

## Site-Specific Mutagenesis of a Highly Conserved Region of the Herpes Simplex Virus Type 1 DNA Polymerase Gene

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Seven point mutations were introduced into region I of the herpes simplex virus type 1 DNA polymerase, which is most highly conserved among DNA polymerases and has no drug sensitivity markers mapped to it. The functional consequences of these mutations were studied in an in vitro transcription-translation system in which T7 transcripts of cloned polymerase genes were used to generate enzymatically active polypeptides in reticulocyte lysate. Analysis of labeled polypeptides on sodium dodecyl sulfate-polyacrylamide gel electrophoresis failed to show any alterations of stability caused by these mutations. The mutations G885R, D886N, T887K, D888A, and G896V lacked polymerase activity and failed to be stimulated by cotranslation of the herpes simplex virus 65-kilodalton DNA-binding protein, whereas R881G and S889A retained both polymerase activity and the capacity to be stimulated by the 65-kilodalton DNA-binding protein.

Herpes simplex virus (HSV) type 1 (HSV-1) DNA polymerase serves as a useful model for the study of viral DNA replication and its inhibition by antiviral drugs. It is now clear that HSV polymerase is a member of a large family of DNA polymerases which includes those from bacteriophages, other animal viruses, and replicative polymerases of lower eucaryotes (*Saccharomyces cerevisiae*) and higher eucaryotes (human) (1-3, 8, 13, 15, 19, 20, 26, 28, 31, 32). The basis for grouping these proteins in one family is that they share regions of striking sequence similarity and that these regions occur in the same order in all members. The sequence relatedness of these proteins suggests that they may share basic molecular mechanisms and that functional sites may reside within the conserved regions and can help to direct studies aimed at understanding the roles of particular sites in polymerase function.

Little is known, at present, about functional domains within HSV polymerase, in contrast to several of the prokaryotic DNA polymerases. The structure of *Escherichia coli* polymerase I has been solved (22), and much is understood about how various domains within that protein contribute to enzymatic activity (9, 14, 22). Although several enzymatic activities are associated with HSV polymerase (deoxynucleoside triphosphate binding, DNA chain elongation, a 3' exonuclease, and pyrophosphate binding) (6, 16, 25), the localization of these activities within the 140-kilodalton (kDa) protein molecule is not apparent. Recent characterization of altered drug sensitivity markers in HSV polymerase has revealed several mutations occurring in three of the six most highly conserved regions of the polymerase gene, *pol* (12, 18, 20, 29). Because of clustering of mutations in regions II and III it has been hypothesized that amino acids in these regions interact directly with deoxynucleoside triphosphates and their analogs (12, 20). Region I (Fig. 1), the most highly conserved region, is of particular interest, since no drug resistance mutations have been found in it. Also, it has been shown that a 78-residue deletion spanning region I abolishes enzyme activity and is lethal to the function of the gene in a biological complementation assay

(7). Because of the significant evolutionary conservation of region I and the fact that no function has been assigned to it, the construction of mutants with alterations at the sites of conserved residues should be helpful in learning the role of region I in polymerase function. Also, because DNA polymerase enzymatic activity can be detected by in vitro transcription-translation of cloned HSV *pol* genes, this allows the study of the effects of mutations introduced into the *pol* gene on enzymatic activity. This system allows for the screening of mutants with intact enzymatic activity which may lack biological activity due to intracellular instability or failure to interact properly with other elements of the viral replicative machinery. Seven point mutations were introduced into *pol* region I by the strategy shown in Fig. 2 by

HSV	881	R	I	I	Y	G	D	T	D	S	I	F	V	L	C	R	G
EBV	750	R	V	I	Y	G	D	T	D	S	L	F	I	E	C	R	G
CMV	905	R	V	I	Y	G	D	T	D	S	V	F	V	R	F	K	G
VZV	840	K	V	I	Y	G	D	T	D	S	V	F	I	R	F	K	G
VAC	724	R	S	V	Y	G	D	T	D	S	V	F	T	E	I	D	S
AD2	865	K	S	V	Y	G	D	T	D	S	L	F	V	T	E	R	G
HU $\alpha$	997	E	V	I	Y	G	D	T	D	S	I	M	I	N	T	N	S
YPI	991	L	V	V	Y	G	D	T	D	S	V	M	I	D	T	G	C
T4	613	F	I	A	A	G	D	T	D	S	V	Y	V	C	V	D	K
$\phi$ 29	451	R	I	I	Y	C	D	T	D	S	I	H	L	T	G	T	E
PRD1	423	R	P	L	Y	C	D	T	D	S	I	I	C	R	D	L	K

FIG. 1. Conserved region I of HSV-1 DNA polymerase. Amino acid residues 881 through 896 from HSV-1 DNA polymerase were aligned with amino acid sequences of other DNA polymerases to show sequence similarities between human viruses (HSV-1, Epstein-Barr virus [EBV], cytomegalovirus [CMV], varicella zoster virus [VZV], vaccinia virus [VAC], and adenovirus type 2 [Ad2]), human DNA polymerase- $\alpha$ , yeast DNA polymerase I, and three bacteriophage DNA polymerases (T4,  $\phi$ 29, and PRD1). The number of the amino terminal-proximal residue is indicated to the left of the peptide sequence (1-3, 5, 8, 13, 15, 17, 19, 20, 24, 26, 28, 30-32).

