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Engineered Herpes Simplex Virus DNA Polymerase Point Mutants: the Most Highly Conserved Region Shared among α-Like DNA Polymerases Is Involved in Substrate Recognition

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Eucaryotic, viral, and bacteriophage DNA polymerases of the α-like family share blocks of sequence similarity, the most conserved of which has been designated region I. Region I includes a YGDTDS motif that is almost invariant within the α-like family and that is similar to a motif conserved among RNA-directed polymerases and also includes adjacent amino acids that are more moderately conserved. To study the function of these conserved amino acids in vivo, site-specific mutagenesis was used to generate herpes simplex virus region I mutants. A recombinant virus constructed to contain a mutation within the nearly invariant YGDTDS motif was severely impaired for growth on Vero cells which do not contain a viral polymerase gene. However, three recombinants constructed to contain mutations altering more moderately conserved residues grew on Vero cells and exhibited altered sensitivities to nucleoside and PP_i analogs and to aphidicolin. Marker rescue and DNA sequencing of one such recombinant demonstrated that the region I alteration confers the altered drug sensitivity phenotype. These results indicate that this region has an essential role in polymerase function in vivo and is involved directly or indirectly in drug and substrate recognition.

The mechanisms by which polymerases recognize nucleic acid templates and nucleotide substrates and catalyze faithful replication are largely unknown. Clues to common strategies among polymerases can be discerned in structure-function relationships among these enzymes. Eucaryotic DNA polymerases α and δ and many other DNA polymerases of viruses and bacteriophages share at least six discrete regions of sequence similarity that define a family of α-like polymerases (7, 48). These regions occur in the same spatial order in the various family members and have been designated I through VI, in decreasing order of similarity (32, 45). Region I, which was first noted among DNA polymerases of herpes, pox, and adenoviruses (2, 18, 22, 38), is characterized both by a Tyr-Gly-Asp-Thr-Asp-Ser motif that is almost invariant within the α-like polymerase family and by adjacent residues that are more moderately conserved (Fig. 1). Region I shares similarity with a sequence motif found in RNA-directed RNA and DNA polymerases (2). The extensive conservation at this site strongly suggests functional importance and a similarity in the mechanisms of replication by a wide variety of polymerases, but the function of region I remains unknown.

The DNA polymerase (Pol) encoded by herpes simplex virus (HSV) serves as an excellent prototype for the α-like polymerase family especially because of the ability to study polymerase functions in vivo in the authentic context of the virus-infected cell by using genetic and pharmacological tools. HSV is sensitive to a number of nucleoside and PP_i analogs and to aphidicolin, all of which act via inhibition of HSV Pol (16, 19, 20, 30, 35, 37, 40). This has allowed the isolation of HSV DNA polymerase (*pol*) mutants with altered sensitivities to these inhibitors. Since these drugs mimic natural deoxynucleoside triphosphate or PP_i substrates and/or inhibit Pol competitively with these substrates, *pol* mutations that confer altered drug sensitivity change amino acids involved directly or indirectly in interactions with the drugs and cognate substrates. Since certain of these drugs, such as the nucleoside analog acyclovir (ACV), are valuable in treating herpesvirus infections, studies of drug-resistant mutants can also have clinical implications. Indeed, such *pol* mutants are a problem of increasing importance in the clinic (15, 41).

To date, mapping and sequencing of HSV *pol* mutations conferring altered drug sensitivity (21, 22, 24, 27, 32, 44) have provided the most information regarding the function of certain regions of sequence similarity shared by α-like polymerases. One mutation alters an amino acid in sequence similarity region V (Fig. 1 and 2). Three mutations alter a portion of HSV Pol termed region A, which shares similarity with viral polymerases that are sensitive to certain antiviral drugs but does not share similarity with DNA polymerase α. By far, however, most of the mutations alter amino acids in sequence similarity regions II and III, which has led to the proposal that these regions directly participate in drug and substrate recognition (21).

