

Interaction of herpes simplex virus 1 origin-binding protein with DNA polymerase α

(DNA replication/UL9 protein/protein-protein interactions)

SAM S.-K. LEE*, QUN DONG†, T. S.-F. WANG†, AND I. R. LEHMAN*

Departments of *Biochemistry and †Pathology, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT The herpes simplex virus 1 (HSV-1) genome encodes seven polypeptides that are required for its replication. These include a heterodimeric DNA polymerase, a single-strand-DNA-binding protein, a heterotrimeric helicase/primase, and a protein (UL9 protein) that binds specifically to an HSV-1 origin of replication (*ori_S*). We demonstrate here that UL9 protein interacts specifically with the 180-kDa catalytic subunit of the cellular DNA polymerase α -primase. This interaction can be detected by immunoprecipitation with antibodies directed against either of these proteins, by gel mobility shift of an *ori_S*-UL9 protein complex, and by stimulation of DNA polymerase activity by the UL9 protein. These findings suggest that enzymes required for cellular DNA replication also participate in HSV-1 DNA replication.

In vivo studies of the replication of the linear 152-kb herpes simplex virus 1 (HSV-1) genome have demonstrated that it circularizes shortly after infection and then enters a rolling circle mode of DNA replication (1). However, the existence of three origins of replication, *ori_L* and the diploid *ori_S*, as well as an HSV-1-encoded protein (UL9 protein) that binds specifically to these origins (for review, see ref. 2), suggests that origin-dependent, theta-type DNA replication, analogous to that observed for the simian virus 40 (SV40) minichromosome, may also occur. A recent analysis *in vivo* of the replication of plasmids containing an HSV-1 origin (*ori_S*) in HSV-1-infected cells by two-dimensional gel analysis (3) has identified "bubble arcs" indicative of theta-type DNA replication (K. Kelly and I.R.L., unpublished data).

The HSV-1 genome encodes seven polypeptides that are essential for its replication (2, 4). These include, in addition to the UL9 protein, a heterodimeric, highly processive DNA polymerase, a single-strand-DNA-binding protein (ICP8), and a heterotrimeric helicase/primase. We have recently found that a complex consisting of the HSV-1 DNA polymerase, helicase/primase, and ICP8, isolated from Sf21 insect cells multiply infected with baculoviruses recombinant for the genes encoding these enzymes, can promote the rolling circle replication of a 3-kb plasmid that is independent of *ori_S* and the UL9 protein (5). This reaction may reflect the rolling circle phase of viral DNA replication.

The UL9 protein, which exists in solution as a homodimer of 83-kDa subunits (6, 7), binds to specific sequences within *ori_S* (sites I, II, and III) (8–11). The two high-affinity sites (sites I and II) are separated by an 18-nt A+T-rich spacer. The UL9 protein also has DNA-dependent ATPase and DNA helicase activities (6, 7, 12, 13). These activities are also known to be associated with T antigen, the SV40 encoded initiator protein (for review, see refs. 2, 14, and 15), suggesting that the UL9 protein serves an analogous function in the initiation of DNA replication at *ori_S* and its larger homologue, *ori_L*.

Studies of SV40 DNA replication *in vitro* have shown that the SV40 T antigen interacts specifically with the cellular DNA polymerase α -primase (Pol α) to form a complex that initiates DNA replication at the SV40 origin (16–19). We have therefore inquired whether the HSV-1-encoded UL9 protein forms an analogous complex with Pol α . We demonstrate here that the UL9 protein interacts specifically with the catalytic subunit of the human Pol α . This finding suggests that a cellular enzyme, Pol α , participates in the initiation of HSV-1 DNA replication at an HSV-1 origin.

MATERIALS AND METHODS

Enzymes. The HSV-1-encoded DNA polymerase catalytic subunit, UL42, and UL9 proteins, as well as the 180-kDa catalytic subunit of the human Pol α were overexpressed in the baculovirus-infected Sf21 cell system and purified as described (5, 12, 20, 21). The purity of these proteins was confirmed by SDS/PAGE (22); the UL9 protein consisted of a single polypeptide that migrated with a molecular mass of 83 kDa; the Pol α catalytic subunit contained a 180-kDa polypeptide, as well as several smaller degradation products.

