Aph^r10 parent to aphidicolin, arabinosylthymine, DHPG, and acycloguanosine (not shown), while showing wild type sensitivity to phosphonoacetic acid (rather than hypersensitivity). These results suggest that the suppressor mutant carries both the original Aph^r10 mutation and a second mutation which alters its phosphonoacetic acid sensitivity but does not affect its sensitivity to the other drugs.

Mapping experiments confirm that the suppressor mutant carries at least two DNA polymerase mutations. Marker rescue experiments were carried out with wild type viral DNA and one of two overlapping fragments from the Aph^r10-Su1 DNA polymerase gene (either EcoRI M or BamHI Q; see Figure 1). Aphidicolin-resistant recombinants were recovered and tested for sensitivity to phosphonoacetic acid. As shown in Table 3, only a fraction of the aphidicolin-resistant progeny exhibited normal sensitivity to phosphonoacetic acid. The remainder resembled the hypersensitive Aph^r10 parent (data not shown). Since the normal and hypersensitive phenotypes could be separated during recombinant formation, we concluded that more than one mutation was involved.

From each mapping experiment (with the M and Q fragments), we examined the drug sensitivities of one recombinant which showed normal phosphonoacetic acid sensitivity. These recombinants were identical to the Aph^r10-Su1 parent in their response to arabinosylthymine, DHPG, and acycloguanosine (data not shown). Consequently, we concluded that the mutations responsible for these phenotypes occur within the overlap of the two fragments used for mapping.

This overlap region (equivalent to the large EcoRI fragment shown in Figure 1) was sequenced. Two mutations were found, the original Aph^r10 mutation and a new mutation, called Su1, shown in Table 2.

Computer Analyses. The amino acid sequence surrounding the sites of our mutations was subjected to computer analysis to gain insight into possible structural and functional features. Residues 440 to 965 in the HSV-1 sequence were aligned with DNA polymerase sequences from other herpes viruses (Epstein Barr, varicella zoster and human cytomegalo). As shown in Figure 3a, this alignment reveals three short sequences which appear to have been inserted into (or deleted from) one or more of the sequences. These inserts (called A, B, and C) may flank subdomains within the substrate binding site. Insert B has been noted previously (38,39). We also identified a region downstream of the Su1 mutation which shows a weak

TABLE 3: Marker Rescue of Aphidicolin Resistance and Phosphonoacetic Acid Sensitivity

DNA Polymerase Fragment from Aph ^r 10-Su1	Percent of Aphidicolin Resistant Progeny	Fraction of Aphidicolin Resistant Progeny Which Showed Normal Sensitivity to Phosphonoacetic Acid
no fragment	≤ 0.03	—
EcoRI M	0.15	2/9
BamHI Q	0.15	2/6

The marker rescue protocol is described in Materials and Methods and Table 1. The DNA fragments (see Figure 1) were either the EcoRI M fragment from a digest of mutant viral DNA or the BamHI Q fragment which had been cloned into pBR322. Aphidicolin resistant progeny from the initial marker rescue experiments were plaque purified and tested for their sensitivity to 25 µg/ml phosphonoacetic acid. Viruses showing normal sensitivity gave 5–20 per cent survival at that drug concentration.

alignment with the sequence from DNA polymerase I of *Escherichia coli* (Figure 3c). Below we discuss the relevance of this alignment to the function of the Su1 residue.

Secondary structure predictions for the four herpes polymerase sequences were also determined (data not shown). Of particular interest was the finding that beta-turns were predicted at positions flanking the inserts A,B and C (described above) and in four regions which are highly conserved within polymerase genes related to that of HSV-1 (1,3,17). These predictions seem worthy of mention because they were found in at least three of the four sequences in each case. Their significance is discussed below.

DISCUSSION

Our studies of aphidicolin-resistant derivatives of the HSV-1 DNA polymerase were undertaken to gain insight into the mechanism of aphidicolin inhibition and of dNTP substrate recognition. Because our mutants affect the polymerase's response to dNTP substrates, we suggest that the aphidicolin binding site overlaps the substrate binding site within this enzyme. We also discuss the relationship of this site to binding by other inhibitors (e.g. phosphonoacetic acid) and to sequences which are highly conserved within polymerase genes. Our results should serve as a model for DNA polymerases from eukaryotic cells (e.g. human), since the herpes enzyme sequence is related to these cellular counterparts. *The Putative dNTP Binding Site in the HSV-1 DNA Polymerase:* A region within the HSV-1 DNA polymerase which binds dNTP substrates is suggested by the location of sequence changes in polymerase mutants which exhibit abnormal substrate specificity. These mutations occur in viral derivatives which were selected as resistant to various inhibitors but which show abnormal responses to nucleotide analogue inhibitors (e.g. our aphidicolin-resistant mutants show altered sensitivity to arabinosyladenine and thymine, acycloguanosine, and DHPG; 28). Since these analogues mimic dNTP substrates, it is likely that the resistance mutations affect binding to normal substrates as well as to drugs. In some cases, abnormal substrate binding has been demonstrated (9–11). As shown in Figure 3a and b, these mutations lie within a stretch of 365 amino acids in the central third of the HSV-1 polymerase sequence (1–3). The large size of this region suggests that the protein folds so as to bring together distantly spaced residues to make a functionally active dNTP binding site.

