Journal of Biochemistry and Molecular Biology, Vol. 31, No. 6, November 1998, pp. 585–589

JOURNAL OF
Biochemistry
and
Molecular Biology

Overexpression, Purification, and Characterization of the Herpes Simplex Virus-1 DNA Polymerase-UL42 Protein Complex

Byeong Doo Song[†] **and I. Robert Lehman***

Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305, USA

Received 7 August 1998, Accepted 9 September 1998

The herpes simplex virus type-1 (HSV-1)-encoded DNA polymerase consists of two subunits, the products of the UL30 and UL42 genes. UL30 and UL42 were coexpressed in Sf9 cells infected with recombinant baculoviruses carrying the two genes. The UL30 and UL42 gene products remained tightly associated throughout the purification, which led to a near homogeneous heterodimer composed of the DNA polymerase and UL42 protein. The DNA polymerase-UL42 protein heterodimer, purified from the recombinant baculovirus-infected Sf9 cells, showed the same high degree of processivity of deoxynucleotide polymerization as the enzyme purified from the HSV-1 infected primate cells. Like the latter, it contained a 3′-5′ exonuclease activity that specifically hydrolyzes an incorrectly matched nucleotide at the 3′ terminus of a primer, thereby contributing to the fidelity of DNA replication.

Keywords: Exonuclease, Fidelity, HSV-1 Pol-UL42, Processivity.

Introduction

The herpes simplex virus type-1 (HSV-1) genome is a linear 153 kb DNA duplex. Of the approximately 75 open reading frames, the products of seven have been shown to be necessary and sufficient for replication of the viral genome. These gene products are a single-strand DNA-binding protein (ICP8), a heterotrimeric helicase-primase (Crute *et al.*, 1988; Crute and Lehman, 1989), an origin-binding protein (Elias *et al.*, 1986; Olivo *et al.*, 1988), a DNA polymerase, and a duplex DNA binding protein (UL42 protein) (Elias *et al.*, 1986; Marsden *et al.*, 1987; Gallo *et al.*, 1988; Parris *et al.*, 1988). The 65 kDa UL42 protein exists in a 1:1 complex with the 136 kDa UL30 DNA polymerase polypeptide (Park *et al.*, 1982; Crute and Lehman, 1989; Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990). The UL42 protein interacts with origin-binding protein (UL9 protein) (Monahan *et al.*, 1998) and the DNA polymerase subunit interacts with UL8 protein of the heterotrimeric helicase-primase (Mardsen *et al*, 1997). In addition to DNA polymerase activity, the DNA polymerase-UL42 protein complex has a proofreading activity that can function as a 3′-5′ exonuclease. The mutations in the Exo III motif of the DNA polymerase incorporated into the viral genome produced increased mutation frequencies (Hwang *et al.*, 1997).

We have coexpressed the genes for the DNA polymerase and UL42 protein using the baculorvirus-Sf9 insect cell system and purified the heterodimeric enzyme to near homogeneity in milligram quantities. The baculovirus-expressed enzyme complex is identical in its processivity and proofreading capacity to that of the complex isolated from HSV-1 infected mammalian cells.

Materials and Methods

Enzymes and DNA Oligonucleotides were purchased from Oligos Etc. Activated calf thymus DNA was prepared by treatment of calf thymus DNA (Sigma, St. Louis, USA) with DNase as previously described (Uyemura and Lehman, 1976) to generate DNA fragments approximately 200 bp in length. Heparin-Sepharose was prepared as described (Davison *et al.*, 1979). Restriction enzymes were purchased from New England Biolabs (Beverly, USA). Polynucleotide kinase, exonuclease-free T7 DNA polymerase, and Sequenase version 2.0 were obtained from Amersham Life Sciences (Arlington Heights, USA).

Buffers Buffer A contained 40 mM Hepes, pH 7.5, 2 mM

[†]Present Address:
School of Construction & Urban Environmental Engineering, Handong University, Heunghae, Pohang, Kyongbuk, 791-940, Korea

* To whom correspondence should be addressed.
Tel: 1-415-723-6164; Fax: 1-415-723-6783
E-mail: shjang@biho.taegu.ac.kr

EDTA, 2 mM EGTA, 1 mM DTT, 10 mM mercaptoethanol, 10 mM sodium bisulfite, pH 7.5, 10% glycerol, 2 μg/ml leupeptin and pepstatin A, 1 mM PMSF, and 1 mM aminobenzamidine·2HCl. Buffer B contained 40 mM Hepes, pH 7.5, 2 mM EDTA and EGTA, 5 mM sodium bisulfite, 1 μg/ml leupeptin and pepstatin A, and 10% glycerol.