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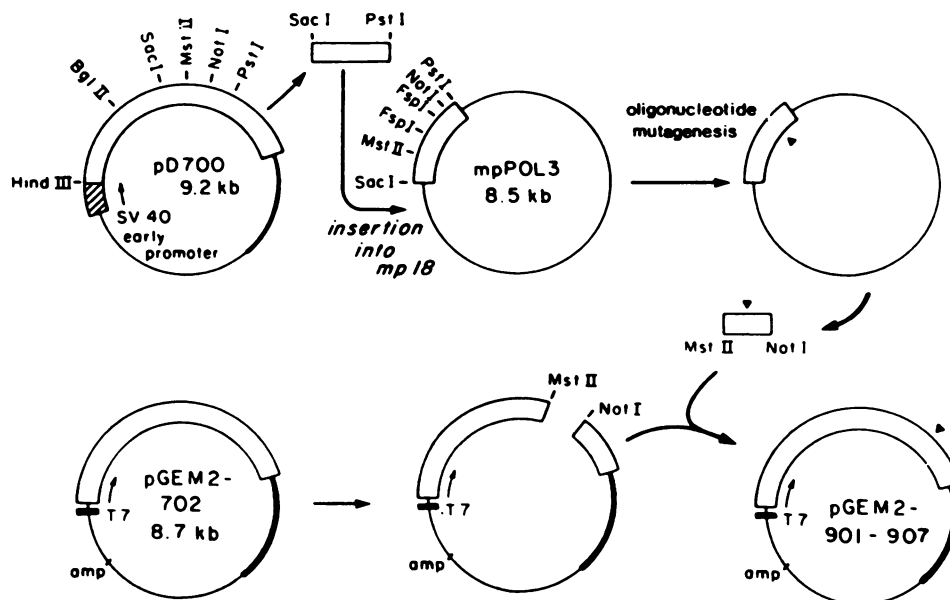


FIG. 2. Mutagenesis strategy. A fragment of the HSV-1 *pol* gene containing region I was subcloned into mp8 (7). Oligonucleotides were synthesized on an Applied Biosystems machine, gel purified, and shown to prime correctly in a dideoxy-sequencing reaction. Mutagenic 17-mers were synthesized as follows: 901/R881G/C3044G, 5' GATGATGCCCATGGAAT; 902/G885R/G3056A, 5' CGTGCCCTGTAGATGA, 903/D886N/G3059A, 5' GTCCGTGTTCCCGTAGA; 904/T887K/C3063G, 5' TGGAGTCCCTGTCCCGG; 905/D888A/A3066C, 5' AGATGGAGGCCGTGTCC; 906/S889A/T3069G, 5' AAAGATGGCGTCCGTGT; 907/G896V/G3090T, 5' CCGTGAGGACGCGGCAC. The mutagenic oligonucleotides were of negative polarity with respect to the translation frame of the *pol* gene, as was the 16-mer used as a primer to conveniently sequence the region I DNA, 5' CAGCGCGCGAGATG (position 3139). The numbering system used was that of Quinn and McGeoch (26). Mutagenesis was carried out by the method of Nakamaye and Eckstein (21) with an Amersham oligonucleotide-directed in vitro mutagenesis system. The introduction of mutations into mpPOL3 was confirmed by dideoxy sequencing and a 746-base-pair *MstII-NotI* subfragment of mpPOL3 was inserted into a complementary 7.9-kilobase *MstII-NotI* fragment of the T7 expression vector pGEM2-702. The resulting plasmids were named pD901 through pD907. Verification of the construction was carried out by direct dideoxy sequencing of the resulting plasmids, which were used as templates for T7 RNA polymerase transcription.

using oligonucleotide-directed mutagenesis (21). (Plasmid numbers and mutations were as follows: pD901, R881G; pD902, G885R; pD903, D886N; pD904, T887K; pD905, D888A; pD906, S889A; pD907, G896V.) The mutations were introduced into a previously described M13 vector, mp8, containing a 3.5-kilobase *PstI-SacI* fragment of *pol* (mpPOL3) (7), which was used for verification of the mutagenesis by DNA sequencing (27). After mutagenesis, the 746-base-pair *MstII-NotI* subfragments containing wild-type and mutant *pol* sequences were inserted into a complementary 7.7-kilobase *MstII-NotI* fragment of pGEM2-702, and the constructions were verified by direct dideoxy sequencing of the plasmids (4).

