Identification of an intrinsic 5' → 3' exonuclease with ribonuclease H activity

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The herpes simplex virus-1 DNA polymerase is a heterodimer of Mr 190,000 which consists of the products of the UL30 (Pol) and UL42 genes. The 136-kilodalton Pol gene product contains an intrinsic ribonuclease H activity that specifically degrades RNA•DNA heteroduplexes or duplex DNA substrates in the 5' → 3' direction. It can therefore catalyze the excision of the RNA primers that initiate the synthesis of Okazaki fragments at a replication fork during herpes DNA replication.

This activity is similar to the 5' → 3' exonuclease of Escherichia coli DNA polymerase I in that it can act on duplex DNA as well as on RNA•DNA hybrids (15).

EXPERIMENTAL PROCEDURES

Materials—All reagents were obtained from Sigma unless otherwise noted. Unlabeled polynucleotides ((rA)₄₀₀, (dA)₄₀₀, (dT)₄₀₀, and poly(rU)) were from the Midland Certified Reagent Co. (Midland, TX). Unlabeled deoxynucleoside 5'-triphosphates, ribonucleoside 5'-triphosphates, and Sephadex G-50 (fine) were from Pharmacia LKB Biotechnology Inc. 3H-Labeled oligo(rA) (38–137 residues, 30 pM/mmol) and (3H)²P]ATP (400 Ci/mmol) were from Amersham Corp. (6–13P]ATP (900 Ci/mmol) was from Du Pont-New England Nuclear. Polynucleotide kinase was from Bio-Rad. M13 single-stranded DNA and activated calf thymus DNA were prepared as described (17, 18). Antiserum raised against the carboxyl-terminal portion of the HSV-1 DNA polymerase and an internal decapetide of the predicted UL42 product were generously provided by Drs. Donald Coen (Harvard University) and Deborah Parrish (Ohio State University), respectively.

Buffers—The buffers used were prepared as described previously (14). Buffer A contained 40 mM TAPS, pH 8.5, 3.0 mM MgCl₂, 10% (v/v) glycerol, 100 μg/ml bovine serum albumin, 2.0 mM dithiothreitol.

Nucleic Acids—The (dT)₄₀₀·[²P]oligo(rA) that served as the RNA H substrate was prepared by annealing (dT)₄₀₀ to [²H]oligo(rA) in a 1.3:1 (nucleotide) ratio. (dT)₄₀₀·[²P]poly(rA) (24,000 cpm/pmol RNA) and the [²P]labeled transcript of M13 ssDNA (6,000 cpm/pmol RNA) were made by incubating either (dT)₄₀₀ or M13 ssDNA with E. coli RNA polymerase holoenzyme in the presence of [α-³²P]ATP as the labeled nucleotide triphosphate as described (19). The 5' → 3' labeled substrates were generated by first labeling (rA)₄₀₀ or (dA)₄₀₀ with [³²P]dATP using [²H]dATP and polynucleotide kinase (17). The [²P]·(rA)₄₀₀ was then annealed to (dT)₄₀₀ in a 1:3 (nucleotide) ratio; the [²P]·(dA)₄₀₀ was annealed to the (dT)₄₀₀ in a 2:3 (nucleotide) ratio. The products were then elongated with Drosophila DNA polymerase α as described (16) using [³²P]dATP (10 Ci/mmole) as the labeled deoxyribonucleoside triphosphate. The two substrates were deproteinized by phenol and chloroform extraction and desalted by centrifugation through Sephadex G-50 (fine) (20).

The specific activities were 750 cpm of ³²P and 9,800 cpm of [²H]dATP (10 Ci/mmole) for the (dT)₄₀₀·(rA)₄₀₀ and 260 cpm of [³²P] and 29,000 cpm of [²H]dATP (10 Ci/mmole) for the (dT)₄₀₀·(dA)₄₀₀. Concentrations of polynucleotides are expressed as mol of nucleotide.