Cells *Spodopotera frugiperda* cells (Sf9 cells, a generous gift from M. Summers, Texas A & M University) were maintained at 27°C in Grace's medium (GIBCO, Rockville, USA) supplemented with 0.33% TC yestolate (Difco, Rockville, USA), 0.33% lactalbumin hydrosylate (Difco), and 10% heat inactivated fetal bovine serum (GIBCO).

Purification and quantitation of oligonucleotides The 30 mer and 50 mer oligonucleotides were purified by electrophoresis through a 15% polyacrylamide gel, electroeluted from the gel with the Elutrap (Schleicher & Schuell, Keene, USA), concentrated with a Speedvac, and desalted by ethanol precipitation or by Bio-Spin 6 (Bio-Rad, Hercules, USA) gel filtration. The 30 mer was 5′-^{32}P labeled with polynucleotide kinase and [γ-^{32}P] ATP, and annealed to the 50 mer. The measured specific activity (cpm/pmol) was then used to calculate the concentration of the 30/50 mer. Based on the concentration and the A_{260}, the extinction coefficient was calculated to be 1.9×10^6. The same extinction coefficient was used for 30/50 mer containing a terminal deoxyadenylate (29A/50 mer). The concentration of the 30/50 mer was also determined by measuring labeled deoxynucleotide incorporation in the presence of an excess of HSV-1 DNA polymerase. The reaction mixture (100 μl) incubated at 37°C contained 40 mM Hepes, pH 7.5, 150 mM KCl, 12 mM $MgCl_2$, 0.1 μM 30/50 mer (based on the A_{260}), 2 μM [α-^{32}P] dATP, 0.5 μCi/μM, and 50–100 nM HSV-1 DNA polymerase. Aliquots (10 μl) were taken at 1 min intervals. The reaction was stopped by the addition of 10 μl of 0.5 M EDTA, pH 8.0, and [^{32}P] dAMP incorporation was measured as described below. Both methods agreed within 10%. The oligonucleotides used are shown in Scheme I.

```
30/50mer   GCCTCGCAGCCGTCCAACCAACTCTACCCT
           CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC

29A/50mer  GCCTCGCAGCCGTCCAACCAACTCTACCC(A)
           CGGAGCGTCGGCAGGTTGGTTGAGATGGGATGAGTTTGAAGTAGGTACAC
```

Construction of a recombinant baculovirus containing the HSV-1 DNA polymerase (UL30) gene Plasmid pTH1 containing the UL30 gene (Hernandez and Lehman, 1990) was treated with *Nde*I and the 3′ recessed end produced was filled in with T7 DNA polymerase (Sequenase). The resulting blunt ended linear DNA was digested with *Xba*I. A fragment of the appropriate size was isolated and ligated into the pVL1393 (Invitrogen, Carlsbad, USA) that had been treated with *Sma*I and *Xba*I. The resulting recombinant plasmid, pBK22, was used to generate the recombinant baculovirus AcMNPV/UL30, BKV22, according to the instructions provided by the manufacturer (Invitrogen). Confirmation that the UL30 gene was out of frame with the polyhedrin gene was obtained by dideoxy sequencing using the oligonucleotide 5′-ATGATAACCATCTCGCAA-3′, which corresponds to a sequence 74 bases upstream of the start codon of the polyhedrin gene as a primer.

The recombinant baculovirus containing the UL42 gene (AcMNPV/UL42) was kindly provided by Dr. Mark Challberg (NIH).