The resulting set of T7 expression vectors containing *pol* gene mutations were used as templates for in vitro T7 RNA polymerase transcripts capable of programming in vitro translation in rabbit reticulocyte lysates. In vitro-translated *pol* has been shown to possess DNA polymerase enzymatic activity when assayed by standard methods on an activated calf thymus DNA template (7). The in vitro *pol* RNA was translated in rabbit reticulocyte lysates in the presence of [<sup>35</sup>S]methionine for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis fluorography or in the presence of nonradioactive amino acids for enzyme assays. The major product of the *pol* gene migrates as a 140-kDa polypeptide (7). Included in these assays was the mutant T7-7.1, which specifies an HSV-1 DNA polymerase with a 67-residue amino-terminal deletion expressed as a fusion with the six amino-terminal residues of phage T7 gene 10. As previously reported, this 135-kDa polypeptide possesses up

to fivefold the polymerase activity of the wild-type polypeptide when these activities were assayed by in vitro transcription-translation (7). The mechanism for this enhancement is not understood at present. Wild-type and mutant polypeptides are displayed in Fig. 3; no gross differences between wild-type and mutant polypeptides can be detected. Because incorporation of [<sup>35</sup>S]methionine into the in vitro translation product is linear over the 90-min labeling period and no significant differences in proteolytic degradation could be detected, the results suggest that the *pol* region I point mutants are equally well translated as the wild-type *pol* and that none is preferentially degraded in reticulocyte lysate. When assayed for polymerase activity, mutants 901 and 906 were found to possess significant DNA polymerase activity (Fig. 4). Mutants 902, 903, 904, 905, and 907 lacked any detectable DNA polymerase activity.

The constructed *pol* region I mutants were then characterized with respect to their interaction with the HSV-1 65-kDa DNA-binding protein (DBP). The 65-kDa product of the HSV-1 UL42 open reading frame is a DNA-binding protein (65-kDa) that is essential for viral DNA replication (11, 23). Recently, it has been shown that cotranslation of *pol* and the 65-kDa DBP in reticulocyte lysates results in a specific stimulation of *pol* activity (10). The exact mechanism of this stimulation is unknown, but it is hypothesized that the 65-kDa DBP is a DNA polymerase accessory protein (10, 11, 23). The effect of cotranslation with the 65-kDa DBP was examined with the *pol* region I mutants in an effort to detect augmented activity in the mutants found to be lacking in activity. Only the active mutants 901 and 906 were

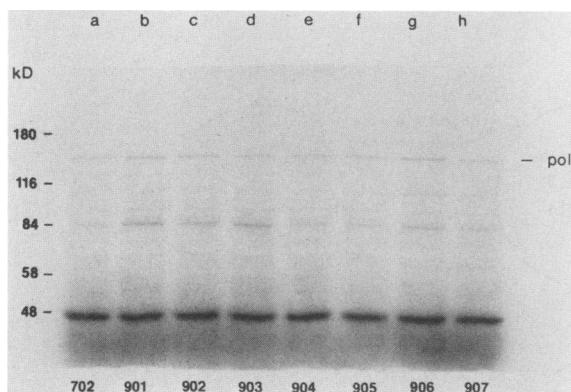


FIG. 3. HSV-1 *pol* region I mutant in vitro translation products. Plasmid templates were linearized at a unique *Xba*I site distal to the coding region, and RNA was prepared by in vitro transcription of the linearized templates with T7 RNA polymerase (Promega Biotech) by previously described methods. The *pol* RNA was translated for 90 min at 30°C in rabbit reticulocyte lysate (Promega Biotech) containing [<sup>35</sup>S]methionine, and the translation products were displayed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Lanes: a, pGEM2-702 (wild type); b, pD901; c, pD902; d, pD903; e, pD904; f, pD905; g, pD906; h, pD907.

stimulated by the 65-kDa DBP (Table 1). The degree of stimulation noted in previous studies varied between 2- and 10-fold (10). None of the active mutants, including the amino-terminal deletion T7-7.1 (67 amino-terminal residues deleted), failed to be stimulated by the 65-kDa DBP. Because none of the inactive mutants was stimulated, it is likely

