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Polymerization Activity of an α -Like DNA Polymerase Requires a Conserved 3'-5' Exonuclease Active Site

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Most DNA polymerases are multifunctional proteins that possess both polymerizing and exonucleolytic activities. For *Escherichia coli* DNA polymerase I and its relatives, polymerase and exonuclease activities reside on distinct, separable domains of the same polypeptide. The catalytic subunits of the α -like DNA polymerase family share regions of sequence homology with the 3'-5' exonuclease active site of DNA polymerase I; in certain α -like DNA polymerases, these regions of homology have been shown to be important for exonuclease activity. This finding has led to the hypothesis that α -like DNA polymerases also contain a distinct 3'-5' exonuclease domain. We have introduced conservative substitutions into a 3'-5' exonuclease active site homology in the gene encoding herpes simplex virus DNA polymerase, an α -like polymerase. Two mutants were severely impaired for viral DNA replication and polymerase activity. The mutants were not detectably affected in the ability of the polymerase to interact with its accessory protein, UL42, or to colocalize in infected cell nuclei with the major viral DNA-binding protein, ICP8, suggesting that the mutation did not exert global effects on protein folding. The results raise the possibility that there is a fundamental difference between α -like DNA polymerases and *E. coli* DNA polymerase I, with less distinction between 3'-5' exonuclease and polymerase functions in α -like DNA polymerases.

DNA polymerases are central to the replication of genetic material. Much of our information regarding DNA polymerases has come from detailed structural, enzymological, and genetic studies of *Escherichia coli* DNA polymerase I (Pol I) and its relative, T7 DNA polymerase (33, 61). However, most eukaryotic cellular and viral replicative DNA polymerases and certain bacterial and bacteriophage DNA polymerases are members of the α -like polymerase family and share only very limited sequence similarity with Pol I (1, 39, 45, 60, 68). A fundamental question is: Do these enzymes carry out their functions similarly to or differently from Pol I?

DNA polymerases usually contain both polymerase and exonucleolytic activities either on a single polypeptide or as separate subunits. The 3'-5' exonuclease activities associated with polymerases frequently perform proofreading functions, thereby reducing replication errors and mutation rates. A key feature of Pol I is that its polymerase and 3'-5' exonuclease activities reside on a single polypeptide (37) as separate domains with the active sites about 3 nm apart (47). This physical separation translates into functional independence, as revealed by analysis of mutations, including fairly gross deletions, of Pol I and T7 DNA polymerase that inactivate 3'-5' exonuclease activity without major deleterious effects on polymerase activity and processivity (11, 12, 19, 61).

Interestingly, α -like DNA polymerases share sequence similarities with active sites in the 3'-5' exonuclease domain of Pol I (2, 39, 45, 60). Bernad et al. (2) have termed these segments Exo I, Exo II, and Exo III (Fig. 1). Exo I and Exo

II lie within region IV of sequence homology among α -like DNA polymerases (75), while Exo III lies roughly 50 to 150 amino acids downstream in any of the polymerases. That these segments are important for 3'-5' exonuclease activity has been confirmed by mutational analyses of the α -like polymerases of bacteriophages ϕ 29 and T4. Several bacteriophage T4 mutations resulting in either altered mutation rates, reduced 3'-5' exonuclease activity, or both reside in these segments or elsewhere in a region extending from 90 amino acids upstream of Exo I to 20 amino acids downstream of Exo III (52-55). Bernad et al. (2) have shown that mutagenesis of specific residues within Exo I and Exo II of ϕ 29 DNA polymerase results in an enzyme that retains polymerase activity but is deficient in 3'-5' exonuclease activity. These results have led to the hypothesis that α -like DNA polymerases, like Pol I, are modular in organization with relative functional and structural independence of polymerase and exonuclease domains (2, 52, 53).

Herpes simplex virus (HSV) encodes a DNA polymerase (HSV Pol) that is a member of the α -like polymerase family and contains an intrinsic 3'-5' exonuclease activity (41). Because of the ease with which polymerase function can be studied by pharmacological, genetic, and molecular techniques in the authentic context of the virus-infected cell, HSV Pol has been valuable for determining the functional relevance of regions of sequence similarity shared among α -polymerases. In particular, regions I, II, III, V, and A (Fig. 1) are considered to be involved in substrate binding and polymerizing activity, as concluded from the clustering of mutations in these regions that result in alterations in the sensitivity of HSV to various antiviral drugs and aphidicolin, which mimic and/or compete with deoxynucleoside triphosphate (dNTP) and pyrophosphate substrates (22, 28, 31, 35, 38, 42, 67).