Cells and Viruses—R∆305 (21), a thymidine kinase deletion mutant of HSV-1 [F], was used to infect roller-bottle cultures of Vero cells using a multiplicity of infection of 5–10 plaque-forming units/cell.

Purification of HSV-1 DNA Polymerase—The HSV-1 DNA polymerase was purified from nuclear extracts of HSV-1 infected Vero cells (14) essentially as described (22) except that heparin-agarose was used in place of DNA-cellulose, and gel filtration through Superose 12 (14) was substituted for glycerol gradient sedimentation. Approximately 400 μg of nearly homogenous HSV-1 DNA polymerase was obtained from 60 g (wet weight) of HSV-1-infected Vero cells.

Enzyme Assays—DNA polymerase activity was assayed in reaction mixtures (25 μl) that contained 40 mM HEPEs, pH 7.5, 150 mM ammonium sulfate, 10% (v/v) glycerol, 3.0 mM MgCl₂, 100 μg/ml

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bovine serum albumin, 2.0 μg of activated calf thymus DNA, 2.0 mM dithiothreitol, and 25 μM each of dATP, dGTP, dCTP, and dTTP. The dTTP was labeled with 3H at 4.0 Ci/mmol. The reaction was started by the addition of 1.0 μl of enzyme fraction; incubation was for 20 min at 34 °C. RNase H activity was assayed essentially as described (23). The standard reaction mixture (25 μl) contained Buffer A, and RNase H substrate was added as indicated: 20 μM (dT)ₙ₀₀₀₀ [³²P]oligo(rA), 10 mM ³²P-labeled M13 ssDNA transcript annealed to M13 ssDNA, 480 μM (dT)ₙ₀₀₀₀ [³²P,³⁻³H] (rA)ₙ₀₀₀₀ or 480 μM (dT)ₙ₀₀₀₀ [³⁻³P,³⁻³H] (dA)ₙ₀₀₀₀. Unless otherwise indicated, 0.5 μg of the Superose 12 fraction of herpes DNA polymerase was added to the standard reaction mixture (25 μl) for 20 min at 34 °C. RNase H activity was assayed essentially as described (23). The standard reaction mixture (25 μl) for both DNA polymerase and RNase H activities was added to start the reaction. After 20 min, acid-soluble nucleotide was determined (23). For the Mg⁺⁺ and pH profiles, Mg⁺⁺ was varied as indicated, and pH was varied by changing the reaction buffer (40 mM TAPS, pH 8.5) to one of the following: MES, pH 5.5 and 6.0; ADA, pH 6.5; MOPS, pH 7.0; HEPEs, pH 7.5; HEPPs, pH 8.0; TAPS, pH 8.5; CHES, pH 9.0 and 9.5; and CAPS, pH 10.0.

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described (24). When necessary, gels were fixed and either stained with silver (25) or Coomassie Brilliant Blue (24). Western immunoblot analysis was carried out as described (26, 27). In situ renaturation of RNase H activity was performed as described (19), casting the SDS-polyacrylamide gel (10 cm × 8 cm × 0.75 mm) with 10 nlml of the (dT)ₙ₀₀₀₀ [³²P(poly(rA)) RNase H substrate. After electrophoresis, the gel was soaked in several changes of 40 mM TAPS, pH 8.5, 50 mM NaCl, 3.0 mM EDTA, 1.0 mM EGTA as described (28). HSV-1 DNA polymerase that had been purified through the Superose 12 step (50 μg) was diluted 2-fold in the above buffer and layered onto the gradient. Protein standards (bovine serum albumin, aldolase, and catalase) were applied to a parallel gradient. Centrifugation was at 4 °C for 24 h at 50,000 rpm in an SW 60 rotor. A total of 25 fractions were collected and assayed for DNA polymerase (1.0 μl) and RNase H (4.0 μl) activity.