On the other hand, no spontaneous drug-resistant mutants were recovered with alterations in region I. We therefore undertook experiments to engineer region I mutants of HSV. Analysis of these mutants has shed light on region I function.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as described previously (47). DP6 cells were derived from Vero cells and contain the HSV *pol* gene. Virus HP66 was derived from wild-type strain KOS and contains a 2.2-kb *pol* deletion and an insertion containing the *Escherichia coli lacZ* gene

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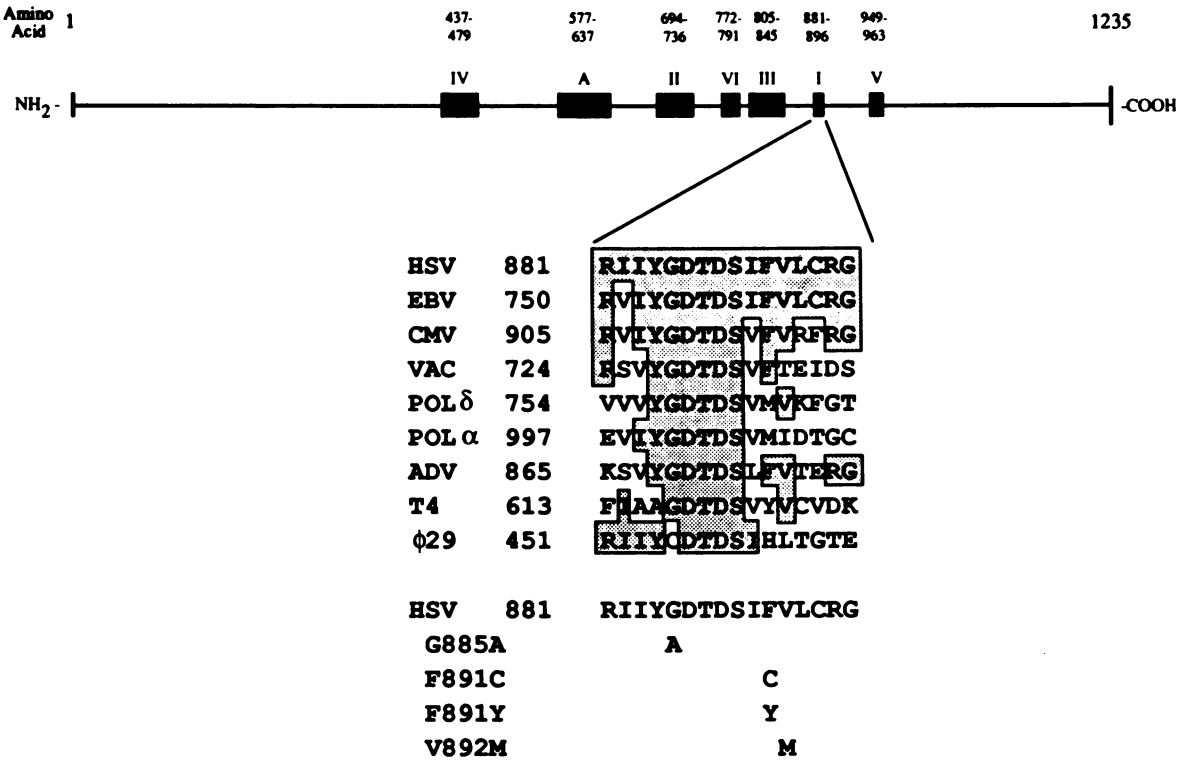

FIG. 1. Region I of sequence similarity among DNA polymerases. The top line is a schematic of the predicted HSV Pol polypeptide with the locations of regions of sequence similarity shown. Below are shown the amino acid sequences of region I from various DNA polymerases. Amino acids shared between HSV and other polymerases are shaded. The following polymerases are represented: HSV (22, 38); Epstein-Barr virus (EBV) (3); human cytomegalovirus (CMV) (29); vaccinia virus (VAC) (18); yeast Pol III, the analog of mammalian DNA polymerase δ (POLδ) (7); human DNA polymerase α (POLα) (48); adenovirus (ADV) (23); T4 (42); and φ29 (50). At the bottom of the figure are the alterations in region I discussed in Results.

under the control of the HSV thymidine kinase promoter. The derivation of the DP6 cell line and the use of virus HP66 for identifying *pol* recombinants are described in detail elsewhere (36).