To understand the functional importance of one of the

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FIG. 1. The Exo II region of homology shared among diverse DNA polymerases. (A) Schematic of the HSV Pol polypeptide. The locations of regions I to VI of sequence similarity shared among α -like polymerases compiled by Wang et al. (68), region A (22), and the UL42 binding domain (14) are shown as open boxes; the Exo I, II, and III segments of Bernad et al. (2) are depicted as dark boxes. The numbers above the line refer to amino acid residues; the vertical lines below refer to the locations of mutations that confer resistance or hypersensitivity to polymerase substrate analogs. (B) Amino acid sequences of several DNA polymerases in the Exo II segment (2). Numbers refer to amino acid residues. Conserved residues are shaded. Polymerase sequences: *E. coli* Pol I (32), T7 (15), T5 (39), ϕ 29 (78), T4 (60), adenovirus (AdV) (25), human DNA polymerase α (HUM) (75); yeast DNA polymerase δ (CDC2) (4); vaccinia virus (VV) (36, 65); Epstein-Barr virus (EBV) (1), and HSV (23). (C) Mutations described in this report.

reported homologies with the 3'-5' exonuclease active site of Pol I, we have engineered mutations in this region, recombined them into HSV, and examined their effects on polymerase functions in vitro and in vivo. Our results lead us to consider the possibility that α -like DNA polymerases differ fundamentally from *E. coli* Pol I in the relationship between polymerase and 3'-5' exonuclease activities.

MATERIALS AND METHODS

Plasmids and oligonucleotides. pDP1 (42) consists of HSV *pol* sequences extending from about 150 bp upstream of the major open reading frame to about 150 bp downstream, cloned into pUC19. MD18.1 contains the insert of pDP1 cloned into M13mp18 (77) which had been digested with *Hind*III and *Sal*II. All oligonucleotides were purchased from Genetic Designs, Inc., Houston, Tex.

Cells and viruses. African green monkey kidney (lines Vero and CV-1) and human osteosarcoma (line 143) cells were propagated and maintained as described previously

(71). DP6 cells, which contain the HSV *pol* gene and permit the growth of otherwise nonviable HSV *pol* mutants, were propagated and maintained similarly except that they were passaged in G418 (500 μ g/ml; GIBCO Laboratories) once each month. HSV wild-type strain KOS, recombinant virus HP66, and mutants E460D, V462A, and G464V (this report) were grown and titered as described previously (9). The derivation of the DP6 cell line and the use of recombinant virus HP66 for identifying *pol* recombinants are described in detail elsewhere (40, 42).

Mutagenesis. Oligonucleotide-directed site-specific mutagenesis was performed on MD18.1 by the method of Taylor et al. (63), which relies on the resistance of phosphorothioate-modified DNA to digestion by some restriction enzymes. All reagents necessary for this procedure were purchased as a kit from Amersham. The procedure was adapted by using degenerate oligonucleotides contaminated at each residue with 1.2% of each of the other three deoxyribonucleotides during synthesis. The wild-type sequence of the oligonucleotide used in mutagenesis is CCCGAGTTCGTGACTGGGTACAAC. The underlined sequence refers to an *Nci*I re-

striction site that was altered as an internal control without altering the sequence of the encoded protein.

Marker rescue. Marker rescue experiments were performed essentially as described previously (7), with the following exceptions. DP6 cells were used in all cases. A 5-fold molar excess of bacteriophage RF or a fragment derived from pDP1 or a 25-fold molar excess of single-stranded bacteriophage DNA was cotransfected with 1 μ g of infectious DNA. After all cells were infected, virus was harvested and the progeny virus was assayed for titer on Vero cells and DP6 cells (9).

Southern hybridization analysis. To screen virus for proper recombination of mutant sequences, the method of Southern (59) was used. Approximately 18 h after infection of DP6 cells at a multiplicity of infection of 3, DNA was harvested by a modification of the Hirt method (30). Briefly, 6×10^6 infected cells were lysed directly in 1 ml of 0.6% sodium dodecyl sulfate (SDS), and sodium chloride was added to 1 M. After 18 h at 4°C, chromatin was pelleted by centrifugation in a microfuge for 15 min. The supernatant containing HSV DNA was then extracted with phenol and chloroform, followed by ethanol precipitation and RNase digestion by standard methods.

DNA was digested with either *Nci*I (New England BioLabs, Beverly, Mass.) or *Eco*RI (American Allied, Aurora, Colo.) according to the manufacturer's directions and subjected to agarose gel electrophoresis. The resulting digests were transferred to GeneScreen Plus (New England Nuclear, Boston, Mass.) by capillary blotting, cross-linked with UV radiation (8), and hybridized according to the directions supplied by New England Nuclear with a 32 P-labelled oligonucleotide probe. To detect *Eco*RI fragments containing the 3' end of the *pol* gene, an oligonucleotide with the sequence CGCGGGTCTGGGCCACG was used. To detect *Nci*I fragments containing region IV, an oligonucleotide with the sequence CGACCTCCCCGAATCCC was used.

