Herpes Simplex Virus Type 1 DNA Polymerase

MUTATIONAL ANALYSIS OF THE 3'-5'-EXONUCLEASE DOMAIN*

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Like true DNA replicases, herpes simplex virus type 1 DNA polymerase is equipped with a proofreading 3'-5'-exonuclease. In order to assess the functional significance of conserved residues in the putative exonuclease domain, we introduced point mutations as well as deletions within and near the conserved motifs’ exonuclease (Exo) I, II, and III of the DNA polymerase gene from a phosphonoacetic acid-resistant derivative of herpes simplex virus-1 strain ANG. We examined the catalytic activities of the partially purified enzymes after overexpression by recombinant baculovirus. Mutations of the motifs’ Exo I (D368A, E370A) and Exo III (Y577F, D581A) yielded enzymes without detectable and severely impaired 3'-5'-exonuclease activities, respectively. Except for the Exo I mutations, all other Exo mutations examined affected both exonuclease and polymerization activities. Mutant enzymes D368A, E370A, Y557S, and D581A showed a significant ability to extend mispaired primer termini. Mutation Y557S resulted in a strong reduction of the 3'-5'-exonuclease activity and in a polymerase activity that was hyperresistant to phosphonoacetic acid. The results of the mutational analysis provide evidence for a tight linkage of polymerase and 3'-5'-exonuclease activity in the herpesviral enzyme.

In view of the evolutionary preservation of fundamental mechanisms of DNA replication among prokaryotic and eukaryotic DNA polymerases (for a review, see Refs. 1, 2), the herpes simplex virus DNA polymerase of type 1 (HSV Pol) is an attractive model enzyme for studies of structural and functional organization of eukaryotic DNA polymerases (3). Like true DNA replicases, HSV Pol has an associated 3'-5'-exonuclease that functions as proofreading activity (4–6). HSV Pol belongs to the family of α-like DNA polymerases (7). It has been shown to be more closely related to DNA polymerase δ (8, 9), which is the most conserved exonuclease replicative DNA polymerase (10), suggesting that the viral enzyme derives from an ancestor of the latter polymerase. Like this major DNA polymerase, HSV Pol forms a heterodimeric complex with an auxiliary protein, the phosphorylated 65-kDa double-stranded DNA binding protein encoded by the ul42 gene (ul42 protein) (11). The ul42 protein increases the DNA polymerase processivity by its interaction with the C terminus of HSV Pol and is essential for virus replication (12–15). The 136-kDa core subunit provides the polymerase and proofreading function (6, 11, 16). The close relationship between HSV Pol and DNA polymerase δ is further documented by the identical response to common replication inhibitors (9, 17).

Three-dimensional structural studies, together with site-directed mutagenesis and biochemical analyses, have led to the identification of the catalytic residues responsible for polymerization and 3'-5'-exonucleolytic activity of the Klenow fragment of Escherichia coli Pol I (for a review, see Ref. 2). Corresponding amino acids involved in metal binding and catalysis (Asp-355, Glu-357, Asp-424, Asp-501, Tyr-497) of the Pol I 3'-5'-exonuclease activity were first identified by sequence similarities and site-directed mutagenesis using the φ29 DNA polymerase (18), and subsequently three sequence motifs (Exo I, Exo II, and Exo III) containing the Pol I homologous residues were proposed to be important for the 3'-5'-exonuclease function in prokaryotic and eukaryotic DNA polymerases. Despite the low overall homology between Pol I- and α-like DNA polymerases, the Exo motifs with correspondingly arranged conserved residues were found in herpesviral and δ-polymersases (7–9). Thus far, within the exonuclease domain of eukaryotic DNA polymerases, no homologs have been identified for the Pol I residues Leu-361 and Phe-473 which are predicted to be critical for the base positioning (19, 20).

From site-directed mutagenesis studies of the exonuclease domain with different prokaryotic (18, 20–25) and eukaryotic DNA polymerases (26, 27), it is evident that the putative exonuclease domain embodies the proofreading function (for a review, see Ref. 7). This function seems to be organized in a quite separate domain in prokaryotic enzymes and in yeast, since exonuclease-minus mutants are readily obtained that display fully functioning polymerase activity. In addition, the structural and functional independence of these two domains in Klenow fragment was demonstrated by the ability of the polymerase domain to retain activity when cloned separately (28) and by antibody neutralization studies (29). In the case of HSV Pol, mutational studies (30) as well as investigations carried out by limited proteolysis of HSV Pol (31) provided no evidence for a similar structural independence of 3'-5'-exonuclease and polymerase domains.

In order to demonstrate the possible structural and functional interrelationship of exonuclease and polymerase domains of HSV Pol more directly, in this report we have chosen to mutate directly the putative catalytic residues of the conserved motifs’ Exo I, II, and III of the exonuclease domain and to examine functionally the consequences of the mutations on the enzymatic activities using recombinant baculovirus tech-
nology. The results show that in vitro it is possible to generate HSV Pol enzymes with active polymerase but with no or weak exonuclease activity by mutating the proposed catalytic residues, suggesting a functional independence for the polymerase activity. On the other hand, defined mutations in the exonuclease domain also had a strong impact on the polymerizing function of the enzyme, providing evidence for the involvement of exonuclease domain residues in essential functions of the polymerase forward reaction.