Mutagenesis. Site-specific mutagenesis was performed by using the method of Taylor et al. (43) with an M13 clone containing a portion of *pol*. The phage vector M13mp18 (49) was modified as follows to include a unique *Nco*I site in the polylinker region. Replicative form M13mp18 was digested with *Sal*I, treated with the Klenow fragment of *E. coli* Pol I, and then ligated to *Nco*I linkers. After digestion with *Nco*I, the vector was treated with ligase and was used to transform *E. coli*. The resulting replicative form DNA was digested with *Kpn*I and *Nco*I and ligated to the 623-bp *Kpn*I-*Nco*I fragment from pDP1, which encodes Pol amino acids 696 to 907 (Fig. 2). Two oligonucleotide preparations (bases 2976 to 2993 and 2997 to 3014 [22]) were used for site-specific mutagenesis. Oligonucleotides 5′-GTGTCCCCGTAGATGA TG-3′ and 5′-CACAGCACAAATATGGAG-3′ bound to the coding strand of *pol* and the underlined bases were synthesized with a phosphoramidite mixture containing 50% of the correct base and 50% of an equimolar mixture of the remaining three incorrect bases. Clones containing region I mutations but no others were identified directly by DNA sequencing by using previously described procedures (21). The 511-bp *Bsu*36I-*Nco*I fragments containing the region I mutations were recovered and ligated to the 6-kb *Bsu*36I-*Nco*I fragment from pDP1 to generate full-length clones containing altered *pol* genes.

Marker rescue construction of mutants and mutation mapping. Marker rescue was performed as described previously (8) by using infectious HP66 DNA and region I mutant plasmid DNAs or by using infectious F891C DNA and the indicated fragments from plasmid pDP1 (36) containing wild-type strain KOS *pol* sequences. To determine the sequence alteration conferring altered drug sensitivity in mutant F891C, the *Bam*HI Q fragment from this virus, which corresponds to the largest *Bam*HI fragment depicted in Fig. 2 (encoding amino acids 1 to 1072), was cloned into pGEM7Zf (+) (Promega Biotec), and the pertinent region within the resulting double-stranded plasmid DNA was sequenced with a universal sequencing primer and *pol*-specific oligonucleotide primers (21) and Sequenase (U.S. Biochemicals) according to the manufacturer's instructions.

Drug assays. Plaque reduction assays to determine relative sensitivities of recombinant and wild-type viruses to antiviral drugs were performed as described by Coen et al. (11). ACV was kindly provided by D. Barry, Burroughs Wellcome Co., and was prepared as described previously (14). Phosphonoacetic acid (PAA) was generously provided by S. Schmidt, Abbott Laboratories, and prepared as described by Coen et al. (13). Aphidicolin was provided by M. Suffness, National Cancer Institute, and prepared as described previously (12).

RESULTS

Strategy for engineering recombinant HSV with region I mutations. To examine the role of region I, we initiated