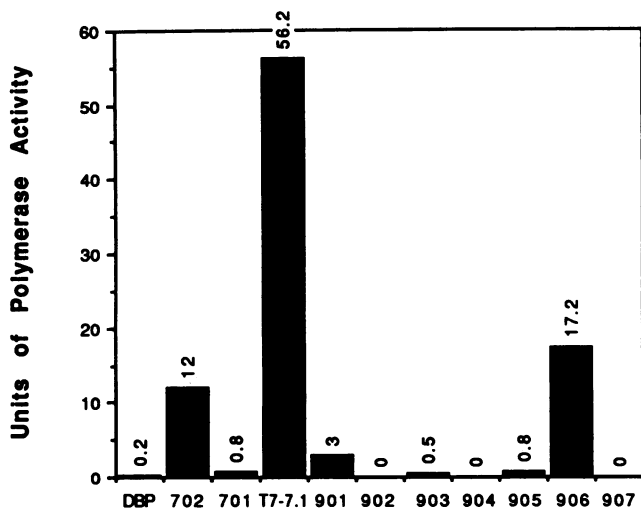


FIG. 4. DNA polymerase activity of in vitro translation products. RNA (4 μg) obtained from linearized templates by in vitro T7 transcription was translated in 25 μl of rabbit reticulocyte lysate for 90 min at 30°C. The 25-μl translation mixtures were assayed for DNA polymerase activity by measurement of acid-precipitable radioactivity incorporated at 37°C for 20 min by using [α-<sup>32</sup>P]dCTP as described previously (7). DBP, 65-kDa DBP obtained from the plasmid LBN19a (10); 701, a deletion mutant of HSV-1 *pol* lacking residues 881 through 959 (7); T7-7.1, a deletion mutant of HSV-1 *pol* lacking the amino-terminal 67 residues (7). One unit of polymerase activity is defined as 1 fmol of [α-<sup>32</sup>P]dCMP incorporated into an activated calf thymus DNA template at 37°C per h per ml of rabbit reticulocyte lysate.

TABLE 1. Effect of 65-kDa DBP on the activity of region I *pol* mutants<sup>a</sup>

Mutant	Polymerase activity (U)		Fold stimulation
	-65-kDa DBP	+65-kDa DBP	
701	0	0	
702 (wild type)	22.4	76.2	3.4
901	13.1	40.3	3.1
902	0	0	
903	0	0	
904	0	0	
905	0	0	
906	19.8	54.0	2.7
907	0	0	
T7-7.1	58.1	118.1	2.0

<sup>a</sup> Runoff transcript RNA (4 μg) prepared with T7 RNA polymerase from linearized plasmids was incubated in 30 μl of rabbit reticulocyte lysate either with (+) or without (-) 4 μg of RNA prepared from the plasmid pLBN19a, which encodes the 65-kDa DBP. After translation at 30°C for 90 min, the entire translation mixtures were assayed for DNA polymerase activity on activated calf thymus DNA template, as described in the legend to Fig. 4.

that none of them possessed activity that was just barely subthreshold for the assay, because stimulation would have augmented it to detectable levels. However, the possibility cannot be ruled out that the inactive mutations specifically affected the interaction between the DNA polymerase and the 65-kDa DBP. The simplest interpretation of these results, however, is that mutations in region I that do not abolish polymerase activity in vitro have no obvious effect on the interaction between the polymerase and the 65-kDa DBP.

In summary, a set of mutants in HSV *pol* region I has been constructed with alterations in seven conserved residues (Fig. 5). When evaluated for their effects on enzymatic function in an in vitro transcription-translation system, five of the seven mutants were found to lack any enzymatic activity. Furthermore, these five mutants failed to show any activity when cotranslated with the 65-kDa DBP, which has been shown to be capable of between 2- and 10-fold stimulation of polymerase activity in this system (10). None of the mutants appeared to be significantly less stable in rabbit reticulocyte lysate than the wild-type polypeptide. Interestingly, two substitutions retained approximately wild-type levels of activity. The 901 mutant (R881G), with about one-third of the wild-type activity, nevertheless retained the ability to be stimulated by the 65-kDa DBP. The R881 residue happens to be present in six of the family members shown, with another basic residue, lysine, present at that position in two other members (Fig. 1). *S. cerevisiae* DNA polymerase I, human polymerase-α, and bacteriophage T4 DNA polymerase genes lack a basic residue at this position. The other enzymatically active mutant, 906 (S889A), is also capable of stimulation by the 65-kDa DBP. It is surprising that a residue that is conserved in every family member can be dispensable for enzymatic activity. Besides suggesting



FIG. 5. Summary of activities of HSV-1 *pol* region I mutants.

that the serine hydroxyl is not a critical catalytic group in this case, the result also suggests that certain sequence alterations in region I can be accommodated through interactions with other regions of the protein. The demonstration that point mutations at conserved sites within HSV-1 DNA polymerase region I can inactivate polymerase activity is consistent with an important role for the region I domain in polymerase function. It will be especially interesting to observe the effects of these and other constructed point mutations on deoxynucleoside triphosphate analog interactions, exonuclease activity, biological activity in complementation assays, and interactions with other viral proteins involved in DNA replication.

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