EXPERIMENTAL PROCEDURES

Materials—Biochemicals were obtained from Amersham Life Science (Braunschweig, Germany), Nucleotides were purchased from Boehringer LKB (Freiburg). Activated calf thymus DNA was from Sigma and Aldrich (Deisenhofen). Oligonucleotides were synthesized using an Exptide Nucleic Acid synthesizer (Millipore, Eschborn), kindly provided by W. Weinig (Deutsches Krebsforschungszentrum, Heidelberg), and purified with the USB SurePure® Oligonucleotide Purification Kit (Amersham Life Science, Freiburg). Mutagenic oligonucleotides used are presented in Table I. Oligonucleotides spanning nucleotide position 1327–1346, 1845–1831, and 1905–1919, respectively, of the HSV Pol sequence of strain ANG (32) were used as sequencing primers. Restriction enzymes were purchased from Boehringer Mannheim, USB (Cleveland, OH), Life Technologies, Inc (Eggenstein), and Stratagene (Heidelberg) and used as guided by the manufacturer. Random primed DNA probes were obtained from oligonucleotide phosphoanacetic acid (PAA) was purchased from Sigma (Deisenhofen).

Buffers—Buffer A, used during HSV Pol purification, contained 25 m mM sodium phosphate, pH 7.2, 200 m M NaCl, 0.5 m M EDTA, 2 m M mercaptoethanol, 1 m M phenylmethylsulfonflouride, 10 m g leupeptin, and 1 m M pepstatin. Buffer B was the same as buffer A with 0.6 M NaCl. Enzyme storage buffer consisted of 20 m M Hepes/KOH, pH 7.5, 1 m M EDTA, 1 m M dithiothreitol, 40% (v/v) ethyleneglycol. TE buffer (Tris-EDTA) contained 10 m M Tris, pH 8, 1 m M EDTA.

Cells, Plasmid DNA, and Viruses—African green monkey kidney monolayer cells (Rita clone, RC-37, Iddalurgical Products, Rome) were cultivated and infected with HSV strain ANG as described previously (16). Baculovirus expression vector system (BEVS) comprising Autographa californica multiply enclosed nucleic polyhedrosis virus (AcMNPV), baculovirus transfer vectors pVL1392 and pVL1393, as well as the Sf9 cell line (Spodoptera frugiperda IPLB-Sf21AE) was obtained from Max D. Summers and Gale E. Smith (Texas Agricultural Experiment Station, TX). Recombinant baculovirus POL and the corresponding pVL1393 containing the large open reading frame (LORF) of a PAA-resistant (PAA) variant of HSV-1 strain ANG (33) from nucleotide position 326 to 4194 (32) were constructed following the BEVS protocol (34–36). For cloning of HSV Pol LORF subcones of the Clal fragment containing h containing pH1381 (37) were used. In separate cloning steps a BamH1 linker was inserted at nucleotide position 325 by Bal31 nucleotide deletion cloning, and at position 4194 an XbaI linker was introduced by filling-in reaction of the Asp718 site (37). The HSV Pol LORF could thus be transferred as a BamH1/XbaI DNA fragment to pVL1393. Recombinant pUC-8 clone pS5 was a subclone of pH1381 and contained the SalI fragment from nucleotide position 1229–3260 of the PAAs HSV Pol gene.

Site-directed Mutagenesis—SalI fragment from pS5 was cloned in phagemid vector pMaS5 (38) yielding pMaSS, and its single-stranded DNA was subjected to standard site-directed mutagenesis procedure (39) using the U-DNA mutagenesis kit as instructed by the manufacturer (Boehringer Mannheim) together with the mutagenic oligonucleotides listed in Table I. Oligonucleotides were designed in order to generate restriction enzyme site polymorphisms allowing screening of mutant clones by DNA restriction endonuclease mapping. All of the mutations were confirmed by DNA sequence analysis, performing as described in the guidelines of the T7 sequencing kit (Pharmacia Biotech, Freiburg).

Construction of Recombinant Baculovirus with Mutated HSV Pol Gene—Characterized NheI/SalI or NheI/MstII DNA subfragments of phagemid vector pMaSS with the desired 3′-5′ exonuclease mutations were individually used to replace the corresponding fragment in pVL1393 by recombination baculovirus transfer vector pPOL. The presence of introduced mutations was confirmed by restriction endonuclease mapping. For generation of recombinant baculoviruses, transfer vector derivatives of pPOL containing the mutated genes as stated were individually cotransfected in Sf9 cells with modified linearized AcMNPV baculovirus DNA (BaculoGold® DNA) following the protocol of BaculoGold® Transfection Kit (Pharmingen, San Diego). Recombinant plaques were screened for plaque expression by staining with a monoclonal antibody raised in rabbit (m.o.i. of 10). After removal of inoculum, fresh medium was added, and cells were cultivated for 48 h. Then medium was removed, cells were washed twice with ice-cold phosphate-buffered saline (Life Technologies, Inc., Eggenstein), transferred to centrifuge tubes by up and down pipeting, and sedimented at 1000 × g for 15 min at 4°C. Cell pellets were kept at −70°C until use.

Immune Detection of HSV Pol Expression—Expression of HSV-1 DNA polymerases in recombinant baculovirus-infected cell extracts was monitored by immunoblot analysis. Culturally grown Sf9 cells (7 × 10^6 cells/25-cm² flasks) were infected for 48 h with recombinant baculovirus at a m.o.i. of 5 and harvested as described above. Pelleted cells were resuspended in 150 μL of double-distilled water, disrupted by sonication. Nucleic acid was centrifuged at 10,000 × g. The concentrated sample buffer was added to three parts of supernatant, and after boiling for 5 min aliquots of cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (44) and electroblotted on nitrocellulose membranes (BA-S 85; Schleicher & Schuell, Dassel). Immunostaining was performed with a 10,000-fold diluted polyclonal rabbit anti-(HSV Pol) serum EX3, directed against the carboxyl terminus (amino acid residues 1072–1235) of HSV-1 ANG DNA Pol, as described previously (16).
Loss of Sst

achieved by heating 1–4 μl to 80°C for 5 min. Hybridization of primer and template was performed by heating a sample of 45 μl of hybridization mixture (45) to 70°C and subsequent incubation at room temperature for 5 min. Oligonucleotides were designed such that restriction endonuclease analysis. Deletion mutant D368 to A ATGTGCTTCGCTATCGAGTGCAAGGCG was subcloned into pH1381 into phagemid vector pMa as described under "Experimental Procedures." Mutagenic oligonucleotides were designed such that restriction endonu-