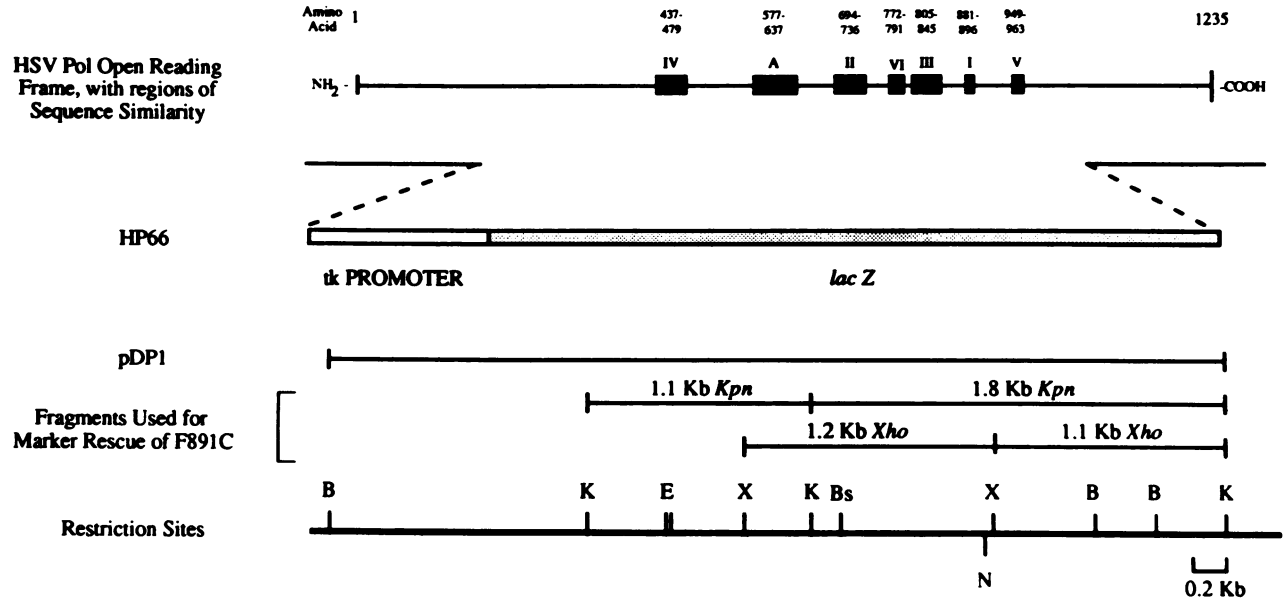

FIG. 2. HSV *pol* locus. The top line indicates the major HSV *pol* open reading frame with the location of regions of sequence similarity (21, 48) shown. Lower lines show the structures of virus HP66, plasmid pDP1, and DNA fragments used for marker rescue. B, *Bam*HI; Bs, *Bsu*361; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; X, *Xho*I; tk, thymidine kinase. The 1.1-kb *Xho*I fragment was generated by cutting pDP1 with *Xho*I and with *Eco*RI, which cuts in the plasmid polylinker just outside the HSV insert.

studies to recover HSV mutants containing lesions in region I of the *pol* gene. A bacteriophage M13 vector containing the 623-bp *Kpn*I-*Nco*I fragment that encodes Pol amino acids 623 to 907, including region I (Fig. 2), was used for site-specific mutagenesis with oligonucleotide pools that encoded alterations in region I. Four alterations in region I were chosen for further study and are designated by the one-letter amino acid code for the wild-type residue, the amino acid number, and the new amino acid. The mutations were G885A (GGG-GCG), F891C (TTT-TGT), F891Y (TTT-TAT), and V892M (GTG-ATG). The M13 phage DNAs were sequenced to ascertain that these mutations were the only ones created within the 623 bp of HSV sequences, and the mutant fragments were inserted into otherwise wild-type *pol* sequences from plasmid pDP1 (36) (Fig. 2).

HSV recombinants were then engineered to contain the mutations by using these region I mutant plasmids in marker rescue experiments with HSV mutant HP66 (36) (Fig. 2). HP66 lacks 2.2 kb of *pol* sequences, which have been replaced by a *lacZ* gene under the control of the HSV thymidine kinase promoter. As a result, HP66 does not grow on Vero cells but can grow on DP6 cells, which contain the wild-type HSV *pol* gene. On DP6 cells, HP66 forms blue plaques in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) because of the *lacZ* insertion. Recombinant viruses can then be generated by marker rescue in which mutant *pol* sequences replace the *lacZ* gene. The recombinants are identified by the ability to form white plaques in the presence of the indicator X-Gal, indicating the loss of *lacZ*.

HSV mutant G885A contains a lethal *pol* mutation. DP6 cells were transfected with infectious HP66 viral DNA alone, in combination with a plasmid containing wild-type *pol* (pDP1), or in combination with a plasmid containing the mutation G885A. The progeny were plated on both Vero and DP6 cells (Table 1). Plasmid pDP1 rescued the lethal mutation in HP66 efficiently; 7.6% of the progeny grew on Vero cells. The plasmid with the G885A alteration failed to achieve marker rescue efficiencies greater than those found with HP66 viral DNA alone (<0.002%). However, 1.5% of the progeny from this transfection formed white plaques in the presence of X-Gal on DP6 cells (data not shown), indicating that recombination had occurred and resulted in the loss of *lacZ*. Several of these white plaques were picked and screened for the replacement of *lacZ* sequences by *pol* sequences by Southern blot hybridization. One such recombinant was tested for its ability to grow on Vero cells versus DP6 cells. It formed plaques on DP6 cells at least 620-fold more efficiently than on Vero cells (data not shown). Because DP6 cells contain no intact HSV gene other than *pol*, this demonstrates that HSV mutant G885A contains a lethal *pol* mutation and indicates that the G885A mutation altering region I inactivates an essential *pol* function.