DNA sequencing. DNA sequencing was by the dideoxy synthesis method (57) as previously described (22, 23), using synthetic oligonucleotide primers designed to hybridize with the HSV *pol* gene.

Dot blot hybridization analysis. A modification of the method of Gadler et al. (20) was used to assay replication of HSV DNA in cell culture. DP6 or Vero cells growing in 96-well trays (Falcon) were infected at a multiplicity of infection of 3 and incubated for 18 h at 37°C. The medium was then aspirated, each well was rinsed twice with 200 μ l of trypsin diluent, and the cells were lysed with 200 μ l of 0.5 M NaOH. The lysed cells were then neutralized with 20 μ l of 10 M ammonium acetate and spotted onto GeneScreen II Plus which was prewet with 1 M ammonium acetate–20 mM NaOH in a manifold apparatus (Schleicher & Schuell, Keene, N.H.). The filters were washed with 1 M ammonium acetate–20 mM NaOH (100 μ l per well) and removed from the manifold. The filters were then soaked in 4 $\times$ SSPE for 2 min, UV cross-linked for 5 min (8), and hybridized to a 32 P-labelled oligonucleotide, CGACCTCCCCGAATCCC, complementary to the HSV *pol* gene, according to the directions supplied by New England Nuclear.

DNA polymerase assays. 143 cells in 100-mm dishes were mock infected or infected with wild-type or mutant HSV at a multiplicity of infection of 10 and incubated at 37°C. Eight hours after infection, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS), scraped into PBS, and sedimented by centrifugation at 1,000 $\times$ g for 10 min. The cell pellet was resuspended in 200 μ l of 20 mM Tris-HCl (pH 7.5)–1 mM EDTA–100 mM NaCl–10

mM β -mercaptoethanol. Cells were disrupted by sonication in a cup horn sonicator, and cell debris was removed by centrifugation in a microfuge at 13,000 $\times$ g for 10 min. DNA polymerase was assayed under conditions that favor detection of HSV Pol versus cellular polymerases. The reaction mix contained, in a final volume of 50 μ l, 20 mM Tris-HCl (pH 7.5); 100 mM (NH₄)₂SO₄; 0.1 mM EDTA; 0.5 mM dithiothreitol; 10% glycerol; 500 μ g of activated salmon sperm DNA per ml, prepared as described previously (69); 60 μ M each dATP, dGTP, and dTTP; 5 μ M [32 P]dCTP (1,600 cpm/pmol); and 5 μ l of cell extract (0.75 μ g of protein, as determined by using a Bio-Rad assay kit and bovine serum albumin as a standard). Reactions were started by addition of cell extract and were incubated at 37°C for 20 min. Reactions were terminated by addition of trichloroacetic acid to a final concentration of 10%. The resulting precipitate was collected on a Whatman GF/C filter, and the acid-precipitable radioactivity was estimated in a liquid scintillation counter. To ensure that results were obtained in the linear range of the assay, a standard curve with dilutions of wild-type virus-infected extract in mock extract was performed in parallel.

Western immunoblot analysis. A 30- μ l sample of each cell extract prepared for DNA polymerase assays was incubated under DNA polymerase assay conditions as described above. Following incubation, samples were boiled in SDS sample buffer, electrophoresed through SDS-polyacrylamide gels, and then transferred to nitrocellulose as described by Towbin et al. (64). The nitrocellulose was then reacted with a 1:1,000 dilution of a polyclonal rabbit antiserum (PP5) directed against a β -galactosidase–polymerase fusion protein expressed in bacteria (76). Bound antibodies were detected with a 125 I-labeled goat anti-rabbit conjugate (ICN) and visualized by autoradiography. Filters were then stripped of bound antibodies by incubation in 0.1 M glycine-HCl (pH 2.5) twice for 10 min each time at room temperature and reprobed with polyclonal rabbit antiserum 3-83, directed against ICP8 (34), followed by detection with labeled goat anti-rabbit conjugate as described above. The autoradiographic signals were quantified by densitometric scanning. To ensure that signals were within the linear range of the assay, a dilution series of KOS-infected cell extract diluted with mock-infected cell extract was simultaneously applied to the gel.

Assay of HSV Pol-UL42 complex formation. Assays of complex formation between HSV Pol and UL42 were performed as described by Digard and Coen (14) except that the anti-UL42 antiserum used was 13809, a polyclonal rabbit serum prepared against purified UL42 (43).