cease recognition sites were deleted. Recombinant phagemid clones containing the desired mutations were then screened by restriction endonuclease analysis, and the mutations were verified by DNA sequencing. The changes introduced were intended to neutralize functionally interacting side chains, e.g. carboxyl and OH groups were changed to methyl and phenyl groups, respectively. In addition, 19 residues located between the Exo II and Exo III motifs of HSV Pol (position 539–557) were deleted, and likely candidate residues for a role in base positioning, such as the tyrosines in position 538 and 557, were changed from aromatic to aliphatic side chains. Sequence analysis revealed that from seven mutations six were successfully introduced by the chosen site-directed mutagenesis protocol (39) but that one phagemid clone (Y538S) contained an extra mutation downstream from the oligonucleotide primer at nucleotide position 1937 (32). This was a conversion of A → C that resulted in the conservative amino acid substitution, isoleucine to leucine, and yielded the amino acid sequence KKLLSSSKL. For the engineering of recombinant baculoviral transfer vector pVL1393 with mutated HSV Pol genes, the LORF of HSV Pol of strain ANG from nucleotide position 325 to 4190 (32) was subcloned into pVL1393, yielding pPOL, cleaved with NheI and MstII, and ligated with mutation bearing NheI-SalI and NheI-MstII fragments from mutated recombinant phagemids. Presence of introduced mutations was confirmed by restriction endonuclease analysis. Deletion mutant A19 with a deletion of amino acids 539–557 was constructed directly from baculoviral transfer vectors pY538S and pY557S by inserting the unique SalI fragment, spanning from nucleotide position 2009–2133 (32) of pY557S into the SalI-cleaved pY538S. Altogether nine baculoviral transfer vectors were successfully established, one containing the unmodified HSV-1 ANG PAAr Pol gene used as a reference, eight containing besides the PAAr mutation the individual point mutations listed in Fig. 1, and a 19-amino acid deletion. The presence of the mutation was verified in each recombinant transfer vector and baculovirus DNA by DNA sequencing and, since the nucleotide change of the PAAr mutation was not conserved in all of the individual recombinant DNA sequences, the nucleotide sequences were determined. For overexpressing the mutated HSV Pol genes, the recombinant baculoviral transfer vectors were individually co-transfected in S9 cells with baculovirus DNA and recombinant baculovirus DNA was selected and propagated as described under "Experimental Procedures." Recombinant baculovirus POL containing the unmodified PAAr HSV Pol was raised using the wild type baculovirus (AcMNPV, Ref. 34). For the generation of recombinant baculovirus with mutated HSV Pol genes, the standard virus of the BaculoGold transfection kit (Pharmingen) was employed. Stocks of three times plaque-purified virus were used for preparation of infected cell extracts. As can be seen from the analysis of infected cell extracts by SDS-poly-

Expression and Purification of HSV Pol Exonuclease Mutants—For overexpressing the mutated HSV Pol genes, the recombinant baculoviral transfer vectors were individually co-transfected in S9 cells with baculovirus DNA and recombinant baculovirus DNA was selected and propagated as described under "Experimental Procedures." Recombinant baculovirus POL containing the unmodified PAAr HSV Pol was raised using the wild type baculovirus (AcMNPV, Ref. 34). For the generation of recombinant baculovirus with mutated HSV Pol genes, the standard virus of the BaculoGold transfection kit (Pharmingen) was employed. Stocks of three times plaque-purified virus were used for preparation of infected cell extracts. As can be seen from the analysis of infected cell extracts by SDS-poly-


table 1

| Mutation | Oligonucleotides (5′–3′) | New restriction site |
|----------|-------------------------|---------------------|
| D368 to A | ATGGCTTCGCGTATCGAGTGCAAGGCG | Loss of EcoRV and BsmI |
| E370 to A | ATGGCTTCGCGTATCGAGTGCAAGGCG | SphI, loss of BsmI |
| D471 to A | CAACCTGCTTCGCGCC | Loss of BsrI |
| Y538 to S | GCTTGGAGCGTCCAAGCTCAGGCG | SstI, loss of XhoI |
| Y577 to S | AGAAAGACCTGCTCAGCTGCAATCC | SstI, loss of NruI |
| Y577 to F | ATCGGGCAAGTTCTGCTACAAGG | Loss of SstI |
| D581 to A | AACTGATACAGGGTTCTGCTGG | Loss of TfiI |

* Intended mutations are represented by double underlining, and silent changes to delete restriction sites are indicated by single underlining.

EXONUCLEASE ASSAY—Exonucleolytic activities were determined in the absence of dTTP under the conditions of the DNA polymerase assay. Reaction mixtures (100 μl) were incubated for 20 min at 37°C with 25 μg of activated calf thymus DNA containing 0.15 μg (6.7 × 10^5 cpμg) of 32P-labeled exonuclease substrate. Reactions were terminated by chilling in ice and by adding 20 μl of a mixture of 0.25 M EDTA, pH 8.5, and 5 mg/ml bovine serum albumin, and 20 μl of 100% trichloroacetic acid. After centrifugation (13,000 × g, 20 min, 4°C) the radioactivity of supernatant fractions (100 μl) was determined with 3 ml of Unisolve 100 (Unisolve, flashlight liquid scintillation counter (Pharmacia-LKB Wallac, Freiburg)).