Antibodies. Rabbit polyclonal antiserum directed against the UL9 protein was raised by subcutaneous injection of the purified protein (Josman Laboratories, Napa, CA). The specificity of the antiserum was determined by immunoblot analysis and immunoprecipitation. Monoclonal antibody directed against the 180-kDa catalytic subunit of Pol α (SJK 237) was prepared as described (23). Both antibodies were purified on protein A-agarose (GIBCO/BRL) (24), and their purity was verified by SDS/PAGE. For ELISAs, antibodies directed against the UL9 protein were dialyzed against a buffer containing 20 mM potassium phosphate, pH 7.2, and 50 mM NaCl and coupled with activated horseradish peroxidase (HRP) (Pierce) according to the manufacturer's instructions. The specificity of these HRP-conjugated antibodies was confirmed by ELISA using purified UL9 protein. Chicken antibody directed against the Pol α (18) and rabbit antibodies to HSV-1 DNA polymerase and the UL42 protein (20) were used for immunoblot analysis.

Preparation of ³²P-Labeled *ori_S*-Containing DNA Fragment. The plasmid pCG5 (25) containing *ori_S* was treated with *Hind*III and *Eco*RI to produce a 138-bp *ori_S*-containing fragment. The fragment was purified with Qiaex beads (Qiagen, Chatsworth, CA) following electrophoresis through a 1.8% agarose gel. It was then labeled at the 3' terminus by incubation with the exonuclease-free Klenow fragment of *Escherichia coli* DNA polymerase I (United States Biochemical/Amersham) in the presence of 50 μ M dATP, dGTP, and dTTP, and [α -³²P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq). Unincorporated nucleotides were removed with a Bio-Spin 6 column (Bio-Rad).

Immunoprecipitation Assay. The 180-kDa subunit of Pol α (300 ng; 1.7 pmol) was incubated with increasing amounts of UL9 protein (0–1000 ng; 0–6.25 pmol) in buffer A [50 mM Hepes-KOH, pH 7.9/10% (vol/vol) glycerol/2 mM MgCl₂/1 mM dithiothreitol/120 mM KCl/0.1% Nonidet P-40/1% bovine serum albumin (BSA)] for 1 h at 4°C. After incubation, 5 μ g of monoclonal antibody (SJK 237) directed against Pol α was added to the reaction mixtures, which were incubated for an additional 2 h at 4°C. The mixtures were centrifuged for 15 min at 15,000 \times g, and the supernatants were transferred to new tubes. To precipitate the immune complexes, 20 μ l of 10% (vol/vol) protein A-agarose in buffer B (50 mM Tris-HCl, pH 8.0/150 mM NaCl/0.5% Nonidet P-40) was added, and the mixture was incubated for 1 h. The immune complexes were then collected by brief centrifugation, and the unbound proteins were removed. The pellets were resuspended in 0.5 ml of buffer B, followed by a brief centrifugation. This step was repeated five times. The pellets were dissolved in SDS/PAGE sample buffer (22), and analyzed by SDS/8% PAGE. UL9 protein and Pol α were detected by immunoblot analysis using the ECL kit (Amersham). In the reciprocal experiment, 300 ng (1.88 pmol) of UL9 protein was incubated with increasing amounts of 180-kDa Pol α subunit (0–1000 ng; 0–5.56 pmol) or HSV-1 DNA polymerase catalytic subunit (0–1000 ng; 0–7.4 pmol) in buffer A for 1 h at 4°C. Polyclonal antibody (3 μ g) directed against the UL9 protein was added and the immune complexes were collected, washed, and analyzed by immunoblotting as described above.

ELISA. The ELISA was performed according to Dornreiter *et al.* (16) with modified washing conditions. ELISA plates were coated with the 300 ng (1.88 pmol) of UL9 protein suspended in 40 μ l of buffer B for 1 h at room temperature. After washing, the ELISA wells were blocked with 5% (wt/vol) BSA in buffer B for 2 h at room temperature. Increasing amounts of 180-kDa Pol α subunit in 40 μ l of buffer B were added to the ELISA wells and shaken gently for 1 h at 4°C. The unbound proteins were removed by washing four to six times with buffer B before being incubated with antibody. For detection of 180-kDa Pol α subunit, 1 μ g of monoclonal antibody (SJK 237) in buffer B containing 3% (wt/vol) BSA was incubated with the plates for 1 h at room temperature, followed by 30 min incubation with HRP-protein A (1:3000) (Amersham) in buffer B containing 3% (wt/vol) BSA. The reaction was developed with tetramethylbenzidine base (TMB, GIBCO/BRL) for 10–20 min and quantitated at 450 nm with a microtiter plate reader (Bio-Rad). In the reciprocal experiment, 300 ng (1.7 pmol) of 180-kDa Pol α subunit were immobilized on ELISA plates and blocked with 5% (wt/vol) BSA. After washing, increasing amounts of UL9 protein were added to the ELISA plates. HRP-conjugated anti-UL9 antibody (1:1000) was added for the detection of UL9 protein. The amount of bound UL9 protein was quantitated as described above.