Assay of localization of HSV Pol and ICP8 in infected cells. Double-label immunofluorescence assays of the intracellular distribution of HSV Pol and ICP8 in infected CV-1 cells were performed as described by Bush et al. (5), with the following modifications. Infected cells were fixed for 10 min in 2% formaldehyde and then for 2 min in ice-cold acetone. They were then reacted for 30 min at room temperature with a mixture of a 1:10 dilution of rabbit anti-HSV Pol antiserum and PP5 (76) and with a 1:30 dilution of mouse monoclonal anti-ICP8 antibody 39S (58), for 30 min with a mixture of goat anti-mouse immunoglobulin conjugated with rhodamine and goat anti-rabbit immunoglobulin conjugated with biotin, and then for 30 min with fluorescein isothiocyanate-avidin (Cappel/Organon Teknika).

RESULTS

Mutagenesis of Exo II sequences. Mixed oligonucleotides were designed to target an eight-amino-acid stretch containing both highly conserved and nonconserved amino acids within the Exo II segment defined by Bernad et al. (2), which lies within region IV of homology among α -like DNA polymerases (Fig. 1). As an internal control, the oligonucleotides also contained a mutation that destroyed an *NciI* recognition site without changing the amino acid sequence. These oligonucleotides were used to mutagenize single-stranded DNA from the HSV *pol* construct MD18.1. Mutagenized DNA was transfected into *E. coli*, and plaques were picked and screened for alterations within the targeted segment by DNA sequencing. We describe results with three engineered mutations here: E460D, V462A, and G464V (the first letter denotes the wild-type amino acid, the number denotes the residue position, and the last letter denotes the mutant amino acid). The glutamic acid at residue 460 is relatively nonconserved, the valine at residue 462 is at a position that is usually nonpolar, and the glycine at residue 464 is highly conserved among the α -polymerases and is also found at the corresponding position in Pol I. These residues lie just upstream of a conserved aspartic acid residue (position 471 in HSV) (Fig. 1), which, when mutated in *E. coli* Pol I and ϕ 29, greatly decreased exonuclease activity without apparent effects on polymerase activity (2, 12, 13). The engineered mutations are fairly conservative, causing relatively subtle changes in amino acid side chains.

Recombination of mutant sequences into a wild-type HSV background. To insert the mutant sequences generated in bacteriophage M13 clones into a wild-type HSV background, the technique of marker rescue was used. To permit propagation of viruses containing lethal *pol* mutations, the DP6 cell line, which expresses HSV Pol and complements otherwise nonviable HSV *pol* mutants, was used (40, 42). HSV mutant HP66 (40, 42) was used to facilitate screening. 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, on which it forms blue plaques in the presence of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) or Blue-Gal.

HSV recombinants were then isolated by cotransfecting HP66 infectious DNA with the various mutant bacteriophage DNAs. Progeny from these transfections were plated onto DP6 cells and stained with X-Gal or Blue-Gal, and white plaques were picked. Small viral stocks were grown from each plaque pick and used to prepare viral DNA. This DNA was then examined by Southern hybridization analysis (Fig. 2). *EcoRI* digests were analyzed to verify that recombination took place at the proper site, and *NciI* digests were analyzed to verify that the recombined segment lacked the *NciI* site in the targeted area.

As can be seen in the example shown in Fig. 2, the *EcoRI* fragment that hybridizes with an oligonucleotide derived from the 3' end of the *pol* gene was 2.6 kbp in HP66 and was 4.1 kbp in the recombinants analyzed in lanes B and C and in the wild-type HSV parental strain, KOS (the *EcoRI* M fragment). Figure 2b shows that there is a shift in the size of an *NciI* fragment hybridizing with an oligonucleotide that recognizes the fragment containing region IV, from 250 bp in KOS to 325 bp in the recombinants in lanes B and C. HP66 lacks homologous sequences and thus did not hybridize with the probe. Results of these and similar analyses showed that the recombinant viruses E460D, V462A, and G464V all

FIG. 2. Southern hybridization analysis of viral DNAs. Viral DNA from plaque isolates or from KOS or HP66 was digested with *EcoRI* (a) or *NciI* (b), subjected to electrophoresis on a 0.7% (a) or 1.2% (b) agarose gel, transferred to GeneScreen II Plus overnight, and hybridized to a 32 P-labeled oligonucleotide specific for the *EcoRI* fragment containing the 3' end of the *pol* gene (a) or region IV (b). Lanes A to D represent DNA from four plaque isolates from a marker rescue experiment with HP66 and pG464V.

contain mutant sequences at the proper location. These viruses were plaque purified three times and used to prepare high-titer stocks.