PRIMER-TEMPLATES FOR PRIMER-EXTENSION ASSAYS—Oligonucleotides used for the construction of primer-templates illustrated in Figs. 5–6 were obtained and purified as described above. DNA primers were labeled at the 5′-end in reaction mixtures (20 μl) containing 1 μg of oligonucleotide, 50 μCi of [γ-32P]ATP (5000 Ci/mmol), 10 units of T4 polynucleotide kinase (Boehringer Mannheim), and 1 × linker-kinase buffer (Boehringer Mannheim) for 30 min at 37°C followed by an incubation of 10 min at 68°C according to a standard procedure (45). 3′-End-labeled primers were synthesized in reaction mixtures (20 μl) containing 7 μg of oligonucleotide, 1 μCi of [α-32P]dATP (3000 Ci/mmol), 30 units of terminal deoxynucleotidyltransferase, and 1 × One-Phor-All buffer, as specified by the supplier (Biotech Pharmacia, Freiburg), for 10 min at 37°C. Unincorporated radioactivity was removed by passing through Sephadex G-50 spin columns, and oligonucleotides were concentrated by precipitation with 1-butanol (45). Purity was examined by denaturing 12% polyacrylamide gel electrophoresis (46) and quantified by measuring the absorbance at 260 nm. Hybridization of primer and template was achieved by heating 1–4 μg of radiolabeled primer with equimolar amounts of the complementary unlabelled 45-mer template oligonucleotide in TE buffer for 10 min at 70°C followed by slow cooling to room temperature over approximately 2 h. The primer-templates were kept at −20°C before use.

PRIMER-EXTENSION ASSAYS—Reaction mixtures contained in a final volume of 10 μl (25 ng (4.5 × 10^3) of 5′-32P-labeled or 80 ng (1.5 × 10^3) of the 3′-[γ-32P]dTMP-labeled primer-template, 50 mM Tris-Cl, pH 8.0, 5 mM of bovine serum albumin, 0.5 mM dithiothreitol, 7.5 mM MgCl2, 50 mM ammonium sulfate, and 0.25 mM each of dATP, dCTP, dGTP, and dTTP. Reactions were initiated by the addition of 4 μl of the stated enzyme preparations. After incubation for 3 min at 37°C, reactions were terminated by adding 5 μl of a formamide/dye mixture, containing 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, and 20 mM EDTA, pH 8. Aliquots (5 μl) of each reaction mixture were boiled briefly, chilled rapidly, and then subjected to electrophoresis on 12% DNA sequencing gels, containing 8 wt% urea (46). The gels were prerun to a temperature of 55°C, and the samples were then electrophoresed for 2 h at 30 watts (constant power). Gels were covered with Saran wrap and directly autoradiographed on x-ray films (Kodak X-Omat).
FIG. 1. Mutations of the 3′-5′-exonuclease domain of HSV Pol. HSV Pol protein is schematically depicted with the domains for exonuclease (EXO) and polymerase (POL) functions. The enlarged section of the exonuclease domain shows the location of the Exo I, II, and III motifs and a sequence alignment, performed as described previously (9), between E. coli Pol I (E. coli Pol I) (64), HSV Pol of strain ANG (HSV-1) (32), human (δ.hu) (65), yeast (δ.sc) (8, 66), and bovine (δ.bo) δ-polymersases (67). Numbers refer to the first and last residue of the sequence. Catalytic residues of E. coli Pol I (19, 20, 59) are underlined, and the amino acid changes of the HSV Pol gene carried out by site-directed mutagenesis are specified.

| Motif | ExoI | ExoII | ExoIII |
|-------|------|-------|--------|
| E. coli | 354 | FDTEDSL 418 QWL-KYRGR 467 KITTEEAG | 495 GRYAASD |
| δ.hu | 315 | FDIEC-AGO 395 QNYIQFDELP 467 KLYSTIYAV 486 KEDVCSHII 509 AVYCLKD |
| δ.sc | 320 | FDIEC-AGO 400 HYTNPNFDP 473 KLYSTIYAV 491 KEDUVSHII 514 AVYCLKD |
| δ.bo | 314 | FDIEC-AGO 394 QNYIQFDELP 467 KLYSTIYAV 485 KEDVCSHII 508 AVYCLKD |
| HSV-1 | 367 | FDIECAG 464 QYIINFDPW 534 KLEYKLYNY 552 KDELYRDY 575 GEYIQGD |

| Mutation: | | | |
|-----------| | | |
| D368A | A | | |
| E370A | A | | |
| D471A | A | | |
| Y538E | S | | |
| Y558E | S | | |
| Y577F | | F | |
| D501A | | | A |

acrylamide gel electrophoresis (Fig. 2A), a 136-kDa polypeptide of the size of the native HSV Pol (16) was overproduced by the majority of the recombinant baculovirus-infected cells as compared with mock- and AcMNPV-infected cells. An exception were cell extracts infected by recombinant baculovirus with the Y538S mutation that contained an overproduced polypeptide of about 120 kDa. Restriction as well as sequencing analysis provided no explanation for this compelling size difference of the Y538S enzyme. Furthermore, immunoprecipitation using a set of HSV Pol antibodies directed against the N-terminal, central, and C-terminal protein domains of HSV Pol (16) demonstrated that the target sequences for these antibodies were present on the recombinant enzyme and suggested that there was no apparent deletion or frameshift mutation.

For the functional analysis of the HSV Pol exonuclease mutants, a simple and quick purification scheme was developed as described under “Experimental Procedures.” The purification involved disruption of cells 2 days after infection by ultrasonication, low speed centrifugation, DNA removal by DEAE-celulose fractionation, and heparin-Sepharose chromatography using a combination of linear salt and step gradient elution. Fig. 2B shows the protein elution and salt gradient profiles exemplified for heparin-Sepharose chromatography of POL-infected S99 cells. Two distinct protein moieties were eluted from the heparin-Sepharose column. By immunoblot analysis (Fig. 2C) with HSV Pol antibody EX1051 directed against residues 597–685 (16), a major protein fraction of HSV Pol was detected in shoulder fractions 12–16 of the first protein peak, designated peak I, and a minor fraction coeluted with fractions 28–32 of the second protein peak, designated peak II.

