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Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase–primase composed of the UL5 and UL52 gene products

MARK S. DODSON AND I. R. LEHMAN

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

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ABSTRACT **Herpes simplex virus 1 encodes a helicase–primase that is composed of the products of the UL5, UL8, and UL52 genes. A stable subassembly consisting of only the UL5 and UL52 gene products has been purified to near homogeneity from insect cells doubly infected with baculovirus recombinant for these two genes. The purified subassembly has the DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase, and DNA primase activities that are characteristic of the three-subunit holoenzyme. The purified UL8 gene product, although required for viral DNA replication, neither exhibits these enzymatic activities nor stably associates with either the UL5 or the UL52 gene product.**

Herpes simplex virus 1 (HSV-1) encodes seven proteins that are required to initiate and sustain DNA replication at any one of three origins of replication (ori_L and diploid ori_S) located on its 153-kilobase linear genome (reviewed in ref. 1). Three of these proteins, encoded by the UL5, UL8, and UL52 genes (2–4), form a heterotrimer with both DNA helicase and DNA primase activities. The UL5, UL8, and UL52 genes have been cloned into a baculovirus overexpression system (5–7), and we have recently shown that fully active enzyme can be reconstituted *in vivo* by triply infecting insect cells with recombinant baculoviruses expressing each of the subunits (6). Here we describe a stable subassembly of the helicase–primase that is generated in insect cells doubly infected with recombinant baculovirus expressing the UL5 and UL52 genes. Calder and Stow (7) have recently reported that a partially purified extract from such doubly infected insect cells exhibits DNA-dependent ATPase and DNA helicase activities. We show here that the near-homogeneous UL5/UL52 subassembly has all of the activities—DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase, and DNA primase—that are associated with the three-subunit holoenzyme.

MATERIALS AND METHODS

Buffers and Reagents. Buffer A consisted of 20 mM Hepes (pH 7.6), 10% (vol/vol) glycerol, 1.0 mM dithiothreitol, leupeptin at 4 μg/ml, pepstatin A at 4 μg/ml, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM EDTA, and 1.0 mM EGTA. Buffer B consisted of 25 mM Tris·HCl (pH 8.0), 10% (vol/vol) glycerol, 1.0 mM dithiothreitol, leupeptin at 4 μg/ml, pepstatin A at 4 μg/ml, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM EDTA, and 1.0 mM EGTA. Buffer C consisted of 20 mM Hepes (pH 7.6), 10% (vol/vol) glycerol, 1.0 mM dithiothreitol, leupeptin at 2 μg/ml, pepstatin A at 2 μg/ml, and 1.0 mM EDTA. The sources of the chemical reagents have been described (2–4).

Enzymatic Assays and Gel Electrophoresis. DNA-dependent nucleoside triphosphatase assays were performed as described (2, 8). One unit of enzyme hydrolyzes 1.0 nmol of nucleoside triphosphate per hr (2). The coupled primase assay was used to follow primase activity during purification of the UL5/UL52 subassembly (3, 9). One unit of primase incorporates 1.0 pmol of [^{3}H]dATP per hr (3). DNA helicase assays were performed as described (2). The unwound products were visualized by autoradiography and quantitated by scanning with a Molecular Dynamics 300A computing densitometer. SDS/polyacrylamide gel electrophoresis and silver staining were performed as described (10, 11).

Cells, Viruses, and Enzymes. *Spodoptera frugiperda* (Sf9) cells and stocks of *Autographa californica* nuclear polyhedrosis virus (AcNPV) recombinant for the HSV-1 UL5, UL52, and UL8 genes were maintained and propagated as described (6, 12). The recombinant three-subunit HSV-1 helicase–primase holoenzyme was overexpressed and purified from infected Sf9 cells (6).

Overexpression and Purification of the UL5/UL52 Subassembly from Doubly Infected Sf9 Cells. Infection of Sf9 cells and the preparation of cytosolic extracts from infected cells have been described (6). Sf9 cells (1.2×10^9) were infected with 1.2×10^{10} plaque-forming units of each recombinant baculovirus. Clarified cytosolic extract (31.5 ml) was applied to a column of heparin-agarose (15-ml bed volume) equilibrated with buffer A containing 60 mM NaCl. The column was washed with 15 ml of the same buffer, and a gradient (180 ml) from 60 to 600 mM NaCl in buffer A was applied. Fractions (3.5 ml) were assayed for DNA-dependent ATPase and DNA primase activity. Peak fractions were pooled (13.5 ml) and diluted with 16.9 ml of buffer B containing 100 mM NaCl.