Gel-Retardation Assay. Reaction mixtures (50 μ l) containing 50 mM Hepes-KOH (pH 7.8), 0.5 mM dithiothreitol, 100 mM NaCl, 7 mM MgCl₂, 50 μ g of acetylated BSA, 10% (vol/vol) glycerol, the indicated amounts of 180-kDa Pol α subunit, and UL9 protein (200 ng; 1.25 pmol) were preincubated for 30 min on ice. ³²P-labeled restriction fragment containing the Ori₅ sequence (0.2 pmol) was added, and the mixtures were incubated at 37°C for 15 min. Four percent polyacrylamide gel electrophoresis of the reaction mixtures was performed at room temperature with Tris/glycine buffer (50 mM Tris/380 mM glycine/2 mM EDTA) at 30 V. After electrophoresis, the gel was dried on Whatman DE 81 filters and visualized by autoradiography.

DNA Polymerase Assay. Reaction mixtures (50 μ l) contained 20 mM Tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 200 μ g of acetylated BSA per ml, 10 mM MgCl₂, UL9 protein as indicated, 0.55 unit of 180-kDa Pol α subunit; 50 μ M dATP,

dGTP, and dTTP, 25 μ M dCTP, 0.5 μ Ci of [α -³²P]dCTP (3000 Ci/mmol), and 0.01 pmol of single-stranded M13mp18 DNA annealed to the 17-nt universal primer (United States Biochemical/Amersham) in a 1:1 molar ratio. Incubation was for 2 h at 37°C, and reactions were stopped by spotting onto Whatman DE 81 filter papers. The filters were washed five times with 0.5 M Na₂HPO₄, twice with distilled water, and twice with 95% ethanol. Incorporation of ³²P into acid-insoluble material was measured by scintillation.

RESULTS

180-kDa Catalytic Subunit of Human Pol α Interacts with the HSV-1 UL9 Protein. Physical interaction between SV40 T antigen and Pol α appears to be required for the initiation of DNA replication at an SV40 origin (18, 19, 26). Similarly, the E1 initiator protein of bovine papilloma virus interacts with the 180-kDa catalytic subunit of Pol α (27). We therefore inquired whether an analogous interaction occurs between Pol α and the HSV-1 origin-binding protein, the UL9 protein.

To examine the interaction between the 180-kDa Pol α subunit and the UL9 protein, a fixed amount of the 180-kDa Pol α subunit was incubated with increasing amounts of the UL9 protein, and the mixture was treated with monoclonal antibody directed against the 180-kDa Pol α subunit. The immune complexes were subjected to SDS/PAGE and then blotted onto nitrocellulose filters. When the filters were probed with antiserum directed against the UL9 protein, progressively more UL9 protein was bound to the 180-kDa Pol α subunit as increasing amounts of the UL9 protein were added (Fig. 1). When the immunoblots were reprobed with chicken antiserum directed against the 180-kDa Pol α subunit, the amount of 180-kDa Pol α subunit remained constant (Fig. 1). In control experiments, the UL9 protein did not interact with either monoclonal Pol α antibody or protein A-agarose (Fig. 1). The 180-kDa Pol α subunit-UL9 protein interaction was also examined with antibody directed against the UL9 protein. In this experiment, a fixed amount of UL9 protein was incubated with increasing amounts of the 180-kDa Pol α subunit. The immune complexes formed upon addition of