To prove whether peak I fractions of the heparin-Sepharose chromatography comprising the bulk of the HSV Pol protein also contained corresponding functional activities, peak I and peak II fractions of heparin-Sepharose chromatographies of AcMNPV- and POL- and D368A-infected cell extracts were analyzed for ammonium sulfate-dependent DNA polymerase and exonuclease activity. The results of this comparison are shown in Fig. 3. In each of the three chromatograms, peak II fractions exhibited both a salt-stimulated DNA polymerase and exonuclease activity. From the similar ratios of polymerase to exonuclease activities and the identical optimum of 100 mM ammonium sulfate for both activities, it was concluded that peak II represented mainly the baculoviral DNA polymerase. The differences observed in the relative activities were explained by coeluting portions of HSV Pol protein that were detected by immunostaining as shown in Fig. 2C. Peak I fractions from POL- and D368A-infected cells contained a DNA polymerase activity that was more than 20-fold stronger than that of the AcMNPV control and sensitive to ammonium sulfate. The salt sensitivity of the POL peak I fraction was reversed to that of the wild type holoenzyme by the addition of purified u42 protein (47) as previously shown (48). This provided a further indication that the peak I polymerase activity represented recombinant HSV Pol. When the exonuclease activity was followed up using 3′-end-labeled activated calf thymus DNA as substrate, the POL peak I fraction exhibited about 5-fold stronger activity than that of both the AcMNPV control as well as the exonuclease mutant D368A and the respective salt sensitivity of the polymerizing activity. From this result, we concluded (i) peak I fractions contained the main portion of recombinant HSV Pol, (ii) the mutated HSV Pol D368A most likely induced an exonuclease-deficient enzyme, and (iii) the eluted purification scheme was sensitive enough to monitor DNA polymerase and exonuclease activities of the mutated HSV Pol enzymes when expressed in insect cells by recombinant baculovirus.

Functional Analysis of HSV Pol Exonuclease Mutants—With the purification scheme described nine recombinant HSV Pol as well as baculoviral and cellular DNA polymerases were partially purified, and representative activity profiles after heparin-Sepharose chromatography are represented in Fig. 4. Column fractions were individually assayed for DNA polymerase and exonuclease activity in the presence and absence of 100 mM ammonium sulfate. As can be seen from the DNA polymerase activity profiles, only in recombinant baculovirus-infected insect cells expressing the reference enzyme POL or mutated HSV Pol was a strong additional activity eluted between fractions 12 and 18 coinciding with the before-mentioned peak I (Figs. 2 and 3). The catalytic activities of each of the recombinant HSV Pol were calculated after heparin-Sepharose chromatography from fractions 12–18 as shown in Fig. 4 and are summarized in Table II. For comparison, the data are represented as percentage of the respective POL activity after subtracting the endogenous activity of the corresponding AcM-
NPV peak I fractions. HSV Pol with a mutation in motif Exo I (D368A, E370A) showed about the same or slightly higher polymerizing activity than the reference enzyme POL, but its exonucleolytic activity, determined as monophosphate released from 3'-terminally labeled activated calf thymus DNA, was significantly smaller than the endogenous activity. This indicated that the accomplished mutations of the Exo I motif caused a severe inhibition of the proofreading capability of HSV Pol. Mutations Y538S and Δ19 reduced both catalytic activities to the level of the endogenous activities. Polymerase and exonuclease activity of the Exo II mutant enzyme D471A dropped by 3 and 5-fold, respectively. Exo III mutations Y577F and D581A led to a 1.4- and 1.2-fold lower polymerase and a 6- and 16.7-fold lower exonuclease activity, respectively, as compared with POL. Mutation Y577S reduced the polymerase and exonuclease activity by 1.4- and 2.4-fold, respectively.

In order to obtain a more refined view of the altered catalytic activities, DNA polymerase and exonuclease activities were determined under coupled assay conditions using 5'-end-labeled synthetic templates with matching and mismatching primers as shown in Fig. 5A. Analysis of the reaction products on DNA sequencing gels demonstrated (Fig. 5B, C) that with a correctly base-paired primer, except for the endogenous control (AcMNP peak I), all of the examined recombinant enzymes were capable of extending the 17-mer oligonucleotide to full template size. With a primer having one 3'-terminal base mismatch, except for the control, each of the recombinant HSV Pol performed a significant filling-in reaction that was strong for the reference enzyme POL and E370A, weaker with D471A and D368A, and less efficient with the remainder of the mutant enzymes. A comparable result was obtained using a primer-template with two 3'-terminal base mismatches (Fig. 5B), although the filling-in reaction was less efficient with the mutant enzymes. In the absence of dNTP, preferentially 3'-mispaired primer-templates were exonucleolytically degraded by the reference enzyme POL and to a lesser extent by the D471A enzyme. Weak exonuclease activity higher than the background was observed with the enzymes Y557S and D581A. The mutant enzymes D368A, E370A, and Y577F displayed no exonuclease activity with any of the primer-templates tested (Fig. 5c).

To unequivocally determine the mismatch elongation reaction, the DNA polymerase/exonuclease-coupled assay was repeated using a template containing a primer with a 3'-32P-labeled one base extension (Fig. 6A). Analysis of the reaction products on DNA sequencing gels (Fig. 6, B and C) showed that in the presence of dNTP the POL enzyme efficiently removed the label at the 3'-end by converting the input label into dTMP. Consequently no labeled polymerization product was detectable by autoradiography. A direct comparison of exonuclease activities of POL and Exo I and Exo III mutant enzymes in the presence and absence of dNTP, depicted in Fig. 6C, showed that only the POL enzyme degraded the 3'-mispaired primer-template in the absence of dNTP and that the degradation by POL was faster in the presence of dNTP (Fig. 6B). Generation of dTMP was also seen with the Exo II mutant enzyme D471A but, according to the labeled input DNA, was less efficient. Labeled full-size polymerization products were detected with mutant enzymes D368A and Y557S and to a smaller amount with E370A and D581A. Interestingly, the mutant enzyme Y557S exhibited both a relatively strong exonuclease activity, as seen by the generation of dTMP and by the decrease of label in the position of the 18-mer input band, and in comparison to the Exo I mutant enzymes a similarly strong misincorporation capability.