Overexpression and purification of the DNA polymerase-UL42 protein complex Sf9 cells were seeded into thirty 225-cm^2 flasks and allowed to grow to 60–70% confluence. After removal of the medium, each flask was inoculated with 30 ml of recombinant baculovirus, 15 ml of AcMNPV/UL30, and 15 ml of AcMNPV/UL42, each at 10^8 pfu/ml. After 3 h of incubation, the inoculum was replaced with 35 ml of the fresh medium. The infected cells were incubated for 72 h at 27°C, dislodged by shaking and collected by centrifugation at $1500 \times g$ for 10 min. The cell pellet (10 ml) was resuspended and homogenized with a Dounce homogenizer in 1.5 M NaCl in buffer A. The resulting cell extract was centrifuged for 40 min at 40,000 rpm using the Ti45 rotor. The supernatant was dialyzed against buffer A containing 0.1 M NaCl. The purification procedure used was that described by Crute and Lehman (1989) except that Q-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) was substituted for the gel filtration step. The HSV-1 DNA polymerase lacking the UL42 protein was purified using the same procedure as that used for the DNA polymerase-UL42 protein complex.

DNA polymerase assay DNA polymerase activity was measured in a reaction mixture (20 μl) that contained 40 mM Hepes, pH 7.5, 150 mM KCl, 12 mM $MgCl_2$, 0.2 mg/ml activated calf thymus DNA, 12.5 mM each of dATP, dCTP, dGTP, and [^{3}H] dTTP, 460–480 cpm/pmol. The reaction was started by the addition of 1.0 μl of fractions from column chromatography. Incubation was for 10 min at 37°C. One-half of the reaction mixture (10 μl) was directly spotted onto a DE 81 filter. The filters were washed three times with 0.5 M sodium phosphate, pH 7.5, and then with ethanol before drying. The labeled DNA product was measured by scintillation counting.

Measurement of processivity Singly-primed M13mp18 DNA was prepared by annealing a 5′-^{32}P labeled primer (5′-AAATACCGAACGAAC-3′) to single-stranded M13mp18 DNA. The reaction was then initiated by mixing 5 μl of solution A containing 5 nM enzyme, 1 nM DNA in 20 mM Hepes, pH 7.5, 40 mM KCl, and 5% glycerol with 5 μl of solution B containing 3.4 mg/ml activated calf thymus DNA, 4 mM $MgCl_2$, 40 μM each of dATP, dCTP, dGTP, and dTTP in 20 mM Hepes, pH 7.5, 40 mM KCl, and 5% glycerol. In the controlled reaction, activated calf thymus DNA (3.4 mg/ml) was added to solution A in place of solution B. The reaction was stopped by addition of 2 μl of alkaline gel loading buffer. The products were analyzed by 1% agarose gel electrophoresis under alkaline conditions. Following electrophoresis, the gel was dried and autoradiographed.

Single nucleotide incorporation and exonuclease assay The single nucleotide incorporation reaction was initiated by mixing 37 μl of solution A containing 40 nM HSV-1 DNA polymerase, 1 nM 5′-^{32}P labeled DNA (30/50 mer) in 40 mM Hepes, pH 7.5, 150 mM KCl, 2.5% glycerol with 37 μl of solution B containing 100 μM dATP and 12 mM MgCl2 in 40 mM Hepes, pH 7.5, 150 mM KCl, 2.5% glycerol. The reaction was stopped with 60 μl of 0.5 M EDTA, pH 8.0, using a rapid quench flow apparatus (KinTek Instruments, College Park, USA). For the exonuclease

assay, the dATP of solution B was omitted. The products were analyzed by electrophoresis through a 15% polyacrylamide gel containing 7 M urea at 50–55°C. The gel was dried and the radioactive products were quantitated by scanning with PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

Western-blot analysis and protein assay Western analysis was performed using antisera directed against the HSV-1 DNA polymerase and the UL42 protein (Hernandez and Lehman, 1990). Protein concentrations were determined by the Bradford method with bovine serum albumin as standard (Bradford, 1976).

Results

Purification of recombinant HSV-1 DNA polymerase-UL42 complex from doubly infected insect cells Electrophoresis of the Q-Sepharose fractions through an SDS-polyacrylamide gel showed two distinct bands that coincided with DNA polymerase actvity (Fig. 1) throughout the peak and migrated with apparent molecular weights of 136,000 and 65,000, values that are close to those expected for the proteins encoded by the UL30 and UL42 genes (Crute *et al.*, 1989), respectively. They were also identical in electrophoretic mobility to the corresponding subunits of the enzyme purified from the HSV-1 infected Vero cells (data not shown). Immunoblot analysis using antisera directed against the DNA polymerase and UL42 protein showed that the purified enzyme is, in fact, composed of the UL30 and UL42 gene products (Fig. 2). Approximately 1.2 mg of nearly homogeneous HSV-1 DNA polymerase-UL42 protein complex was obtained.