Mutants E460D and G464V are nonviable *pol* mutants. To test whether particular mutations caused a lethal phenotype, we examined the ability of the recombinant viruses derived described above to grow on various cell lines. Recombinant viruses E460D and G464V were viable on DP6 cells but not on Vero or human 143 cells (plating efficiencies, <0.01%; wild-type virus exhibits similar plating efficiencies on the different cell lines). Because the only HSV gene present in DP6 cells is *pol* (42), this result demonstrates that the lethal phenotypes of these mutants are due to *pol* mutations.

To verify that the lethal phenotype of G464V was due to the engineered mutation and not some other mutation, we mapped and sequenced the mutation responsible. Infectious G464V DNA was transfected by itself or with specific wild-type DNA fragments from pDP1, and the progeny were assayed for the ability to form plaques on Vero cells (Table 1). The fragments that rescued the ability of G464V to grow

TABLE 1. Marker rescue of G464V

Fragment	Map location ^a	Efficiency ^b
No plasmid		<0.0013
pDP1	1-1235	9.6
<i>EcoRI</i> -B	443-1235	0.57
<i>EcoRI</i> -A	1-440	<0.001
<i>XhoI</i> + <i>HindIII</i> -B	1-535	0.079
<i>XhoI</i> + <i>HindIII</i> -A+C	535-1235	<0.00001

^a Expressed as amino acid residues in the HSV *pol* open reading frame (24).

^b Expressed as (titer on Vero cells/titer on DP6 cells) $\times$ 100. Values in boldface are considered positive for rescue of growth on Vero cells.

FIG. 3. Dot blot analysis of HSV DNA replication. Eight wells each of Vero and DP6 cells, as indicated, were mock infected or infected with wild-type KOS or recombinant G464V, E460D, or V462A, as indicated. Total DNA was harvested at 0 or 20 h postinfection, as indicated, blotted onto a nylon membrane, and probed with a 32 P-labeled oligonucleotide complementary to the HSV *pol* gene.

on Vero cells overlap in a 277-bp fragment extending from an *Eco*RI site on the upstream edge of region IV (amino acid residue 443) to an *Xho*I site downstream of region IV (residue 535). Preliminary marker rescue experiments with mutant E460D also indicate that its lethal mutation maps within the same 277 bp (24). The 4.1-kbp *Eco*RI M fragment from G464V viral DNA was then isolated and cloned into M13mp19, and the relevant portion was sequenced. The engineered mutation was the only alteration found within the 277-bp *Eco*RI-to-*Xho*I fragment, proving that this mutation causes the lethal phenotype of G464V. Thus, the Exo II segment of region IV contains residues that are critical for HSV growth.

Mutant V462A is viable. In contrast to the other two mutants, V462A was viable on both Vero and 143 cells (plating efficiencies, ca. 100%). Moreover, the mutant plasmid, pV462A, was able to rescue the lethal phenotype of HP66 efficiently (not shown). HSV mutant V462A also was not detectably altered in its sensitivity to three diverse antiviral drugs (21), in its frequency of spontaneous mutation (21), or in its ability to kill mice following intracerebral inoculation (48). The wild-type behavior of this Exo II mutant provides a control for the phenotypes of the nonviable mutants.

The nonviable Exo II mutants fail to synthesize viral DNA. Since the sequences that we had mutagenized were within a conserved 3'-5' exonuclease active site, we wished to determine whether mutants E460D and G464V failed to form plaques because they accumulated too many replication errors or because they failed to synthesize HSV DNA. Using dot blot hybridization, we compared DNA synthesis from the recombinants with that of wild-type virus in both Vero and DP6 cells. As can be seen in Fig. 3, no accumulation of HSV DNA was observed in Vero cells infected with either of the lethal mutants E460D and G464V observed in the complementing DP6 cells infected with these viruses. Recombinant V462A exhibited wild-type levels of DNA in both cell lines, as expected from its viable phenotype. Thus, the lethal Exo II mutations abolish viral DNA synthesis.

TABLE 2. DNA polymerase activities in extracts of wild-type- and mutant-infected cells

Infecting virus	Activity (pmol/20 min) ^a	% Activity ^b
None (mock infection)	50	
KOS (wild type)	800	100
E460D	50	0
V462A	450	53
G464V	30	0

^a Incorporation of radiolabeled TMP into DNA was measured. Each assay contained 0.75 mg of total protein.

^b Calculated as (mutant incorporation - mock incorporation)/(wild-type incorporation - mock incorporation) $\times$ 100.