Since each of the recombinant HSV Pol derived from the PAA' DNA polymerase gene, we have analyzed the effect of PAA on the individual polymerase activities. The results presented in Fig. 7 show that in contrast to the PAA-sensitive wild type enzyme, partially purified from HSV-1 ANG-infected RC-37 cells, the PAA-resistant recombinant enzymes POL, D368A, and D581A exhibited a similar PAA' phenotype being inhibited to about 50% by 100 μg/ml PAA. The Y557S mutant enzyme, on the other hand, was completely resistant to this drug concentration.

**DISCUSSION**

Previous protein sequence alignments (8, 9, 18) and mutational (30) and functional studies (16, 31, 49–51) showed that although the primary sequence of HSV Pol contains distinct domains for exonuclease and polymerase functions, these activities seem to behave less independently than is familiar for the E. coli Klenow model enzyme (2). To obtain a more refined view about how exonuclease and polymerase activities are possibly linked in HSV Pol, we have mutated residues of the Exo
I, II, and III motifs of the putative exonuclease domain of HSV Pol, highly conserved among E. coli Pol I and eukaryotic d-polym- erases (7, 10, 27), and additionally deleted a sequence portion located between Exo II and III motifs. Since it was also our aim to study the effects of the exonuclease mutations on polymerase functions in vivo, we have mutated the HSV Pol gene of a PAA-resistant strain. The PAA‘ phenotype resulting from point mutation A719V in the polymerase domain (33) should later be useful as a selective marker for recombining the exonuclease mutations into wild type HSV.

Using the BEVS (34), the recombinant HSV Pol enzymes were successfully overproduced in Sf9 cells, and with one exception (Y538S) exhibited the size of the authentic HSV Pol polypeptide of 136 kDa (16). The described simple and rapid purification scheme made use of the distinct elution behavior and salt sensitivity of recombinant HSV Pol, baculovirus-in-duced DNA polymerase (52), and cellular DNA polymerases (53) and allowed the simultaneous testing of exonuclease and polymerase activity of the partially purified mutant enzymes (Figs. 3 and 4).

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Using the BEVS (34), the recombinant HSV Pol enzymes were successfully overproduced in Sf9 cells, and with one exception (Y538S) exhibited the size of the authentic HSV Pol polypeptide of 136 kDa (16). The described simple and rapid purification scheme made use of the distinct elution behavior and salt sensitivity of recombinant HSV Pol, baculovirus-in-duced DNA polymerase (52), and cellular DNA polymerases (53) and allowed the simultaneous testing of exonuclease and polymerase activity of the partially purified mutant enzymes (Figs. 3 and 4).

HSV Pol is the first eukaryotic DNA polymerase where all five conserved residues of the Exo motifs were individually mutated, and the consequences of the mutations were function-
ing the conserved Asp and Glu residues of the Exo I motif into Ala was associated with the selective inactivation of the 3'→5' exonuclease activity and with the acquisition of a strong mutator phenotype. Mutation of the Asp residue of the Exo II motif into Ala also resulted in selective inactivation of the exonuclease activity and in a strong reduction of the polymerase activity. In that respect as well as with its inability of mismatch elongation, the Exo II mutant enzyme of HSV Pol (D471A) resembles the correspondingly mutated T4 DNA polymerase that displayed no mutator phenotype in vivo and the least mismatch extension reactions of the examined mutant enzymes. This underlines the importance of the Exo II residue for a processive DNA replication mechanism (55). As seen for the Exo III mutants of HSV Pol, certain mutations of the Tyr and Asp residues of the Exo III motif in T29 DNA polymerase inactivated 3'→5' exonuclease activity almost completely and had a weaker inhibitory effect on the polymerase activity (21).

Even though we cannot rule out that the effects of the Exo mutations of HSV Pol seen in the present analysis were possibly influenced by the PaAA mutation at the polymerase domain, our results show that changing of Exo I and Exo III motif residues (D368A, E370A, D581A), proposed to be involved in metal binding, cause the strongest inhibition of the exonuclease and the least one of the polymerase activity (Table II). Furthermore, only these same mutations permit the polymerase to extend mispaired primer termini (Fig. 6C). It should be noted that recent studies on partially purified enzymes of the KOS strain of HSV-1 carrying the D368A mutation demonstrated that the Exo I mutation in a wild type gene background led to a similar drastic deficiency in exonuclease activity without significantly altering the polymerase activity (58). This suggests that the exonuclease of HSV Pol may indeed utilize the same metal ion-mediated mechanism employed by E. coli Pol I (2).

Other mutations examined such as D471A, Y538S, Y557S, and Y577F affected both exonuclease and polymerase activities of HSV Pol. Mutation of Tyr at position 538, conserved between herpesviral and δ-DNA polymerases (9, 10), into Ser resulted in

| Polymersase | Polymerase Activity | Exonuclease Activity |
|-------------|---------------------|---------------------|
| POL         | 52,031              | 16,349              |
| D368A       | 53,688              | 2,607               |
| E370A       | 57,708              | 3,028               |
| D171A       | 17,398              | 3,028               |
| Y538S       | 2,084               | 2,660               |
| Δ19         | 2,083               | 2,918               |
| Y557S       | 38,120              | 9,032               |
| Y577F       | 37,157              | 5,763               |
| D581A       | 42,734              | 3,828               |
| AcMNPV peak I | 1,443              | 3,628               |
| AcMNPV peak II | 16,619             | 3,798               |