Alterations at residues 891 and 892 are not lethal. In contrast to the results with the G885A plasmid, transfection of DP6 cells with HP66 DNA in combination with any of the three plasmids encoding alterations in amino acids 891 and

TABLE 1. Marker rescue of HP66

Plasmid	Marker rescue efficiency[a]
None	0.004
pDP1	**7.6**
pG885A	<0.002
pF891C	**0.67**
pF891Y	**1.1**
pV892M	**1.9**

[a] Results are the average of two experiments and are expressed as (titer on Vero cells/titer on DP6 cells) × 100. Numbers in boldface are regarded as positive for marker rescue.

892 resulted in at least a 170-fold increase in the percentage of viruses able to grow on Vero cells compared with transfection with HP66 DNA alone (Table 1). Similar percentages of the progeny could form white plaques in the presence of X-Gal on DP6 cells (not shown). Progeny from the transfections with F891Y, F891C, and V892M that formed white plaques on DP6 cells were picked and plaque purified three times for further analysis. They exhibited plating efficiencies on Vero cells relative to DP6 cells ranging from 40 to 100%, which was similar to that of wild-type strain KOS. Thus, the mutations in these viruses did not meaningfully impair HSV growth in cell culture.

The F891C, F891Y, and V892M mutants exhibit altered drug sensitivities, including resistance to ACV. Since *pol* mutations can result in altered sensitivity to inhibitors of DNA replication, we examined the sensitivities of the viable region I recombinant viruses to PAA, ACV, and aphidicolin. We compared the drug sensitivities of the recombinants with those of wild-type KOS virus in plaque reduction assays in which virus is plated on Vero cells at various concentrations of PAA, ACV, and aphidicolin and the number of plaques is counted at each concentration. The concentration of PAA that reduced plaque formation by 50% for virus F891C was sixfold greater than that for wild-type strain KOS (Fig. 3A). Recombinants F891Y and V892M exhibited intermediate levels of resistance. Results with ACV (Fig. 3B) showed a similar trend; virus F891C was again sixfold more resistant to the drug than wild-type KOS, whereas F891Y and V892M were slightly less resistant. F891C was 10-fold more sensitive to aphidicolin than wild-type KOS, whereas F891Y and V892M had sensitivities closer to yet distinct from that of the wild type (Fig. 3C). On the basis of these assays and previously established criteria for altered drug sensitivities (8, 11), we conclude that recombinants F891C, F891Y, and V892M are resistant to PAA (PAA^r), resistant to ACV (ACV^r), and hypersensitive to aphidicolin (Aph^{hs}).

Demonstration that a region I mutation confers altered drug sensitivity. We wished to verify that the altered drug sensitivities were due to a region I *pol* mutation. As a first test, we examined independent recombinants that arose from transfections with the region I mutant plasmids F891C and V892M and found that they were indistinguishable in terms of their resistances to PAA and ACV and hypersensitivity to aphidicolin from mutants F891C and V892M (not shown). This ensures that their phenotypes were not due to adventitious mutations.

As a second test, we performed marker rescue experiments using DNA fragments from plasmids containing wild-type *pol* sequences and infectious DNA from mutant F891C. This mutant was chosen because its aphidicolin hypersensitivity was great enough to permit marker rescue mapping of the Aph^{hs} mutation. The wild-type fragments tested for marker rescue included region I of *pol* as well as sequences outside of this site (Fig. 2). The progeny of transfections with mutant F891C DNA alone formed no plaques in the presence of 0.1 or 0.3 μg of aphidicolin per ml. Reproducibly, the 1.8-kb *Kpn*I and the 1.2-kb *Xho*I fragments that span region I were able to rescue F891C efficiently for growth in the presence of 0.1 or 0.3 μg of aphidicolin per ml (Table 2). The 1.1-kb *Kpn*I and 1.1-kb *Xho*I fragments, which do not include region I, were not able to rescue the Aph^{hs} marker. Thus, the marker conferring increased sensitivity to aphidicolin in F891C mapped to the 683 bp between the *Kpn*I and *Xho*I sites that includes region I.

