Evidence that the Nuclease Activities Associated with the Herpes Simplex Type 1 DNA Polymerase Are Due to the 3'-5' Exonuclease

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We investigated nuclease activities associated with the catalytic subunit of herpes simplex virus type 1 DNA polymerase. We confirm that a 3'-5' exonuclease copurifies with this enzyme. Previous reports suggested that a 5' DNase was intrinsic to the polymerase. Our preparation lacks such activity.

Herpes simplex virus type 1 (HSV-1) DNA polymerase is essential for viral replication and is a target for antiviral drugs (e.g., acyclovir). The catalytic subunit of this heterodimeric enzyme shows extensive sequence similarity to other herpesvirus and cellular polymerases (1).

A 3'-5' exonuclease is intrinsic to the HSV-1 polymerase (7, 8, 11) and is inactivated by a mutation within the catalytic subunit (4). RNase H activity has been detected in HSV-1 polymerase preparations from infected cells (holoenzyme) and an overexpression system (catalytic subunit) (2, 8). A 5'-3' exonuclease found in one holoenzyme preparation was proposed to account for this RNase activity (2). However, Knopf and colleagues failed to detect any 5' exonuclease associated with the polymerase holoenzyme and presented evidence that RNase activity could result from the 3'-5' exonuclease (5, 6). Because a 5' RNase H might remove RNA primers during HSV-1 replication, we further examined the nucleases associated with the HSV-1 DNA polymerase catalytic subunit. We confirm that the 3' exonuclease is intrinsic to this enzyme but suggest that a 5' exonuclease is lacking.

Polymerase purification. For these studies, we overexpressed the HSV-1 polymerase catalytic subunit, using a baculovirus vector, and purified it by phosphocellulose chromatography (4), single-stranded DNA-agarose chromatography (8), and sedimentation in a 15 to 35% glycerol gradient (8). Purification steps were performed with buffer C (4) plus 20% glycerol. The polymerase was stored at -20°C in buffer plus 60% glycerol. Specific activities (polymerase units per milligram) recovered at each step (Table 1) were determined by measuring incorporation of radioactive deoxynucleoside triphosphates into salmon sperm DNA (4). As shown in Table 1, the polymerase is sensitive to phosphonoacetic acid, a known inhibitor of herpesvirus polymerases. In contrast, DNA polymerase I (large fragment) from *Escherichia coli* was relatively resistant.

Associated nucleases. As previously observed (7), this polymerase copurifies with a nuclease which digests 3'-labeled DNA and presumably serves to edit misincorporated nucleotides during replication. The data in Fig. 1 confirm that this nuclease activity cosedimented with the polymerase activity during glycerol gradient purification. Exonuclease assays (4) measured production at 37°C of acid-soluble material from

partially duplex substrates, consisting of 3'-labeled oligomers annealed to M13 single-stranded DNA.

The HSV-1 polymerase also copurified with an RNase H activity through single-stranded DNA-agarose or heparin-agarose chromatography (not shown). However, this RNase H separated from the polymerase during glycerol gradient purification (Fig. 1). RNase H activity was measured in assays identical to the exonuclease assay described above except that the substrate was oligo(rA)-poly(dT).

To test whether the RNase H might be a polymerase breakdown product, glycerol gradient peaks were subjected to a Western blot (immunoblot) analysis. Proteins were separated by electrophoresis in a sodium dodecyl sulfate (SDS)-polyacrylamide (7%) gel (9), transferred to a nitrocellulose membrane (9), and probed with chick antibodies made against the HSV-1 polymerase. This process involved sequentially soaking the membrane in 20 mM Tris-HCl (pH 7.6)-137 mM NaCl supplemented with dried milk (5%), then anti-polymerase antibody, then anti-chicken, horseradish peroxidase-linked rabbit immunoglobulin, and finally ECL solution (Amersham Life

TABLE 1. HSV-1 DNA polymerase purification and inhibitor sensitivities^a

DNA polymerase	Purification step	Polymerase (U/ mg of protein)	% Polymerase activity in presence of 10 µg of phosphonoacetic acid per ml
HSV-1 wild type	Phosphocellulose	5.33×10^2	9.7 (0.044)
	Heparin-agarose	1.40×10^3	
	DNA-agarose	2.67×10^3	
	Glycerol gradient	3.40×10^4	
HSV-1 D368A	Phosphocellulose	6.93×10^2	11.6 (0.023)
	Heparin-agarose	2.98×10^3	
DNA Pol I			95.0 (0.076)