* Data are presented as average peak activities in cpm determined from 10-μl aliquots of the heparin-Sepharose fractions (Fig. 4) and in percentage relative to the reference enzyme POL with the AcMNPV peak I activity subtracted.
an inactive HSV Pol and exhibited an unexplainable size anomaly (Fig. 2A). Deleting of residues 539–557 in mutant enzyme Δ19 also yielded an inactive enzyme. Interestingly, the Y557S mutant enzyme was able to extend mispaired primer termini (Fig. 6B). Furthermore, whereas Exo I and Exo III mutant enzymes showed about a similar dose-response curve as the reference enzyme POL, the Y557S enzyme was completely resistant to PAA up to a concentration of 200 \( \mu g/ml \) examined (Fig. 7). This suggests that one of the Exo mutations (Y557S) together with the Pol mutation A719V exerts a synergistic effect on the PAA resistance of the polymerase activity. These results are in line with those of previous experiments showing that certain mutations in the N-terminal sequences and in the exonuclease domain (V258A, D531N, E460D, V462A, G464V) of HSV Pol affect both polymerase activity and drug sensitivity of the enzyme (30, 59, 60).

There is increasing evidence for a tighter linkage of the catalytic functions in other DNA polymerases (24, 61). It has long been a puzzle that mutations within a polypeptide stretch from residues 258–961, covering 57% of the total protein, affect the sensitivity of HSV Pol toward a variety of antiviral drugs. Moreover, mutations rendering the enzyme resistant to one class of drugs, for example PAA, a non-nucleoside inhibitor, likewise increased the resistance to nucleoside analogs such as acyclovir (62, 63). Recent inhibitor studies revealed that certain drugs and divalent cations may interfere with DNA binding of HSV Pol (9, 36) and consequently affect both polymerase and exonuclease. The relative independence of both catalytic functions of HSV Pol was documented in the present analysis by mutating residues of the Exo I and Exo III motifs that are proposed to be involved in metal binding. These mutations much more substantially affected the exonuclease than the polymerase (Table II). The opposite effect is produced by proteolytic cleavage of recombinant HSV Pol, yielding 70–85-kDa N-terminal fragments without polymerase but intact exonuclease activity (31). This indicates that the polymerase function, which has to undergo more complex interactions during the polymerization reaction, e.g., with DNA and nucleotides, than the exonuclease, is influenced to a greater extent by mutations targeting these complex interactions. Clarification of the underlying structural causes for the observed interrelationship of exonuclease and polymerase activities of HSV Pol has yet to await a successful crystallization of this enzyme. A further important issue to be addressed in future experiments is to
Reactions were performed as described in Fig. 5 but with 80 ng (1.5 \times 10^4 cpm) of the 3'-spaired 32P-labeled primer at 100% activity obtained in the absence of the drug were (58) of the HSV Pol-associated proofreading function. Individual activities obtained in the absence of the drug were (58) of the HSV Pol-associated proofreading function. 

To examine the effects of the exonuclease mutations in vitro, which should provide a clearer picture of the putative essential role (58) of the HSV Pol-associated proofreading function.