Recombinant viruses resulting from the marker rescue experiments using either the 1.8-kb *Kpn*I or the 1.2-kb *Xho*I fragments were plaque purified and tested for their sensitivities to PAA, ACV, and aphidicolin. These viruses were no longer resistant to PAA and ACV or hypersensitive to aphidicolin and instead showed sensitivities to these drugs indistinguishable from those of wild-type virus (data not shown).

These results localized the PAA^r, ACV^r, and Aph^{hs} markers of mutant F891C to the 683 bp between the *Kpn*I and *Xho*I sites of *pol* that include region I. The *Bam*HI Q fragment of virus F891C that includes this region was cloned, and the 683 bp were sequenced. The only alteration from the wild-type sequence was the T to G change resulting in the F891C mutation. We conclude that the F891C mutation altering region I confers resistance to PAA and ACV and hypersensitivity to aphidicolin.

DISCUSSION

Our results show that a virus engineered to contain a G885A alteration in region I was severely impaired for growth on Vero cells, whereas viruses engineered to contain the alterations F891C, F891Y, and V892M could grow on Vero cells but had drug sensitivities different from those of wild-type virus. For mutant F891C, mapping of the Aph^{hs} mutation and sequencing of the relevant *pol* region showed unambiguously that the altered drug sensitivity phenotypes are due to the region I alteration. These in vivo results extend and broaden results from studies of similar mutations in vitro and have implications specifically for drug resistance and replication in HSV and generally for the function of region I in nucleic acid polymerases in a wide variety of organisms.

Comparisons with studies of region I mutants in vitro. We are aware of two studies examining the effects of in vitro enzyme activities of alterations in region I of DNA polymerases, both of which appeared after the present study was completed (6, 17), and of similar studies of an equivalent region in an RNA-directed polymerase (33, 34). Bernad et al. (6) found that various alterations in region I of ϕ29 DNA polymerase decreased one or more polymerase activities without affecting 3′-5′ exonuclease activity. Dorsky and Crumpacker (17) reported that various alterations in region I of HSV Pol including a G885 to Arg substitution also decreased polymerase activity when the *pol* gene was expressed by in vitro transcription-translation. These results are consistent with the findings in vivo with our HSV G885A mutant, but none of the mutant enzymes was examined for altered sensitivity to inhibitors.

Five of six mutant human immunodeficiency virus reverse transcriptases with alterations in the portion thought to resemble region I (1), including one with an alteration at a site that can be considered equivalent to G885, were inactive in vitro (33, 34). The single human immunodeficiency virus mutant that was active enough to examine was altered at a residue that can be considered to be equivalent to Val-892. Its enzyme exhibited two- to threefold increases in 50% inhibition values for azidothymidine triphosphate and phosphonoformic acid in vitro; however, the mutant was described as resembling the wild-type enzyme in sensitivity (34).

Although none of the aforementioned results using an in vitro approach are necessarily inconsistent with ours, we would emphasize that the mutations described in this report were analyzed in the authentic in vivo context of virus-infected cells. Because DNA replication is dependent on