^a HSV-1 DNA polymerases were purified from infections with recombinant baculoviruses carrying HSV-1 DNA polymerase genes with either wild-type or D368A sequences. Wild-type polymerase was purified by phosphocellulose chromatography and then by either heparin-agarose chromatography or single-stranded DNA agarose chromatography following by glycerol gradient sedimentation. The mutant polymerase was purified as shown. At each step, peak polymerase fractions were pooled and analyzed for protein concentration (4) and polymerase activity. Activity was determined by using the salmon sperm DNA polymerase assay described in the text with enzyme dilutions which exhibited linear incorporation with incubation time. One enzyme unit = 10 nmol incorporated in 30 min at 37°C. Reaction mixtures with phosphonoacetic acid were incubated for 30 min and contained the enzyme units indicated in parentheses. DNA Pol I indicates the large fragment of DNA polymerase I (*E. coli*) (Gibco BRL).

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FIG. 1. Glycerol gradient analysis of wild-type HSV-1 DNA polymerase. Sedimentation was performed with HSV-1 DNA polymerase purified through single-stranded DNA-agarose chromatography. Gradient fractions were analyzed, as described in the text, for DNA polymerase (●), 3'-5' exonuclease (□), and RNase H activities (○), using 1 μ l of a given fraction per assay. The substrate in the exonuclease reaction was a 32 P-3'-labeled 25-nucleotide oligomer annealed to single-stranded M13 bacteriophage DNA and was present at 0.17 μ g (0.062 pmol) per assay. The RNase H substrate was 3 H-oligo(rA)₃₈₋₁₃₇-poly(dT) annealed at a nucleotide ratio of 1:3 and was present at 0.037 μ g per assay. Activity is presented as a percentage of the peak fraction for each assay.

Science), which creates a chemiluminescent signal detected on X-ray film. As shown in Fig. 2, a band of the expected size was observed for the polymerase peak, but no similar band or breakdown product was found in the RNase H peak. These results show that RNase H activity is a major contaminant during HSV-1 polymerase purification from baculovirus-infected cells, but they do not rule out the possibility that RNase H with lower activity is still intrinsic to the enzyme. Thus, we suggest that our data are not inconsistent with earlier studies (5, 6, 8, 11) showing that highly purified polymerase preparations can digest RNA substrates.

Nuclease specificity. To determine the nuclease specificity of the polymerase, we measured the polarity of substrate digestion. For this study, we compared wild-type polymerase with a mutant which lacks the 3'-5' exonuclease as a result of an aspartic acid-to-alanine mutation at residue 368 (D368A mutation) (4). The mutant was included because a strong 3' exonuclease might remove radioactivity from substrates designed to detect 5' activity, thus masking the 5' nuclease. Mutant and wild-type polymerases were overexpressed from baculovirus vectors and purified by phosphocellulose chromatography (4) and heparin-agarose chromatography (eluting with 50 to 800 mM NaCl in buffer C plus 20% glycerol). The two enzymes were obtained at comparable yields (Table 1), suggesting that

FIG. 2. Western analysis of peaks from the glycerol gradient in Fig. 1. Samples were subjected to SDS-polyacrylamide electrophoresis, transferred to a nitrocellulose membrane, and probed with an antibody directed against a polymerase fragment comprising all but the N-terminal 67 amino acids of the polymerase. Samples were the pooled polymerase peak, 0.2 μ g (lane 1); pooled RNase H peak, 0.2 μ g (lane 2); HSV-1 polymerase prior to glycerol gradient purification, 1.3 and 0.66 μ g (lanes 3 and 4); and crude extracts from 6.7×10^5 *Spodoptera frugiperda* insect cells infected with recombinant baculovirus (*Autographa californica* nuclear polyhedrosis virus) expressing HSV-1 polymerase (lane 5) or nonrecombinant virus (lane 6). Similar results (not shown) were obtained with a second antibody, EX1051 (10).

the D368A mutation does not interfere with polymerase activity or expression.