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REFERENCES

1. So, A. G., and Downey, K. M. (1992) Crit. Rev. Biochem. Mol. Biol. 27, 129–155
2. Joyce, J. M., and Steitz, T. A. (1994) Annu. Rev. Biochem. 63, 777–822
3. Chalberg, M. D., and Kelly, T. J. (1989) Annu. Rev. Biochem. 58, 671–718
4. Weisshaech, A., Hong, S.-C. L., Auckzer, J., and Muller, R. (1973) J. Biol. Chem. 248, 6270–6277
5. Knopf, K. W. (1979) Eur. J. Biochem. 98, 231–244
6. O'Donnell, M. E., Elias, P., and Lehman, I. R. (1987) J. Biol. Chem. 262, 4252–4255
7. Blanco, L., Bernad, A., and Salas, M. (1992) Gene (Amst.) 112, 139–144
8. Boulet, A., Simon, M., Faye, G., Bauer, G. A., and Burgers, P. M. J. (1989) EMBO J. 8, 1849–1854
9. Knopf, K. W., and Strick, R. (1994) Frontiers of Virology (Becker, Y., and Darai, G., eds) Vol. 3, pp. 87–135, Springer-Verlag, Berlin
10. Cullmann, G., Hindges, R., Berchtold, M. W., and Hubscher, U. (1993) Gene (Amst.) 134, 191–200
11. Crute, J. J., and Lehman, I. R. (1989) J. Biol. Chem. 264, 19266–19270
12. Gallo, M. L., Dorsky, D. I., Crumpacker, C. S., and Parris, D. S. (1989) J. Biol. Chem. 264, 5023–5029
13. Gottlieb, J., Marcy, A. I., Coen, D. M., and Challberg, M. D. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 5976–5987
14. Derbyshe, V., Fremont, P. S., Sanderson, M. R., Beese, L. S., Friedman, J. M., Joyce, J. M., and Steitz, T. A. (1998) Science 280, 199–201
15. Derbyshe, V., Grindle, N. D. F., and Joyce, J. M. (1991) EMBO J. 10, 17–24
16. Soengas, M. S., Esteban, J. A., Lazaro, J. M., Bernad, A., Blasco, M. A., Salas, bodies, and Blanco, L. (1992) EMBO J. 11, 4227–4237
17. Mullen, G. P., Serpersu, E. H., Ferrin, L. J., Loeb, L. A., and Mildvan, A. S. (1991) J. Biol. Chem. 265, 14327–14334
18. Bars, H. M., Hammond, K. A., Kennedy, C. C., Mack, S. L., and Brown, N. C. (1992) Gene (Amst.) 111, 43–49
19. Reha-Krantz, L. J., Stocki, S., Nonay, R. L., Dimayuga, E., Goodrich, L. D., Konigsberg, W. H., and Spicer, E. K. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2417–2421
20. Patel, S. S., Wong, I., and Johnson, K. W. (1991) Biochemistry 30, 511–525
21. Simon, M., Giot, L., and Faye, G. (1991) EMBO J. 10, 2165–2170
22. Morris, A., Bell, J. B., Kunchel, T. A., and Sugino, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 9473–9477
23. Fremont, P. S., Ellis, D. L., Steitz, T. A., and Joyce, C. M. (1986) Proteins Struct. Funct. Genet. 1, 65–72
24. Ruscitti, T., Polavieja, F. D., Kura, A. E., and Linn, S. (1992) J. Biol. Chem. 267, 16806–16811
25. Gibbs, J. S., Weisshart, K., Digard, P., De Bruijn-Kops, A., Knipe, D. M., and Coen, D. M. (1991) Mol. Cell. Biol. 11, 4796–4795
26. Weisshart, K., Kuo, A. A., Hwang, B. C. C., Kumura, K., and Coen, D. M. (1994) J. Biol. Chem. 269, 22788–22796
27. Knopf, C. W. (1986) Nucleic Acids Res. 14, 8225–8226
28. Knopf, C. W. (1987) J. Gen. Virol. 68, 1429–1433
29. Summers, M. D., and Smith, G. E. (1987) Tex. Agric. Exp. Stn. Bull. 1755
30. Bierley, S. (1990) Expression der HSV-1 ANG DNA-Polymerase in Insekten- domännern. PhD thesis, University of Heidelberg
31. Strick, R. (1993) Struktur- und Funktionsanalyse der Herpes Simplex-Virus DNA-Polymerase: Die Enzymfunktionen. doctoral thesis, University of Heidelberg
32. Knopf, K. W. (1988) Die Herpes Simplex-Virus DNA-Polymerase: Vom Gen zur Funktion. Habilitationsschrift, University of Heidelberg
33. Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., and Konigsberg, W. (1986) Protein Struct. Funct. Genet. 1, 65–72
34. Konigsberg, W. H., and Spicer, E. K. (1991) Replication and Functional Control of Bacteriophage Lambda, Chapman and Hall, New York
35. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488–492
36. King, L. A., and Poser, R. D. (1992) The Baculovirus Expression System: A Laboratory Manual, Chapman and Hall, New York
37. Brown, M., and Faulkner, P. (1977) Eur. J. Biochem. 62, 120–129
38. Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., and Konigsberg, W. H., eds) Vol. 3, pp. 87–135, Springer-Verlag, Berlin
39. Ku¨hn, F. J. (1995) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
40. Laemmli, U.-K. (1970) Nature 227, 685–688
41. Brown, M., and Faulkner, P. (1977) Eur. J. Biochem. 62, 120–129
42. Shanafelt, A. B. (1991) BioTechniques 11, 330
43. Sambrook, J., Fritsch, E. F., and Maniatis, F. (1989) Molecular Cloning: A Laboratory Guide, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
44. Hu¨bscher, U. (1993) Frontiers of Virology (Becker, Y., and Darai, G., eds) Vol. 3, pp. 87–135, Springer-Verlag, Berlin
45. Laemmli, U.-K. (1970) Nature 227, 685–688
46. Hart, G. J., and Boehme, R. E. (1992) FEBS Lett. 306, 97–100
47. Hart, G. J., and Boehme, R. E. (1992) FEBS Lett. 306, 97–100
48. Hart, G. J., and Boehme, R. E. (1992) FEBS Lett. 306, 97–100
49. Haffey, M. L., Novotny, J., Bruccoleri, R. E., Carroll, R. D., Stevens, J. T., and Matthews, J. T. (1990) J. Virol 64, 5008–5018
50. Knopf, C. W., and Weisshart, K. (1988) Biochim. Biophys. Acta 951, 298–314
51. Knopf, C. W., and Weisshart, K. (1990) Eur. J. Biochem. 191, 263–273


52. Wang, X., and Kelly, D. C. (1983) *J. Gen. Virol.* **64**, 2229–2236
53. Wang, T. S.-F. (1991) *Annu. Rev. Biochem.* **60**, 513–552
54. Ishino, Y., Iwasaki, H., Kato, I., and Shinagawa, H. (1994) *J. Biol. Chem.* **269**, 14655–14660
55. Reha-Krantz, L. J., and Nonay, R. L. (1993) *J. Biol. Chem.* **268**, 27100–27108
56. Frey, M. W., Nussal, N. G., Capson, T. L., and Benkovic S. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2579–2583
57. Zhu, W., and Ito, J. (1994) *Nucleic Acids Res.* **22**, 5177–5183
58. Hall, J. D., Orth, K. L., Sander, K. L., Swihart, B. M., and Senese, R. A. (1995) *J. Gen. Virol.* **76**, 2999–3008
59. Hall, J. D., Wang, Y., Pierpont, J., Berlin, M. S., Rundlett, S. E., and Woodward, S. (1995) *Nucleic Acids Res.* **23**, 9231–9244
60. Wang, Y., Woodward, S., and Hall, J. D. (1992) *J. Virol.* **66**, 1814–1816

61. Stocki, S. A., Nonay, R. L., and Reha-Krantz, L. J. (1995) *J. Mol. Biol.* **254**, 15–28
62. Coen, D. M. (1991) *Antiviral Res.* **15**, 287–300
63. Knopf, K. W., Kaufman, E. R., and Crumpacker, C. (1981) *J. Virol.* **39**, 746–757
64. Joyce, C. M., Kelley, W. S., and Grindley, N. D. F. (1982) *J. Biol. Chem.* **257**, 1958–1964
65. Chung, D. W., Zhang, J., Tan, C.-K., Davie, E. W., So, A. G., and Downey, K. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 11197–11201
66. Morrison, A., and Sugino, A. (1992) *Nucleic Acids Res.* **20**, 375
67. Zhang, J., Chung, D. W., Tan, C. K., Downey, K. M., So, A. G., and Davie, E. W. (1991) *Biochemistry* **30**, 11742–11750