Processivity of nucleotide polymerization In the absence of the UL42 protein, the HSV-1 DNA polymerase polymerized approximately 500 nucleotides per binding event. The DNA polymerase-UL42 protein complex polymerized 1700 nucleotides per binding event (Fig. 3). It is likely that the processivity of the complex is much greater than 1700 since the enzyme was limited by a strong stop site in the M13mp18 template. The DNA polymerase-UL42 protein complex purified from HSV-1 infected Vero cells showed the same processivity as the baculovirus expressed enzyme (Fig. 3).

3′-5′ Exonuclease activity In the absence of dATP, both the DNA polymerase subunit alone and the DNA polymerase-UL42 protein complex excised a 3′ mismatched nucleotide at a much greater rate than the correct nucleotide (Figs. 4A, 4B). The extent of excision

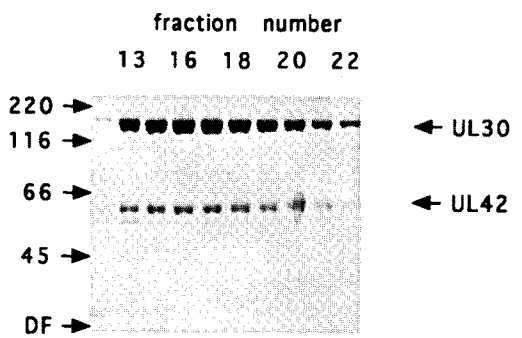

Fig. 1. SDS-polyacrylamide gel electrophoresis of Q-Sepharose Fast Flow fractions of DNA polymerase-UL42 protein. Following electrophoresis of each fraction (4 μl), the polypeptides were visualized by staining with Coomassie blue. The positions of the molecular weight markers are indicated. DF, dye front.

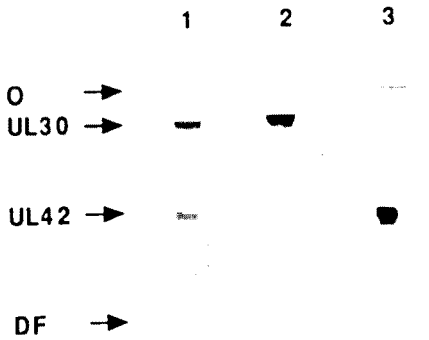

Fig. 2. Immunoblotting of purified DNA polymerase-UL42 protein. The pooled Q-Sepharose fractions (15–20) (Fig. 1), were immunoblotted as described under Materials and Methods. Lane 1 was stained with Coomassie blue. Lanes 2 and 3 were individually probed with 1:100 dilution of the UL30 or UL42 antisera. O, origin. DF, dye front.

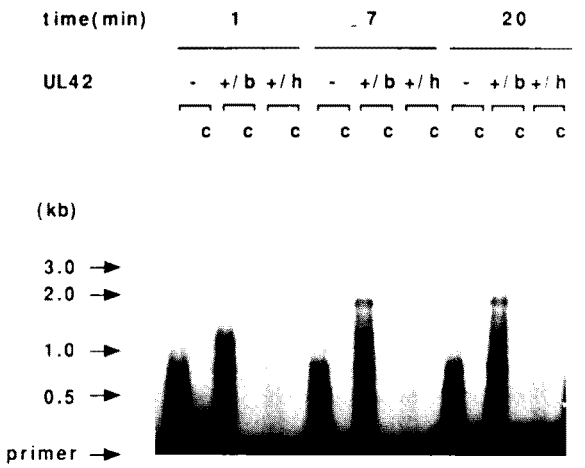

Fig. 3. Processivity of DNA polymerase subunit and DNA polymerase-UL42 protein. The processivity of the DNA polymerase (−), and the DNA polymerase-UL42 protein complex purified from baculovirus infected insect cells (+/b), or from HSV-1 infected cells (+/h) was analyzed as described under Materials and Methods using singly primed M13mp18 DNA as template. C, control reaction in which the enzyme was preincubated with both primed M13mp18 and activated calf thymus DNA. The position of DNA standards is indicated.