The analysis in Fig. 3A confirms that the wild-type polymerase contains a 3' exonuclease which digests single-stranded DNA. Reactions were performed with a 5'-labeled oligomer at 37°C (4), stopped by addition of formamide (27%) and EDTA (5.7 mM), and subjected to electrophoresis (20% polyacrylamide-8 M urea) (9) and autoradiography. The results suggest that digestion with wild-type polymerase (Fig. 3A, lanes 2 to 6) occurred exonucleolytically from the 3' end of the substrates, producing a ladder of bands which became shorter with time. Substrates were rapidly digested from 20 to 11 nucleotides and more slowly to smaller products. The 11-mer may represent a minimum size for efficient binding by the enzyme. The D368A mutant polymerase was drastically impaired in this assay, confirming its nuclease deficiency (Fig. 3A, lanes 7 and 8).

Experiments in Fig. 3B indicate that the 3' nuclease acts on double-stranded substrates and suggest that no 5' exonuclease is present. Reactions were conducted as described for Fig. 3A but with a substrate consisting of M13 DNA annealed to a 25-nucleotide oligomer containing four radioactive guanines within the five residues at its 3' end (i.e., GTGGG-3'). In the presence of wild-type polymerase (Fig. 3B, lanes 2 to 6), oligomers were initially shortened from 25 residues to 21 as radioactive nucleotides were removed from the 3' ends. Further digestion converted the radioactivity to dGMP. No ladder of bands indicative of 5' digestion was seen. The D368A mutant (Fig. 3B, lanes 7 and 8) failed to exhibit any detectable 3' activity, as indicated by a lack of dGMP production, or any 5' activity, as indicated by a lack of a ladder of bands.

Conclusions. We have examined the nucleases associated with the catalytic subunit of the HSV-1 DNA polymerase. We confirm that a 3'-5' exonuclease copurifies with this enzyme and is inactivated by the D368A mutation. Our findings address the question of whether this enzyme also contains 5' exonuclease and RNase H activities. A previous report suggested that HSV-1 polymerase holoenzyme contains a 5' exonuclease which digests duplex DNA and RNA-DNA hybrids (2). We failed to detect any 5' exonuclease in the polymerase catalytic subunit. We used a mutant lacking the 3'-5' exonuclease to better detect such an activity, if present. In our prep-

FIG. 3. Polyacrylamide-urea electrophoresis of HSV-1 DNA polymerase digestion products. Reaction mixtures contained 0.1 pmol (0.47 μ g) of substrate consisting of either a 32 P-5'-labeled 20-nucleotide oligomer (A) or a 32 P-3'-labeled 25-nucleotide oligomer annealed to single-stranded M13 DNA (B). Lanes 1 show unreacted substrates. Reactions with DNA polymerases were performed with 0.059 (A) or 0.11 (B) U of polymerase and either wild-type (lanes 2 to 6 in both panels) or D368A mutant (lanes 7 and 8 in both panels) enzymes. Reaction mixtures were incubated for 0 (panel A, lanes 2 and 7), 2 (panel A, lane 3), 5 (panel A, lane 4), 10 (panel A, lane 5), or 30 (panel A, lanes 6 and 8) min or for 0 (panel B, lanes 2 and 7), 15 (panel B, lane 3), 30 (panel B, lane 4), 45 (panel B, lane 5), or 60 (panel B, lanes 6 and 8) min. Arrows indicate oligomer sizes in nucleotides. The size of the 11-mer was determined on a DNA sequencing gel (not shown). dGMP digestion products were identified by thin-layer chromatography with 0.3 M acetic acid and 1 M LiCl (not shown).

aration, a major contaminating RNase H activity separates from the polymerase during glycerol gradient purification and does not appear to be a polymerase breakdown product. However, RNase H activity has been detected in highly purified preparations of the polymerase catalytic subunit (8, 11) and has been found to comigrate with full-length polymerase in situ assays (2, 11). The polarity of the activity in these studies was not determined. Knopf and coworkers (5, 6) presented evidence that the 3'-5' exonuclease of the HSV-1 polymerase holoenzyme could digest RNA-DNA hybrids and suggested that RNase H activity detected in situ was due to the 3'-5' exonuclease, not to a separate enzyme site. Our finding that the catalytic subunit lacks a 5' exonuclease strongly supports this possibility.

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