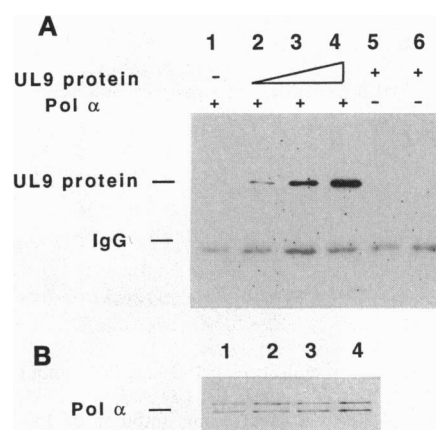


FIG. 1. Coimmunoprecipitation of UL9 protein and 180-kDa Pol α subunit by anti-180-kDa Pol α subunit antibody. (A) A total of 300 ng of 180-kDa Pol α subunit were incubated with increasing amounts of UL9 protein (lane 1, 0 ng; lane 2, 100 ng; lane 3, 300 ng; and lane 4, 1000 ng). Lane 5 contains 300 ng and lane 6 contains 1000 ng of UL9 protein alone. The 180-kDa Pol α subunit-UL9 protein complex was immunoprecipitated by the addition of 5 μ g of 180-kDa Pol α subunit-specific monoclonal antibody and protein A-agarose. The immunoprecipitated proteins were separated by SDS/PAGE, and the UL9 protein was detected by immunoblot analysis using polyclonal antiserum (1:3000) directed against UL9 protein. (B) The immunoblot was reprobbed with a polyclonal antiserum directed against the 180-kDa Pol α subunit (1:1000) (18).

antibody directed against the UL9 protein were subjected to SDS/PAGE and analyzed by immunoblotting as before. As demonstrated in Fig. 2, the immunoblot probed with antiserum to Pol α showed an increasing amount of 180-kDa Pol α subunit bound to the UL9 protein as increasing amounts of Pol α were added. A constant amount of UL9 protein appeared when the immunoblot was reprobed with UL9 specific antiserum. A similar immunoprecipitation experiment was carried out with the UL9 protein and the HSV-1-encoded DNA polymerase catalytic subunit. Addition of UL9 specific antibody to a constant amount of UL9 protein mixed with increasing amounts of the HSV-1 DNA polymerase catalytic subunit failed to precipitate the HSV-1 DNA polymerase (Fig. 2). The same result was obtained when the experiment was performed with the HSV-1 DNA polymerase associated with its processivity-enhancing subunit, the UL42 protein (data not shown). These results indicate that the interaction between UL9 protein and Pol α is specific.

The interaction between the UL9 protein and the 180-kDa Pol α subunit was also investigated by means of a modified ELISA. A fixed amount of the UL9 protein was immobilized on an ELISA plate which was then coated with BSA to prevent nonspecific binding. Increasing amounts of 180-kDa Pol α subunit were added, and, after washing of the plate, 180-kDa Pol α subunit bound to the UL9 protein was detected with the 180-kDa Pol α subunit-specific monoclonal antibody followed by treatment with HRP-protein A. As shown in Fig. 3, the 180-kDa Pol α subunit bound specifically to the immobilized UL9 protein but not to BSA. Furthermore, the 180-kDa Pol α

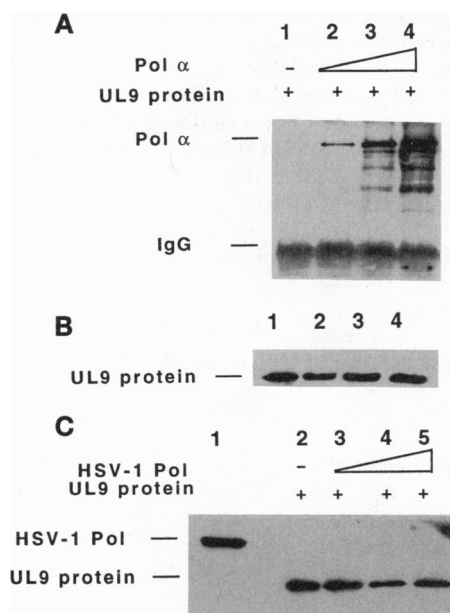


FIG. 2. Coimmunoprecipitation of 180-kDa Pol α subunit and UL9 protein by anti-UL9 protein antibody. (A) A total of 300 ng of UL9 protein were incubated with increasing amounts of 180-kDa Pol α subunit (lane 1, 0 ng; lane 2, 100 ng; lane 3, 300 ng; and lane 4, 1000 ng). The UL9 protein-180-kDa Pol α subunit complex was immunoprecipitated by the addition of 3 μ g of UL9 protein-specific polyclonal antibody and protein A-agarose. Bound 180-kDa Pol α subunit was detected by immunoblot analysis using polyclonal antiserum directed against the 180-kDa Pol α subunit (1:1000) (18). (B) The immunoblot was reprobed with polyclonal antibody directed against the UL9 protein (1:3000). (C) A total of 300 ng of UL9 protein were incubated with increasing amounts of HSV-1 DNA polymerase catalytic subunit (lanes 2–5) (lane 2, 0 ng; lane 3, 100 ng; lane 4, 300 ng; and lane 5, 1000 ng). Lane 1 contains 100 ng of HSV-1 DNA polymerase catalytic subunit. The immunoblot was probed with polyclonal antibody directed against the UL9 protein (1:3000) and the HSV-1 DNA polymerase catalytic subunit (1:2000) (20).