RESULTS

Detection of RNase H Activity in HSV-1 DNA Polymerase—The highly purified HSV-1 DNA polymerase contains a potent RNase H activity (Fig. 1). The initial rate of hydrolysis of the RNA-DNA hybrid (dT)ₙ₀₀₀₀ [³²P]oligo(rA) was more than 10-fold greater than the rate at which the single-stranded oligo(rA) was hydrolyzed. The products of hydrolysis were nucleoside 5'-monophosphates. A double-stranded RNA substrate (poly(rU)) [³²P]oligo(rA) was completely resistant to hydrolysis (data not shown). The RNase H activity was 10-fold less active on RNA-primed M13 ssDNA than on the (dT)ₙ₀₀₀₀ [³²P]oligo(rA) homopolymer pair (data not shown).

Cosedimentation of DNA Polymerase and RNase H Activities

![Fig. 1. Time course of RNase H activity associated with HSV-1 DNA polymerase.](image)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Pmol nucleotide released</th>
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<tr>
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<td>15</td>
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Fig. 1. Time course of RNase H activity associated with HSV-1 DNA polymerase. Assays were performed as described under "Experimental Procedures," except that the period of incubation was varied as indicated. The substrates used were (dT)ₙ₀₀₀₀ [³²P]oligo(rA) (□) or [³²P]oligo(rA) (○). Identical assays performed using poly(rU) [³²P]oligo(rA) detected no activity above background.

HSV-1 RNase H

![Fig. 2. Cosedimentation of HSV-1 DNA polymerase and RNase H activities.](image)

Fig. 2. Cosedimentation of HSV-1 DNA polymerase and RNase H activities.

HSV-1 DNA polymerase was sedimented through a 5–20% sucrose gradient as described under "Experimental Procedures." Fractions from the gradient were assayed for either DNA polymerase (□) or RNase H (○) activity. Results are expressed as pmol of nucleotide incorporated/μl (DNA polymerase activity) or pmol of nucleotide released/μl (RNase H activity) in 20 min. Proteins of unknown sedimentation coefficient were applied to a sucrose gradient to run in parallel. Their positions in the gradient are indicated: catalase, 11.3 S; aldolase, 8.3 S; and bovine serum albumin, 4.3 S. b, aliquots (15 μl) of fractions 5–20 and the material applied to the gradient (4 μl) were analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with silver. The HSV-1 DNA polymerase polypeptide (Pol, Mₙ = 136,000) and the UL42 product, a series of polypeptides of Mₙ = 60,000–64,000 (see also Fig. 3), are indicated. DF, dye front; LO, load.
**FIG. 3. Immunochemical analysis of sucrose density gradient fractions.** The fractions analyzed in Fig. 2 were immunoblotted as described under "Experimental Procedures." Fractions 11, 12, and 13 contain the peak of DNA polymerase and RNase H activities. Polypeptides that were immunoreactive are indicated. a, anti-HSV-1 DNA polymerase (Pol) antiserum; b, anti-UL42 antiserum. DF, dye front; LO, load.

Pol and/or the UL42 products. Their lack of immunoreactivity may represent limitations in the antisera used since neither was raised against a full length polypeptide. Alternatively, although less likely, they may represent as yet unidentified herpes or host-encoded polypeptides that play a role in HSV-1 DNA replication.

As shown in Table I, combination of the Stokes radius (59 Å) and the sedimentation coefficient (7.3 S) yielded a molecular weight of 190,000, assuming a partial specific volume of 0.725. This value corresponds closely to a 1:1 stoichiometry for the Pol and UL42 products (198,000 or 187,000 based on SDS-polyacrylamide gel electrophoresis or sequence, respectively).