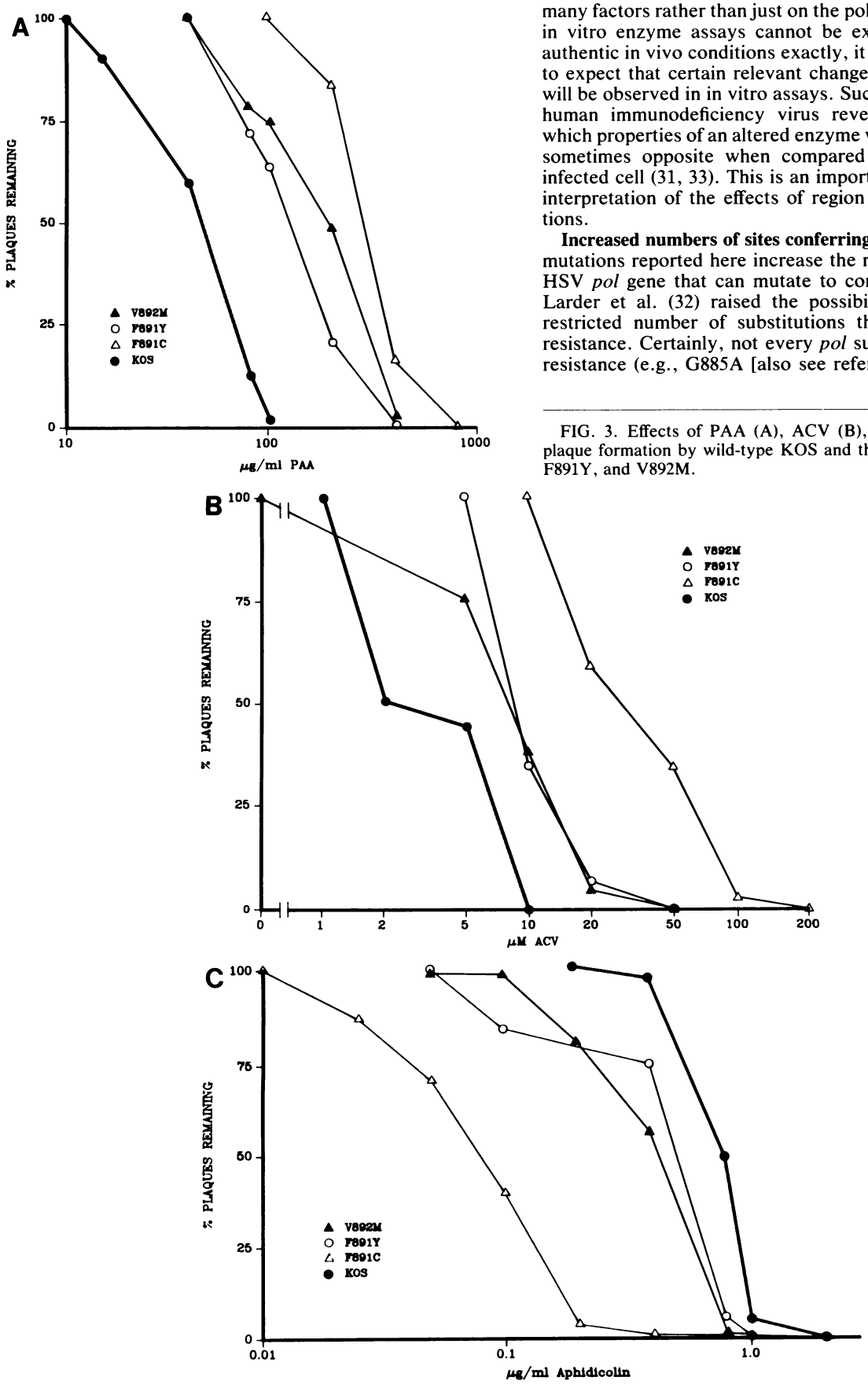

FIG. 3. Effects of PAA (A), ACV (B), and aphidicolin (C) on plaque formation by wild-type KOS and the recombinants F891C, F891Y, and V892M.

many factors rather than just on the polymerase and because in vitro enzyme assays cannot be expected to reproduce authentic in vivo conditions exactly, it may be unreasonable to expect that certain relevant changes in enzyme function will be observed in in vitro assays. Such is the case with the human immunodeficiency virus reverse transcriptase, in which properties of an altered enzyme were not identical and sometimes opposite when compared in vitro and in the infected cell (31, 33). This is an important consideration for interpretation of the effects of region I or other *pol* mutations.

Increased numbers of sites conferring ACV resistance. The mutations reported here increase the number of sites in the HSV *pol* gene that can mutate to confer ACV resistance. Larder et al. (32) raised the possibility that there are a restricted number of substitutions that can confer ACV resistance. Certainly, not every *pol* substitution can confer resistance (e.g., G885A [also see reference 10; J. S. Gibbs

TABLE 2. Marker rescue of Aph^{hs} marker in F891C

Expt no.	Aphidicolin concn (μg/ml)	Progeny[a] of F891C DNA + the following fragment:				
		None	1.8-kb *Kpn*I	1.1-kb *Kpn*I	1.2-kb *Xho*I	1.1-kb *Xho*I
1	0.1	<0.01	**1.7**	<0.02	**0.38**	ND
2	0.3	<0.03	**2.5**	ND	**0.6**	<0.007

[a] Results are expressed as percentage of progeny plating at the indicated concentration of aphidicolin. Values in boldface are regarded as positive for marker rescue. ND, Not determined.

and D. M. Coen, unpublished results]); nevertheless, in combination with earlier studies (21, 24, 27, 32, 44), there have now been reported at least 10 and perhaps as many as 17 that do. How many such substitutions would be likely to cause ACV resistance in a clinical setting (15, 41) is unknown. Certain of the mutations confer only a modest degree of resistance (Fig. 3). It will be interesting to determine the effects of the region I mutations on replication and pathogenicity of HSV in a mammalian host.