The nonviable Exo II mutants are defective for DNA polymerizing activity. Because the nonviable mutants failed to synthesize viral DNA, we investigated whether they induced active viral DNA polymerase activity. Human 143 cells were mock infected or infected with wild-type strain KOS or with each recombinant at a multiplicity of 10. Cell extracts were prepared 8 h postinfection and assayed for polymerizing activity on activated DNA primer-templates under conditions optimal for HSV Pol (Table 2). Wild-type virus and the viable recombinant V462A exhibited polymerase activities 9- to 16-fold over mock-infected values; this variation was due mainly to the difference in the amount of polymerase polypeptide present in the extracts (see below). In contrast, the activities of the two nonviable mutants were severely impaired (<1% of wild-type values when mock-infected values are subtracted). Similar results (70) were obtained with use of *Eco*RI-digested DNA fragments as primer-templates, as employed by Bernad et al. (2). In contrast, recombinants derived by marker rescue of G464V and E460D exhibited wild-type levels of polymerizing activity (70).

To ensure that the mutants expressed wild-type amounts of HSV Pol polypeptide that remained stable during the course of the assay, we performed Western blot analysis on the extracts following polymerase assays. As can be seen in Fig. 4A, extracts from cells infected with each mutant contained a single major polypeptide that reacted with specific anti-HSV Pol antisera (76). This species comigrated with that in wild-type-infected cell extracts. Densitometric scanning showed that the amounts of HSV Pol present in the mutant extracts were $\geq$ 60% those found in wild-type ex-

FIG. 4. Western blot analysis of mock-infected and virus-infected cell extracts. Each lane contained approximately 50 μ g of total protein. Filters were probed successively with polyclonal sera directed against HSV Pol (A) and ICP8 (B). The viruses used are indicated above the lanes.

FIG. 5. Coimmunoprecipitation of wild-type and mutant HSV Pol polypeptides with UL42. In vitro-transcribed mRNAs encoding the various HSV Pol polypeptides were translated in rabbit reticulocyte lysate both with and without mRNA encoding UL42, as indicated, and then analyzed by polyacrylamide gel electrophoresis either before (lanes 1) or after immunoprecipitation with an anti-HSV Pol (lanes 2) or an anti-UL42 antiserum (lanes 3). Positions of the HSV Pol and UL42 polypeptides are indicated.

tracts. The results of Fig. 4B show that roughly equal amounts of early viral proteins are present in each extract, as assessed by Western blot analysis of the major viral DNA-binding protein, ICP8. Densitometric scanning showed that the amounts of HSV Pol polypeptide normalized to the amount of ICP8 varied less than 25%. Thus, there was little if any evidence for degradation of mutant polymerase. We conclude that the defect in DNA polymerizing activity of the mutants was not due to a lack of stable HSV Pol polypeptide.

Exo II mutant polymerase polypeptides associate with a polymerase accessory protein. A possible reason why the Exo II nonviable mutants could be defective in viral growth and DNA polymerizing activity is that the mutations might prevent proper folding of the polymerase in a global fashion. As an initial test of this hypothesis, we assayed mutant polymerase polypeptides for the ability to complex with the HSV UL42 protein, which is an accessory subunit of HSV Pol that increases processivity (26, 29). We used an assay for complex formation that entails in vitro transcription and translation of the *pol* and *UL42* genes followed by immunoprecipitation with antisera specific for each protein (14). Whereas anti-polymerase antisera did not immunoprecipitate UL42-size products when wild-type or mutant *pol* mRNA or wild-type *UL42* mRNA was translated alone, it precipitated UL42 when the two mRNAs were translated together (Fig. 5). Similarly, anti-UL42 antisera did not immunoprecipitate polymerase-size products when any mRNA was translated alone, but precipitated wild-type or mutant polymerase polypeptides when *pol* and *UL42* mRNAs were translated together (Fig. 5). In other experiments, we detected HSV Pol-UL42 complexes in Exo II mutant-infected Vero cells (not shown). Therefore, the Exo II mutations did not prevent proper folding of HSV Pol to an extent that it could not interact with UL42.

The nonviable Exo II mutant polymerases colocalize with ICP8. As a second test for whether the Exo II mutations affected other polymerase functions deleteriously, we investigated whether the mutant polymerases localized properly in HSV-infected cells. In double-label immunofluorescence experiments, Bush et al. (5) have shown that in the absence of viral DNA synthesis, wild-type HSV Pol colocalizes in infected cell nuclei with the major viral DNA-binding protein, ICP8, to nuclear structures called prereplicative sites. These sites, which exhibit a characteristic punctate staining pattern, appear to include viral and cellular components

required for DNA synthesis (5, 10, 50, 72). Proper localization of HSV Pol to prereplicative sites requires ICP8; with HSV ICP8 mutants or with transfected *pol* genes, HSV Pol assumes a diffuse nuclear distribution (5). The nonviable Exo II HSV mutants exhibited colocalization of polymerase with ICP8 in prereplicative sites that was indistinguishable from that of the wild type (Fig. 6). (Perinuclear staining with the anti-polymerase antisera [Fig. 6B, D, and F] is due to nonspecific binding of rabbit antibodies to some component in the Golgi apparatus in infected cells.) We conclude that the Exo II mutations permit proper entry of polymerase into the nucleus and whatever interactions are required for it to colocalize with ICP8 at prereplicative sites. We find no evidence that these mutations lead to deleterious effects on polymerase folding in a global fashion.