**RNase H Activity Is Associated with HSV-1 DNA Polymerase Polypeptide—**To determine which of the polypeptides associated with the HSV-1 DNA polymerase (see Fig. 2) is responsible for the RNase H activity, the enzyme was subjected to SDS-polyacrylamide gel electrophoresis in the presence of the labeled (dT)$_{6000}$. [$^{32}$P]poly(rA) substrate. Following autoradiography, an area of clearing indicative of RNase H activity appeared in the negative of the autoradiogram (Fig. 4A) which corresponded to the position of migration of the

![FIG. 4. In situ assay of RNase H activity. HSV-1 DNA polymerase was denatured, electrophoresed through an SDS-polyacrylamide gel cast with the (dT)$_{6000}$. [$^{32}$P]poly(rA) RNase H substrate, and allowed to renature (see "Experimental Procedures"). The gel was dried and autoradiographed for 3 days. A, negative image of autoradiogram. B, an identical lane of the same gel as in A that was fixed and stained with Coomassie Brilliant Blue. Indicated are the HSV-1 DNA polymerase polypeptide (Pol), $M_r = 136,000$, the UL42 gene product (UL42), and the $M_r = 30,000$ polypeptide found to copurify with the HSV-1 DNA polymerase (see Fig. 2). DF, dye front.
HSV-1 DNA polymerase polypeptide (Mr = 136,000) (Fig. 4B). An Mr = 30,000 polypeptide, presumably a DNA polymerase degradation product, also showed RNase H activity. As shown in Fig. 4B, which depicts a parallel lane of the same gel stained with Coomassie Brilliant Blue and containing the same amount of HSV-1 DNA polymerase as in Fig. 4A, no RNase activity was associated with the UL42 gene product, the cluster of polypeptides centered at Mr = 60,000.

The RNase H Associated with the HSV-1 DNA Polymerase Is Most Active under Conditions of High pH and Low Mg²⁺ Concentration—The RNase H associated with the HSV-1 DNA polymerase was optimally active at alkaline pH. As shown in Fig. 5a, highest activity was at pH 10.0, and there was little (0–10%) activity between pH 6.5 and 7.0, where the enzyme shows substantial DNA polymerase activity. The RNase H was most active at 1 mM Mg²⁺ and was inactive at 10 mM Mg²⁺, the concentration commonly used to measure DNA polymerase activity (Fig. 5b). Essentially the same Mg²⁺ concentration dependence was obtained with the M13 RNA-DNA heteroduplex (data not shown).

The RNase H Activity of the HSV-1 DNA Polymerase Hydrolyzes an RNA-DNA Heteroduplex or DNA Duplex in the 5' → 3' Direction—An RNase H that catalyzes removal of the RNA primers that initiate the synthesis of Okazaki fragments on the lagging strand at a replication fork must act in the 5' → 3' direction. The polarity of hydrolysis of the RNase H associated with the HSV-1 DNA polymerase was therefore examined with two double-labeled substrates. One consisted of 5'-³²P-labeled (dA)₂₀₀ with from 2 to 4 [³H]dAMP residues at the 3' end annealed to (dT)₆₀₀₀. The second substrate was constructed identically except that 5'-³²P(dA)₆₀₀₀ with from 20 to 40 [³H]dAMP residues at the 3' terminus was annealed to (dT)₆₀₀₀₀. The incubation period (25 s) was short enough that removal of ³²P should solely reflect 5' → 3' exonuclease, and removal of ³H should indicate the 3' → 5' exonuclease activity known to be associated with the HSV-1 DNA polymerase (22). With the double-labeled (dT)₆₀₀₀₀ (dA)₆₀₀₀ substrate described under “Experimental Procedures,” O, fraction of ³²P released; •, fraction of ³H released.

Fig. 6. Preferential removal by RNase H of 5'-terminal nucleotides from RNA-DNA heteroduplex. The indicated amounts of HSV-1 DNA polymerase were added to assays performed using the 5'-³²P, 3'-³H-labeled (dT)₂₀₀₀ (dA)₂₀₀ RNase H substrate described under “Experimental Procedures.” O, fraction of ³²P released; •, fraction of ³H released.