This putative binding site encompasses three of the four most highly conserved regions within the group of aphidicolin-sensitive DNA polymerases (solid boxes in Figure 3a). Conserved sites are important, because they presumably indicate residues required for enzyme function. At least two of these sites (middle two boxes in Figure 3a) are likely to be directly involved in substrate binding, since the drug-resistance mutations described above cluster near these sites.

We have noted that secondary structural predictions of the sequences surrounding the conserved sites yield potential beta-turns. Turns (tetrapeptides causing chain reversals) are also frequently conserved in related proteins (36,37) and may act to preserve the overall domain structure of the protein. Hence, the highly conserved regions may serve to maintain the structure as well as the function of the polymerase active site(s).

An additional feature of the putative dNTP binding site in the HSV-1 DNA polymerase is revealed by aligning this sequence with those from other herpes viruses (Figure 3a). This alignment indicates regions where sequences have been inserted into (or deleted from) one or more of the genes (regions A, B and C). Since these inserts appear in only some of the sequences, they may constitute peptide loops involved in functions other than polymerization (e.g. interactions with other replication proteins), and they may serve to

Figure 3: A region within the HSV-1 DNA polymerase involved in dNTP and drug binding. (A) This frame represents an alignment between amino acid residues 440 to 965 from the HSV-1 (hsv) sequence and the corresponding sequences from the DNA polymerases of other herpes viruses: Epstein Barr (ebv), varicella zoster (vzv) and human cytomegalo (cmv) viruses. Shaded boxes represent the polypeptide chain in each case. Regions A, B and C indicate sequences which appear to have been inserted into (or deleted from) one or more of the sequences. Solid boxes indicate the four most highly conserved sites among polymerases related to HSV-1 (1,3,17). Putative binding sites for dNTP substrates, aphidicolin and pyrophosphate are designated based on the locations of mutations which affect viral sensitivity to nucleotide analogues, aphidicolin or phosphonoacetic acid, respectively. The positions of these mutations are indicated by black vertical lines in the HSV-1 sequence (nucleotide analogue

punctuate subdomains within the dNTP binding site. Our finding that the inserts are flanked by sequences which predict beta-turns are consistent with this possibility.

Description of our mutants. Our aphidicolin-resistant mutants are unique among the collection of HSV-1 DNA polymerase mutants. Several previously described mutants are hypersensitive to aphidicolin (see Figure 3b). Only one marginally resistant mutant has been characterized (25), and it occurs at a site different from ours (mutant TP3.2,4.1,4.4 in Figure 3b). Although a resistance mutation from the closely-related virus HSV-2 is identical to one of ours (Aph^r10,16), we failed to observe the mutator phenotype associated with this HSV-2 mutation (26–28). Either the HSV-2 polymerase is sufficiently different from the HSV-1 enzyme to account for this discrepancy, or the HSV-2 mutant contains a second mutation which causes the mutator phenotype.

Above, we argued that two highly conserved regions within the HSV-1 polymerase participate in substrate binding. The mutation in Aph^r12 (and 14) occurs within the second of these conserved regions at a residue which is invariant among the aligned sequences (Figure 3b). The mutation in Aph^r10 (and 16) lies near the first conserved region, within a region which shows similarity to several eukaryotic polymerases. Again, the mutated residue is invariant. The phenotypes of our mutants (altered sensitivity to substrate analogues) and the conserved nature of their mutation sites suggest that the mutated residues are directly involved in substrate binding. Since we found only two sites which conferred high resistance to aphidicolin in four independently-derived mutants, we suspect that these are the only sites that allow full resistance without disrupting polymerase function.

The Su1 mutation in the Aph^r10-Su1 suppressor mutant maps upstream of any previously described mutations which affect drug sensitivity in the HSV-1 DNA polymerase. This mutation appears to affect binding to phosphonoacetic acid and not to aphidicolin or nucleotide analogue inhibitors, since its presence in an Aph^r10 background does not produce any change in sensitivity to these other inhibitors. Since phosphonoacetic acid is thought to be a pyrophosphate analogue (see below), the Su1 residue may interact exclusively with pyrophosphate and not dNTP substrates.