The failure to uncover region I mutations during earlier sequencing studies seems likely to have resulted simply from not having examined enough drug-resistant mutants.

An essential role for region I in vivo. Our results indicate that G885A is a lethal substitution. This in turn indicates that Gly-885 and region I as a whole serve an essential function in vivo during viral replication, as would be expected from the high degree of sequence conservation. Since Gly to Ala is a conservative alteration, one explanation for the detrimental effect of this mutation is that it affects the conformation of Pol at a site important for catalysis. Indeed, secondary structure predictions made by using a variety of programs argue that this conserved region is likely to include a turn (2, 4, 18, 25, 28) to yield a beta-hairpin with a loop containing the conserved Asp and Thr residues (1). The Gly-Ala alteration could disrupt this structure as the Gly and Cys residues found at the position equivalent to Gly-885 in other DNA polymerase sequences have increased probabilities for participating in turn structures, whereas Ala has a decreased probability (9). Although we have ruled out major effects of the G885A mutation on polypeptide stability on the basis of Western immunoblot analysis of G885A-infected cells (unpublished results), we cannot exclude several other alternative explanations for the lethal effects of this mutation. For example, it has been proposed that the role of region I is in interactions with conserved host cell factors (32, 37). However, the results with our other region I mutants (see below) lead us to favor a role in polymerase catalysis, as we and others originally proposed (2, 18, 22).

Region I and substrate recognition. The demonstration that region I alterations confer altered drug sensitivities sheds light on the role of this region in Pol function. Since the drugs have structures similar to those of natural PP_i and dNTP substrates or can compete with these substrates, we infer that region I is involved in substrate recognition. Results from two bacteriophage systems are consistent with this conclusion. Inokuchi and Hirashima (26) have suggested that a region in Qβ replicase similar to region I is important for catalysis but not template activity, on the basis of interference of wild-type Qβ replication by plasmids bearing mutations in these sequences. After the present study was completed, Bernad et al. (6) found that φ29 region I mutations can eliminate initiation activity (adding 5′-dAMP to the φ29 terminal protein bound to φ29 DNA) without effects on 3′-5′ exonuclease activity or interactions with the terminal protein. Although effects on DNA binding were not excluded, Bernad et al. proposed that region I is part of the dNTP-binding site. Thus, these studies (largely by process of elimination) and our studies through altered drug sensitivity phenotypes support the involvement of region I in substrate recognition.

Region I therefore joins regions II, III, V, and A as sites sharing this involvement (21, 24, 27, 32, 44) (Fig. 1 and 2). The finding that region I mutations confer phenotypes similar to those conferred by mutations in other regions supports the model (21) in which these various regions fold together to help form the binding sites for PP_i and dNTP substrates. Indeed, it is tempting to speculate that this portion of the polymerase defines a polymerizing domain, whereas studies of bacteriophage DNA polymerases (5, 39) indicate that region IV defines an active site for a more N-terminal 3′-5′ exonuclease domain and in vitro studies of HSV Pol have identified a more C-terminal binding site for the polymerase accessory protein, UL42 (P. Digard and D. M. Coen, J. Biol. Chem., in press).

As yet, there are too few region I mutations to say that region I participates directly in substrate recognition, as we have proposed for regions II and III (21). Rather, region I mutations may exert their effects on drug sensitivity indirectly. Argos (1) has emphasized the likely importance of the two region I Asp residues; perhaps, the nearby residues altered in F891C, F891Y, and V892M alter the reactivities of these Asp residues. Detailed enzymological studies coupled with X-ray crystallographic solutions of polymerase structure will probably be required to determine the precise role of region I in polymerase function.

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