Fig. 7. Preferential removal by RNase H of 5'-terminal nucleotides from DNA duplex. Assays were performed adding the indicated amounts of HSV-1 DNA polymerase utilizing the 5'-³²P, 3'-³H-labeled (dT)₆₀₀₀₀ (dA)₆₀₀₀ substrate described under “Experimental Procedures.” O, fraction of ³²P released; •, fraction of ³H released.
RNA primers that initiate the synthesis of Okazaki fragments on the lagging strand at a replication fork during herpes DNA replication. This activity is analogous to the 5′–3′ exo-
uclease associated with *E. coli* DNA polymerase I, which acts both in the excision of photoproducts during DNA repair and the removal of RNA primers in the course of the semidiscon-
tinuous replication of the *E. coli* chromosome (15).

The 5′ → 3′ exonuclease is clearly an intrinsic component of the 136-kDa DNA polymerase polypeptide. Thus, the two activities coelute during Superose 12 gel filtration and cose-
iment during sucrose density gradient sedimentation. More decisively, in *situ* analysis by renaturation of the enzyme following SDS-polyacrylamide gel electrophoresis showed the RNase H activity to be associated with the 136-kDa DNA polymerase polypeptide and a 30-kDa polypeptide that may be a proteolytic product of the polymerase. This polypeptide may therefore be analogous to the “small” fragment of *E. coli* DNA polymerase I which contains the 5′ → 3′ exonuclease domain at the amino terminus of the protein (15). The 30-
kDa polypeptide, which is present in HSV-1 DNA polymerase preparations (see Fig. 2b), did not react with antibody raised against the carboxyl-terminal portion of the enzyme. Con-
trolled proteolysis of the herpes polymerase may reveal whether the 5′ → 3′ exonuclease resides in a domain separate from that occupied by the polymerase and 3′ → 5′ exonuclease activities.

The RNase H activity of the HSV-1 DNA polymerase is optimally active at alkaline pH and at low Mg\(^{2+}\) concentrations. Little or no activity can be detected at pH 7.0–7.5 and 10 mM Mg\(^{2+}\), conditions routinely used to assay the DNA polymerase activity. This may, in part, explain why the RNase H activity has not been detected previously in herpes DNA polymerase preparations (22). In addition, the RNase H ac-
tivity was 10-fold more active on RNA-DNA homopolymers such as (dT)\(_{100}\)-oligo(dA) than on RNA-primed M13 ssDNA. We have no explanation for this difference; further analysis of the RNase H should resolve this question.

Several polypeptides coseem with the HSV-1 DNA polymerase (see Fig. 2b). As determined by Western analysis, these include the products of the UL30 (Pol) and UL42 genes, and two polypeptides with molecular weights of 40,000 and 30,000. The latter may be proteolytic products of the DNA polymerase and/or the UL42 product. They do not, however, immunoreact with antisera that were raised against portions of these polypeptides. The possibility does remain, however, that the two polypeptides are the products of unidentified host or HSV-1 genes that play a role in HSV-1 DNA replication.

Physical characterization of the HSV-1 DNA polymerase showed it to be significantly larger than predicted by DNA sequence analysis of the UL30 (Pol) open reading frame. Combination of the Stokes radius (59 Å) with the sedimenta-
tion coefficient (7.3 S) yielded a molecular weight for the herpes DNA polymerase of 190,000, close to that predicted for a 1:1 complex of the UL30 (Pol) and UL42 gene products (198,000 or 187,000 as estimated by SDS-polyacrylamide gel electrophoresis or nucleotide sequence, respectively). The HSV-1 DNA polymerase may therefore consist of two sub-
units: the products of the Pol and UL42 genes, a conclusion that is in agreement with the finding that the UL42 analogue of HSV-2 exists in association with the DNA polymerase (13).

REFERENCES