was also greater for the mismatched than for the matched nucleotide (Figs. 4A, 4B). The DNA polymerase-UL42 protein complex showed somewhat greater exonuclease activity with the mismatched nucleotide than the DNA polymerase subunit alone. In the presence of the next correct nucleotide (dATP), the correctly paired DNA was extended equally well by the DNA polymerase-UL42 protein complex and the DNA polymerase subunit, and little degradation of the DNA substrate was observed (Figs. 5A, 5B). However, with 29A/50 mer containing a 3′ terminal mismatch, little deoxynucleotide incorporation could be detected with either form of the enzyme. Instead, the mismatched nucleotide at the 3′-end of the primer was removed. Its removal was not affected by addition of the next correct nucleotide deoxynucleoside triphosphate (Figs. 5A, 5B).

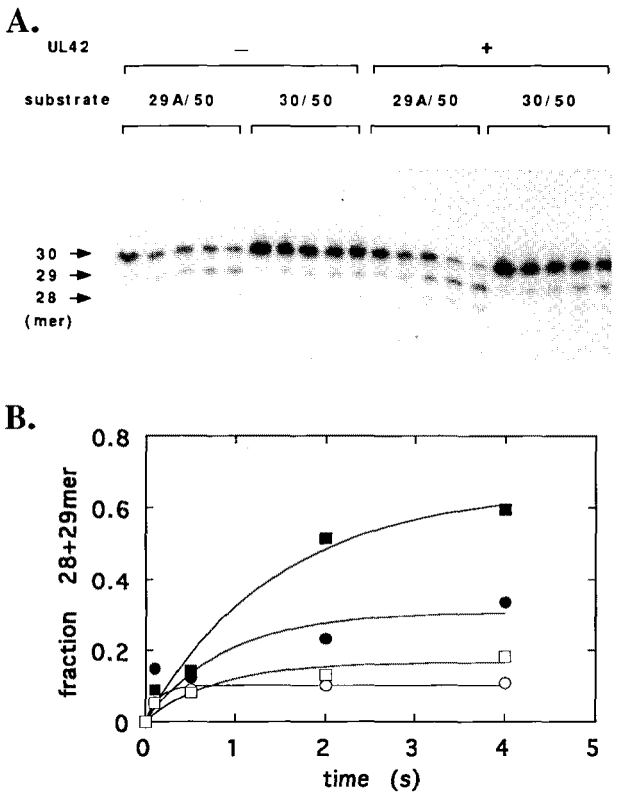

Fig. 4. Exonuclease activity of DNA polymerase subunit and DNA polymerase-UL42 protein. A. Exonuclease activity was measured as described under Materials and Methods with the correctly matched DNA substrate (30/50) or the DNA substrate (29A/50) with a terminal mismatched nucleotide. Each set of five lanes represents five different time points, 0, 0.1, 0.5, 2.0, and 4.0 s (from left to right). The sizes of the substrate and products are indicated. B. Time course of exonuclease activity. Following quantitation of the 30, 29, and 28 mers with PhosphorImager, the fraction of 28+29 mer was plotted against time. Circles represent the DNA polymerase subunit and squares represent the DNA polymerase-UL42 protein. Open symbols, 30/50. Closed symbols, 29A/50.