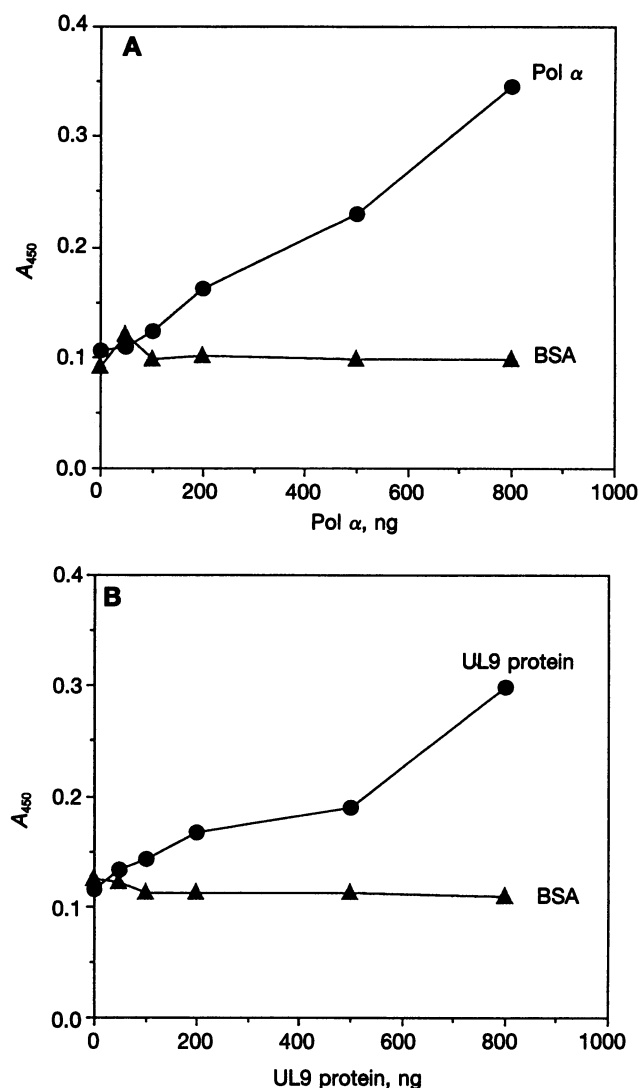


FIG. 3. ELISA analysis of the interaction between UL9 protein and the 180-kDa Pol α subunit. (A) A total of 300 ng of UL9 protein were immobilized on ELISA plates blocked with 5% BSA. Pol α subunit or BSA was added as indicated, and further incubated for 1 h. After the plates were washed with 50 mM Tris-HCl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40, bound 180-kDa Pol α subunit was detected by treatment with 1 μ g of Pol α -specific monoclonal antibody. HRP-conjugated protein A (1:3000; Amersham) was used for detection of immune complexes. (B) A total of 300 ng of 180-kDa Pol α subunit were immobilized in ELISA plates blocked with 5% BSA, and UL9 protein or BSA was added as indicated. Bound UL9 protein was detected with HRP-conjugated anti-UL9 antibody (1:1000) and the chromogenic substrate as described in *Materials and Methods*. The bound proteins were quantitated at 450 nm.

subunit-specific antibody did not interact with the UL9 protein fixed to an ELISA plate. In the reciprocal experiment, the immobilized 180-kDa Pol α subunit was incubated with increasing amounts of the UL9 protein, and the bound UL9 protein was detected with HRP-conjugated UL9 antibody. As shown in Fig. 3, the UL9 protein also interacted specifically with the immobilized 180-kDa Pol α subunit.