DISCUSSION

We have constructed three HSV recombinants containing mutations in the Exo II segment of region IV of homology shared among diverse DNA polymerases. Two of the recombinants, E460D and G464V, failed to grow in tissue culture or to synthesize viral DNA in the absence of a complementing *pol* gene. We have demonstrated by marker rescue and DNA sequencing that the mutation G464V is lethal for viral growth. This replication defect is associated with a profound loss of DNA polymerizing activity and cannot be explained by defects in polymerase synthesis, stability, ability to associate with the accessory protein UL42, or ability to properly colocalize with the DNA-binding protein ICP8. These results have implications for the functions of this region, especially given its homology with sites important for 3'-5' exonuclease function in *E. coli* Pol I and the α -like DNA polymerases of bacteriophages T4 and ϕ 29.

Region IV is essential for viral growth and DNA replication. Our results demonstrate that Exo II, and thus region IV, is essential for viral growth, as might be predicted from the degree of conservation of this region in a variety of DNA polymerases. This finding is consistent with results for T4 and adenovirus, in which certain region IV mutations confer temperature sensitivity or complete failure to grow (46, 51, 54-56, 73). It also demonstrates that region IV plays a critical role in DNA synthesis per se. This finding is consistent with observations for adenovirus and T4 temperature-sensitive mutants that map within region IV or in the general

FIG. 6. Double-label immunofluorescence staining of ICP8 and HSV Pol in infected cells. CV-1 cells were infected with wild-type HSV strain KOS (a and b), E460D (c and d), or G464V (e and f). At 5 h postinfection, the cells were fixed and reacted with anti-HSV Pol antiserum PP5 and anti-ICP8 monoclonal antibody 39S, with biotin-conjugated goat anti-rabbit immunoglobulin and rhodamine-conjugated goat anti-mouse immunoglobulin, and with fluorescein-conjugated avidin. (A) Cells infected with KOS. Image due to reaction with anti-ICP8 antibody. (B) Same field of cells as in panel A; image due to reaction with anti-HSV Pol antiserum. (C) Cells infected with E460D. Image due to reaction with anti-ICP8 antibody. (D) Same field of cells as in panel C; image due to reaction with anti-HSV Pol antiserum. (E) Cells infected with G464V. Image due to reaction with anti-ICP8 antibody. (F) Same field of cells as in panel E; image due to reaction with anti-HSV Pol antiserum.

area, which have been shown to be DNA negative at the nonpermissive temperature (17, 73, 74).

Region IV is essential for virus growth, but mapping of drug resistance mutations (22, 23, 28, 35, 38, 40, 67) indicates that it does not interact with polymerase dNTP or pyrophosphate analogs. Therefore, it might serve as a target for novel antiviral drugs.

Do α -like DNA polymerases contain a distinct 3'-5' exonuclease domain? Comparisons among diverse DNA polymerases identified three segments termed Exo I, II, and III with homology to catalytically important segments of the 3'-5' exonucleolytic activity of *E. coli* Pol I and related proteins (2, 39, 45, 60). In the α -like DNA polymerases encoded by bacteriophages ϕ 29 and T4, mutations in or near Exo I, II, and III, analogous to those in Pol I and T7, decrease 3'-5'

exonuclease activity, evidently without decreasing polymerase activity (although the polymerase assays used would not have detected effects on polymerase processivity) (2, 54). These results have led to the hypothesis that, like Pol I, α -like DNA polymerases contain a distinct 3'-5' exonuclease domain that encompasses Exo I, II, and III (2, 53, 60). Indeed, this hypothesis has formed the basis for several efforts to predict structural features of α -like DNA polymerases (11, 27, 52).