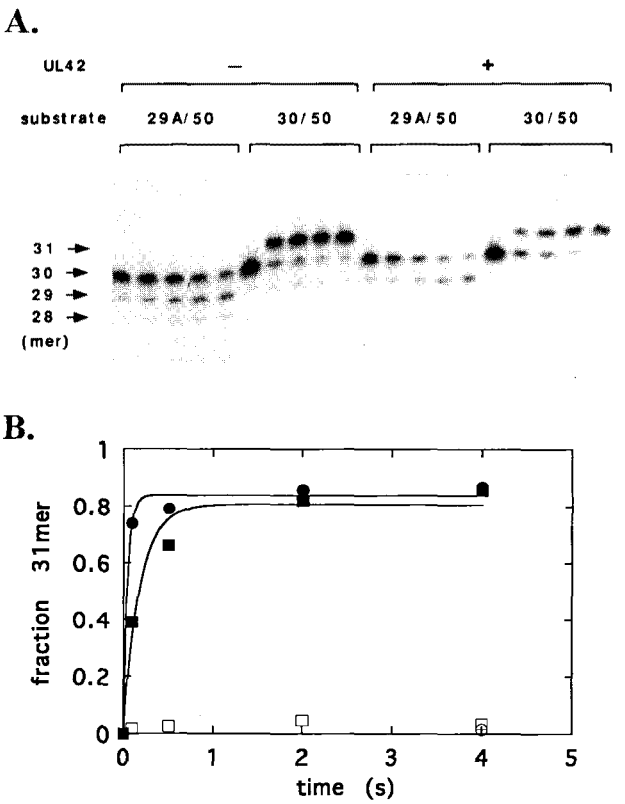

Fig. 5. Single nucleotide incorporation by DNA polymerase and DNA polymerase-UL42 protein. A. Single nucleotide incorporation was performed as described under Materials and Methods with the matched DNA substrate (30/50) or the DNA substrate (29A/50) with a terminal mismatched nucleotide. Each set of five lanes represents five different time points, 0, 0.1, 0.5, 2.0, and 4.0 s (from left to right). The sizes of the substrate and products are indicated. B. Time course of single nucleotide incorporation. Following quantitation of the 31, 30, 29, and 28 mers with PhosphorImager, the fraction of 31 mer was plotted against time. Circles represent DNA polymerase subunit and squares represent DNA polymerase-UL42 protein. Open symbols, 29A/50. Closed symbols, 30/50.

Discussion

Simultaneous infection of Sf9 cells with recombinant baculoviruses carrying HSV-1 UL30 and UL42 genes produced a DNA polymerase-UL42 protein complex in amounts sufficient for detailed enzymological analysis. This approach has been successfully applied to generate a SV40 large T antigen-murine p53 complex (O'Reilly and Miller, 1988), the multisubunit influenza virus RNA polymerase (Kabayachi *et al.*, 1992), and the HSV-1 helicase-primase (Dodson *et al.*, 1989). The HSV-1 DNA polymerase-UL42 protein complex isolated from the baculovirus infected Sf9 cells shows the similar level of processivity of dideoxynucleotide polymerization (>1700 nucleotides polymerized per binding event) as the enzyme

from HSV-1 infected mammalian cells. The DNA polymerase subunit alone has a processivity of about 500 nucleotides, confirming the previous finding that the UL42 protein serves as the processivity enhancing factor. The HSV-1 DNA polymerase subunit is unusual in that it has a relatively high processivity in the absence of a processivity enhancing factor (Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990) . For example, *E. coli* DNA polymerase III*, which lacks the β subunit of DNA polymerase III holoenzyme, incorporates only about 190 nucleotides per binding event, the T7 DNA polymerase has a processivity of 1–50 in the absence of thioredoxin, and DNA polymerase δ without PCNA has a processivity of less than 30. Thus, it is possible that the UL42 protein, which is essential for HSV-1 DNA replication, serves one or more additional functions in HSV-1 DNA replication in addition to increasing the processivity of DNA synthesis. In support of this notion are the findings that the UL42 protein is phosphorylated and occurs in substantial excess over the DNA polymerase subunit in HSV-1-infected mammalian cells. Similarly, both thioredoxin and PCNA are known to have functions in addition to processivity enhancement.

Both the DNA polymerase subunit and the DNA polymerase-UL42 protein complex very efficiently hydrolyze a 3′ terminal mismatched nucleotide, even in the presence of the next correct dideoxynucleoside triphosphate. However, neither enzyme showed significant exonuclease activity with the correctly matched nucleotide. The DNA polymerase-UL42 protein complex shows somewhat greater exonucleae activity with the mismatched terminal nucleotide than the DNA polymerase subunit. The increased proofreading capacity may result from greater intrinsic exonuclease activity of the polymerase upon association with the UL42 protein. Alternatively, it could be due to the slower dissociation of the DNA polymerase from the template-primer, providing the DNA polymerase with a greater time interval with which to excise the mismatched nucleotide.

Acknowledgment This work was supported by Grant AI26538 from the NIH.

References

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–54.