Formation of a *oris*-UL9 Protein-180-kDa Pol α Subunit Complex. To investigate the interaction of 180-kDa Pol α subunit with the UL9 protein bound to its target origin, *oris*, gel mobility-shift experiments were performed with a labeled *oris*-containing restriction fragment. As shown in Fig. 4, the UL9 protein alone bound *oris*, producing two distinct complexes. The labeled complexes disappeared upon addition of a 50-fold molar excess of unlabeled *oris*-containing restriction

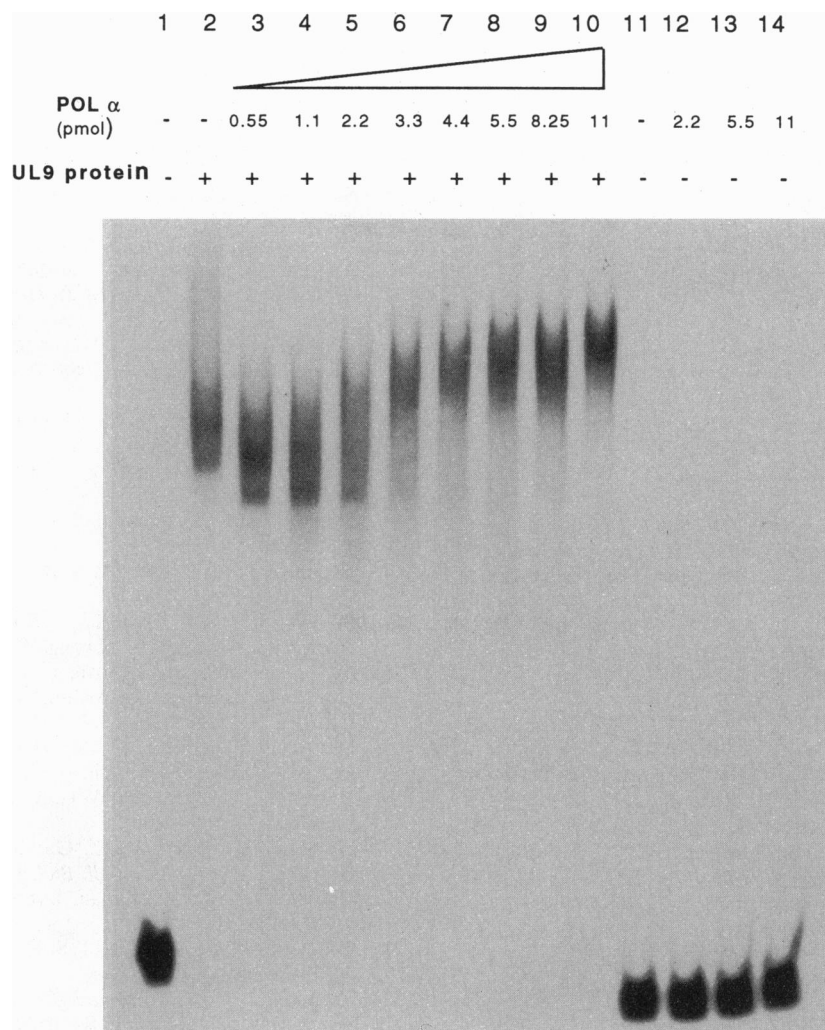


FIG. 4. Interaction of 180-kDa Pol α subunit with UL9 protein bound to HSV-1 *ori_S*. The UL9 protein (1.25 pmol) and the indicated amounts of 180-kDa Pol α subunit were incubated for 30 min at 4°C. ³²P-labeled *ori_S* fragment (0.2 pmol) was then added, and the reaction mixtures were incubated at 37°C for 15 min. The *ori_S*-protein complexes were analyzed by 4% PAGE at room temperature. Lane 1, ³²P-labeled *ori_S*-containing restriction fragment; lane 2, UL9 protein; lanes 3–10, the indicated amounts of 180-kDa Pol α subunit and UL9 protein; and lanes 11–14, increasing amounts of 180-kDa Pol α subunit in the absence of UL9 protein.

fragment, indicating that the binding of UL9 protein to *ori_S* was specific (data not shown). As expected, the 180-kDa Pol α subunit did not interact with the *ori_S*-containing fragment (Fig. 4). To determine whether Pol α can interact with the UL9 protein-*ori_S* complex, the UL9 protein was preincubated with increasing amounts of 180-kDa Pol α subunit at 4°C. The labeled *ori_S*-containing restriction fragment was then added, and the mixture was incubated at 37°C. As shown in Fig. 4, a new complex with retarded mobility appeared as increasing concentrations of 180-kDa Pol α subunit were added, reaching a maximum at a stoichiometry of UL9 protein to 180-kDa Pol α subunit of 1:2.6. At low concentrations of 180-kDa Pol α subunit, the complex showed a higher mobility than that formed in its absence (Fig. 4). We do not know the reason for this effect.