Our results argue against this simple picture. We introduced two different and fairly conservative substitutions into Exo II of the α -like DNA polymerase of HSV that resulted in a drastic impairment of DNA synthesis *in vivo* and DNA polymerizing activity *in vitro*. These results were not due to lack of expression of the HSV Pol polypeptide or its stability under polymerase assay conditions. We found no evidence that the mutations caused global folding defects; the mutant HSV Pol proteins were able to interact with the accessory subunit, UL42, and to interact properly with cellular and viral factors such that they localized correctly within infected cell nuclei. The effects of the Exo II mutations on polymerase activity contrast greatly with the results of experiments in which a variety of point and even gross deletion mutations were introduced into the 3'-5' exonuclease domains of Pol I and T7 DNA polymerase, with little or no effect on polymerizing activity (12, 13, 19, 61). Thus, in HSV Pol, unlike Pol I or T7 DNA polymerase, polymerization activity requires a sequence that is homologous to a conserved 3'-5' exonuclease active site.

It could be argued that the G464V and E460D mutations affect polymerase activity by trapping the DNA primer-template in an otherwise inactive exonuclease active site. Such an interpretation is complicated and would predict that the mutations confer a dominant negative phenotype. The data (e.g., Fig. 3) contradict the prediction. We therefore do not favor such an interpretation.

We do not yet know whether the E460D and G464V Exo II mutations affect 3'-5' exonuclease activity. This determination will likely require purification of the mutant enzymes, since this activity is obscured in HSV-infected cells by other nucleases. Therefore, we do not yet know whether Exo II or any other Exo segment homology is important for the 3'-5' exonuclease activity of HSV Pol, although, given the results in the ϕ 29 and T4 systems (2, 54), we think it likely. Regardless, our data indicate that these results in other systems cannot be extrapolated to imply the existence of an independent 3'-5' exonuclease domain in HSV Pol or α -like polymerases in general. Indeed, we currently favor the hypothesis that HSV Pol does not contain a 3'-5' exonuclease domain with the functional and structural independence of the Pol I domain.

Several other lines of evidence are consistent with our favored hypothesis and suggest that it may apply to other α -like DNA polymerases. Human and yeast DNA polymerase α retain the Exo segment homologies (2), yet whether they contain 3'-5' exonuclease activity remains controversial. Mutations influencing sensitivity to drugs that mimic or inhibit polymerases competitively with dNTP and pyrophosphate substrates have been mapped within or just upstream of the Exo III segment in the α -like DNA polymerases encoded by vaccinia virus (16, 66), HSV (28), M2 (45), and ϕ 29 (44). Several other such mutations have been mapped just downstream of Exo III in HSV (22, 28) (Fig. 1) and vaccinia virus (62), and one of these clearly affects the K_m for dNTPs without effects on the ratio of exonuclease to polymerase activity (13). Indeed, Exo III overlaps region A,

which is proposed to join with regions I, II, III, and V in forming the substrate binding sites of α -like DNA polymerases (22). Nucleoside monophosphates, which inhibit the 3'-5' exonuclease activity of Pol I without affecting polymerization (49), reportedly inhibit the polymerization activity of HSV Pol (18). Numerous T4 and adenovirus mutations within the putative 3'-5' exonuclease domain appear to affect polymerizing activity (6, 52, 54, 55), although it is not clear that these mutations do not exert global folding defects. None of the results presented above has conclusively shown that the polymerizing and exonuclease activities are inseparable; nevertheless, they support our suggestion that many α -like DNA polymerases do not contain polymerase and exonuclease domains as distinct and independent as those of Pol I and its relatives.

It is possible that ϕ 29 DNA polymerase exhibits greater distinction between its polymerizing and exonuclease functions than does HSV Pol. Consistent with this possibility is the report that phosphonoacetic acid, which inhibits these two activities of HSV Pol with equal potency (36), is a much more potent inhibitor of ϕ 29 polymerizing activity than of its 3'-5' exonuclease (3). Moreover, equivalent Exo I mutations have much less severe effects on the 3'-5' exonuclease of T4 DNA polymerase (54) than on that of ϕ 29 (2). There may be considerable variation in the interdependence of 3'-5' exonuclease and polymerase functions among the different α -like DNA polymerases.

Models for the domain structure of α -polymerases. We offer two alternate models for those residues that have been hypothesized to comprise a 3'-5' exonuclease domain of α -polymerases. The first is that this region does exist as a distinct physical domain containing the 3'-5' exonuclease activity, but, unlike the 3'-5' exonuclease domains of Pol I and T7 DNA polymerase, it also performs important functions during the polymerization process. This model may entail movements of DNA, protein, or both during polymerization. The second model is that α -polymerases contain their polymerase and 3'-5' exonucleases on a single physical domain and that the regions containing homology to 3'-5' exonuclease active sites have been conserved to play roles important for both activities. This could include holding template DNA, as suggested by Matsumoto et al. (45). Continued genetic, biochemical, and biophysical analyses should permit resolution of competing models for α -polymerase structure and function.

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