Crute, J. J. and Lehman, I. R. (1989) Herpes simplex-1 DNA polymerase: Identification of an intrinsic 5′-3′ exonuclease with ribonuclease H activity. *J. Biol. Chem.* **264**, 19266–19270.

Crute, J. J., Mocarski, E. S. and Lehman, I. R. (1988) A DNA helicase induced by herpes simplex virus type 1. *Nucleic Acid Res.* **16**, 6585–6596.

Crute, J. J., Tsurumi, T., Zhu, L., Weller, S. K., Olivo, P. D., Challberg, M. D., Mocarski, E. S. and Lehman, I. R. (1989) Herpes simplex virus 1 helicase-primase: A complex of three herpes-encoded gene products. *Proc. Natl. Acad. Sci. USA* **86**, 2186–2189.

Davison, B. L., Leighton, T. and Rabinowitz, J. C. (1979) Purificaton of *Bacillus subtilis* RNA polymerase with heparin-agarose. *J. Biol. Chem.* **254**, 9220–9226.

Dodson, M. S., Crute, J. J., Bruckner, R. C. and Lehman, I. R. (1989) Overexpression and assembly of the herpes simplex virus type 1 helicase-primase in insect cells. *J. Biol. Chem.* **264**, 20835–20838.

Elias, P., O'Donnell, M. E. and Lehman, I. R. (1986) Interaction of origin binding protein with an origin of replication of herpes simplex virus 1. *Proc. Natl Acad Sci. USA* **83**, 6322–6326.

Gallo, M. L., Jackwood, D. H., Murphy, M., Marsden, H. S. and Parris, D. S. (1988) Purification of the herpes simplex virus type 1 65-kilodalton DNA-binding protein: properties of the protein and evidence of its association with the virus-encoded DNA polymerase. *J. Virol.* **62**, 2874–2883.

Gottlieb, J., Marcy, A. I., Coen, D. A. and Challberg, M. D. (1990) The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. *J. Virol.* **64**, 5976–5987.

Hernandez, T. R. and Lehman, I. R. (1990) Functional interaction between the herpes simplex virus-1 DNA polymerase and the UL42 protein. *J. Biol. Chem.* **265**, 11227–11232.

Hwang, Y. T., Liu, B. Y., Coen, D. M. and Hwang, C. B. (1997) Effects of mutations in the Exo III motif of the herpes simplex virus DNA polymerase gene on enzymatic activities, viral replication fidelity. *J. Virol.* **71**, 7791–7798.

Marsden, H. S., Campbell, M. E. M., Haarr, L., Frame, M. C., Parris, D. S., Murphy, M., Hope, R. G., Muller, M. T. and Preston, C. M. (1987) The 65,000-Mr DNA-binding and virion trans-inducing proteins of herpes simplex virus type 1. *J. Virol.* **61**, 2428–2437.

Mardsen, H. S., McLean, G. W., Barnard, E. C., Francis, G. J., MacEachran, K., Murphy, M., McVey, G., Cross, A., Abbotts, A. P. and Stow, N. D. (1997) The catalytic subunit of the DNA polymerase of herpes simplex virus type 1 interacts specifically with the C terminus of the UL8 component of the viral helicase-primase complex. *J. Virol.* **71**, 6390–6397.

Monahan, S. J., Gromstead, L. A., Olivieri, W. and Parris, D. S. (1998) Interaction between the herpes simplex virus type 1 origin-binding and DNA polymerase accessary proteins. *Virology* **241**, 122–130.

Olivo, P. D., Nelson, N. J. and Challberg, M. D. (1988) Herpes simplex virus DNA replication: The UL9 gene encodes an origin binding protein. *Proc. Natl. Acad. Sci. USA* **85**, 5414–5418.

Park, C. S., Wu, F. Y.-H. and Wu, C.-W. (1982) Molecular mechanism of promoter selection in gene transcription. *J. Biol. Chem.* **257**, 6950–6956.

Parris, D. S., Cross, A., Haarr, A., Orr, A., Frame, M. C., Murphy, M., McGeoch, D. J. and Marsden, H. S. (1988) Identification of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. *J. Virol.* **62**, 818–825.

Uyemura, D. and Lehman, I. R. (1976) Biochemical characterization of mutant forms of DNA polymerase I from *Escherichia coli*. *J. Biol. Chem.* **251**, 4078–4084.