The Su1 mutation suppresses the hypersensitivity of the Aph^r10 mutant to phosphonoacetic acid. To explain this phenotype, we suggest that hypersensitivity occurs because the Aph^r10 mutation creates a new interaction with phosphonoacetic acid which

mutations) and by a white vertical line (the Su1 mutation). Names and sources of these mutations are indicated in the legend to frame B. (B) The region in the HSV-1 sequence containing mutations which alter substrate binding specificity (residues 595–965). Letters above the sequence designate the positions of these mutations and indicate resistance (r), hypersensitivity (h) or normal sensitivity (s) to aphidicolin. The mutations are, from left to right: RSC-26 and tsD9 (two mutations at the same position) (3,2,23), PFA^r2 (2), Aph^r10 and Aph^r16 (this manuscript) and Aph^r1 from HSV-2 (27), PFA^r1 (2), TP2.4 (3), PFA^r5 (2), PAA^rC (2), AraA^r13 (2), TP3.2 and TP4.1 and TP4.4 (the same mutation in three mutants) (3), Aph^r12 and Aph^r14 (this manuscript), ACG^r4 (2), TP2.7 (3), PAA^r5 (2), AraA^r9 (2). Numbers below the HSV-1 sequence indicate the residue number which initiates each group of conserved residues. Also shown is an alignment between the HSV-1 (hsv) sequence (43) and other DNA polymerase sequences from Epstein Barr virus (ebv) (44), varicella zoster virus (vzv) (45), human cytomegalovirus (cmv) (46), vaccinia virus (47), baculovirus (48), human cells (DNA polymerase alpha) (17), yeast (DNA polymerase I (49) and DNA polymerase III (50)), yeast killer plasmid pGKL1 (51), maize mitochondria (52), adenovirus-2 (53), ϕ 29 bacteriophage (54), PRD1 bacteriophage (55,56) and T4 bacteriophage (57). Only regions which exhibit high similarity are shown in detail. Residues conserved in 4 or more sequences are identified, and – indicates a single, non-conserved residue. Dots indicate that an unspecified number of residues is not shown. The brackets designate two of the most highly conserved regions and correspond to the middle two solid boxes shown in frame A. (C) Residues 524–588 in the HSV-1 sequence are depicted to show the position of the Su1 mutation and relevant alignments with other DNA polymerases, including DNA polymerase I from *Escherichia coli* (58). Sources of sequences and alignment format are indicated in the legend to frame B.

increases the affinity of the polymerase for the drug. Suppression occurs because the Su1 mutation destroys one of the original interactions between the polymerase and the drug. However, because the new interaction created by the Aph^r10 mutation can compensate for this loss, the overall affinity of the double mutant for phosphonoacetic acid is similar to wild type. We are currently testing the prediction that the Su1 mutation alone should weaken the interaction with phosphonoacetic acid and confer a resistance phenotype.

The map position of Su1 occurs near a region which exhibits a potential alignment between the HSV-1 polymerase sequence and that of DNA polymerase I from *Escherichia coli* (Figure 3c). This alignment includes residues 501 (aspartic acid) and 497 (tyrosine) from the DNA polymerase I sequence which are required for 3'–5' editing nuclease activity (40,41). The occurrence of this alignment near the Su1 site, raises the possibility that this site is part of the exonuclease domain in the HSV-1 protein.

Aphidicolin and phosphonoacetic acid binding sites: In Figure 3a, we propose that aphidicolin binds to the polymerase within the same region proposed for dNTP binding. This overlap appears likely because many mutations which affect nucleotide analogue sensitivity also affect sensitivity to aphidicolin (see Figure 3b). The dNTP and aphidicolin binding sites are not identical, however, since some substrate specificity mutations do not alter aphidicolin sensitivity (Figure 3b). Since aphidicolin has no obvious chemical similarities with dNTP substrates, its mechanism of inhibition is unclear. It may mimic some cellular substance which modulates DNA polymerase activity or may possess obscure structural features shared by dNTP substrates.

The proposed dNTP binding site also appears to overlap the phosphonoacetic acid and pyrophosphate binding sites. Most mutations which affect sensitivity to nucleotide analogues also affect sensitivity to phosphonoacetic acid (2,3,25). Our mutants are hypersensitive (Aph^r10,16) or resistant (Aph^r12,14) to this drug. Phosphonoacetic acid may be a pyrophosphate analogue since it competitively inhibits pyrophosphate exchange by the turkey herpes simplex polymerase (42) and since a related compound, phosphonoformic acid, competitively inhibits pyrophosphate exchange by the HSV-1 enzyme (10). The observation that the Su1 mutation affects sensitivity to phosphonoacetic acid but not other inhibitors suggests that the pyrophosphate binding site may extend downstream of the other binding sites (Figure 3a).

Conclusions: Aphidicolin appears to be a useful tool for probing the substrate binding site in DNA polymerases and for identifying residues which interact with substrates and/or inhibitors. The model which emerges from these studies suggests that the binding sites for aphidicolin, dNTPs and pyrophosphate are in close proximity and overlap substantially. Cumulative knowledge of phenotypes and sequence changes associated with the substrate specificity mutants from HSV-1 should aid in structural speculation about these binding sites.

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This manuscript is dedicated to the memory of Paul Howard-Flanders (former thesis adviser of J.D.Hall)

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