Stimulation of DNA Polymerase Activity of the 180-kDa Pol α Subunit by UL9 Protein. Previous studies have shown that SV40 T antigen stimulates the DNA polymerase activity of the four-subunit Pol α with unprimed and singly primed M13 DNA templates, presumably by facilitating association with the template primer (26, 28). We therefore wished to determine whether association of the 180-kDa Pol α subunit with the UL9 protein would produce an analogous stimulation. As shown in Fig. 5, addition of increasing amounts of UL9 protein to a fixed

amount of 180-kDa Pol α subunit did stimulate its DNA polymerase activity. Maximal stimulation (approximately 3-fold) occurred at a stoichiometry of UL9 protein to 180-kDa Pol α subunit of 1:0.6. These findings suggest that the interaction of the UL9 protein with the 180-kDa Pol α subunit is functionally important in modulating the efficiency of Pol α at the replication fork.

DISCUSSION

The findings reported here suggest that cellular Pol α functions in the replication of the HSV-1 genome. We have demonstrated that the 180-kDa Pol α subunit interacts specifically with the HSV-1-encoded origin-binding protein, either as the free protein or complexed with an HSV-1 origin, *ori_S*. The interaction was demonstrated by immunoprecipitation of the Pol α -UL9 protein complex with antisera directed against either the 180-kDa Pol α subunit or the UL9 protein. The interaction could not, however, be detected by cosedimentation of the two proteins in glycerol gradients or by coelution during gel filtration (S.S.-K.L. and I.R.L., unpublished data). Presumably the interaction is not strong enough to withstand the hydrodynamic forces inherent in these methods. Since the

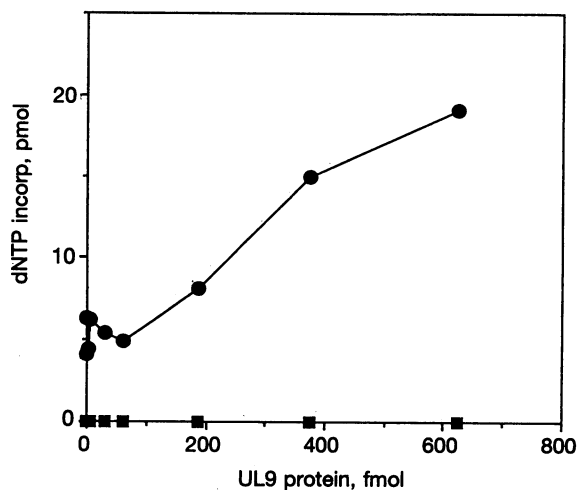


FIG. 5. Effect of UL9 protein on the DNA polymerase activity of 180-kDa Pol α subunit. Increasing amounts of UL9 protein were added to reaction mixtures containing 0.55 unit of 180-kDa Pol α subunit and singly primed M13 DNA template (0.01 pmol) (see *Materials and Methods*). Incubation was at 37°C for 2 h in the absence (■) or presence (●) of 180-kDa Pol α subunit. Incorporation of 32 P-labeled dCTP into acid-insoluble material was measured by scintillation.

Pol α -UL9 protein interaction might be expected to be a transient one, this result is not too surprising.

The SV40 T antigen is a helicase that binds specifically to the SV40 origin of replication in the presence of ATP and interacts with Pol α (16-19). Similarly, Pol α interacts with the E1 initiator protein of bovine papilloma virus (27). Studies of SV40 DNA replication *in vitro* have shown that Pol α and T antigen, together with the cellular single-strand-DNA-binding protein RP-A, initiate DNA replication at the SV40 origin (for reviews, see refs. 14 and 15). Our finding that the HSV-1 UL9 protein binds specifically to the 180-kDa Pol α subunit suggests that, like the SV40 T antigen, the HSV-1 UL9 protein may direct the cellular Pol α to the viral origin(s) to initiate DNA replication. The finding that the HSV-1-encoded UL9 protein interacts specifically with Pol α is not without precedent. Earlier immunostaining studies demonstrated the colocalization of several cellular enzymes, including Pol α and RP-A with the HSV-1-encoded single-strand-DNA-binding protein ICP8 (29).

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