The pooled fractions were applied to a column of DEAE-Sepharose (10 ml) equilibrated with buffer B containing 100 mM NaCl. The column was washed with 10 ml of the same buffer, and a gradient (150 ml) from 100 to 400 mM NaCl in buffer B was applied. Fractions (3.5 ml) were assayed for enzymatic activity as above. Peak fractions were pooled (16.5 ml) and concentrated to 2 ml by centrifugation in a Centriprep 30 concentrator (Amicon). The concentrated DEAE-Sepharose fraction was diluted into 6 ml of buffer C and applied to a column of phosphocellulose (5 ml) equilibrated with buffer C containing 50 mM NaCl. The column was washed with the same buffer, and a gradient (50 ml) from 50 to 400 mM NaCl in buffer C was applied. The peak fractions of enzymatic activity (1.1 ml) were pooled and concentrated to 0.2 ml by centrifugation in a Centricon 30 microconcentrator (Amicon). The concentrated phosphocellulose fraction (0.2 ml) was applied to a prepacked Superose 12 column equilibrated with buffer C containing 200 mM NaCl and 1.0 mM EGTA, and was eluted at 0.2 ml/min. Fractions (0.2 ml) were assayed for DNA-dependent ATPase, DNA-dependent

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Abbreviation: HSV-1, herpes simplex virus 1.

GTPase, DNA primase, and protein. Active fractions were frozen in liquid nitrogen and stored at −80°C.

Overexpression and Purification of UL8 Gene Product from Infected Sf9 Cells. Sf9 cells were seeded into nine 150-cm^2 flasks and grown to near confluency. The growth medium was removed and 5 ml of medium containing 4.8 × 10^8 plaque-forming units of AcNPV/UL8 recombinant baculovirus (6) was added. The infected cells were incubated at 27°C for 1 hr, and 35 ml of fresh medium prewarmed to 27°C was added. The infected cells were incubated at 27°C for 62 hr and then dislodged from the flasks by gentle shaking. The cells (2.8 g) were harvested and a clarified cytosolic extract was prepared (6).

The cytosolic extract (28.6 ml) was applied to a column of heparin-agarose (6.5 ml) equilibrated with buffer A containing 60 mM NaCl, and the flow-through was collected. The column was washed with 7 ml of the same buffer, and the wash was combined with the flow-through fraction. The pool was applied to a column of DEAE-Sepharose (4 ml) equilibrated with buffer A containing 60 mM NaCl. The column was washed with 4 ml of the same buffer and a gradient from 50 to 600 mM NaCl in buffer B was applied. Fractions containing UL8 protein, identified by SDS/10% polyacrylamide gel electrophoresis as an intensely staining band of M_r 70,000, were pooled. UL8 protein was eluted between 100 and 140 mM NaCl. The pool (3.3 ml) was diluted into 2.3 ml of buffer C containing 50 mM NaCl and centrifuged at 1000 × *g* for 5 min. The supernatant was applied to a column of hydroxyapatite (2 ml) equilibrated with the same buffer. The column was then washed with 2 ml of equilibration buffer, and a gradient (26 ml) from 50 to 500 mM ammonium sulfate in buffer C was applied. Fractions (1.1 ml) containing UL8 protein were identified by gel electrophoresis. UL8 protein was eluted at the very beginning of the gradient. The peak fraction (1 ml) was concentrated to 0.2 ml by centrifugation in a Centricon 30 microconcentrator and further purified by Superose 12 chromatography as described above for the UL5/UL52 subassembly. Fractions containing UL8 were identified by gel electrophoresis, frozen in liquid nitrogen, and stored at −80°C. Approximately 0.8 mg of UL8 protein of >95% purity was recovered.

RESULTS

Purification of a UL5/UL52 Subassembly of the Helicase–Primase with DNA-Dependent ATPase, DNA-Dependent GTPase, and DNA Primase Activities. Clarified cytosolic extracts from Sf9 cells doubly infected with baculovirus recombinant for either the UL5 and UL52, UL8 and UL52, or UL8 and UL5 genes of HSV-1 were fractionated by heparin-agarose chromatography, and the fractions were assayed for DNA-dependent ATPase. Enzymatic activity was detected in fractions from cells doubly infected with baculovirus recombinant for the UL5 and UL52 genes, but no activity could be detected in fractions from cells infected with either the UL8/UL5 or the UL8/UL52 combination. SDS/polyacrylamide electrophoresis of the heparin-agarose fractions from each double infection showed that only the UL5 and UL52 gene products were coeluted from the heparin-agarose column, indicating that the UL5 and UL52 proteins can associate to form a stable subassembly (data not shown).

Two peaks of DNA primase activity were eluted from the column (Fig. 1*A*). The first peak was eluted slightly ahead of the DNA-dependent ATPase. The second followed the DNA-dependent ATPase with no overlap between the two activities. Peak heparin-agarose fractions containing the DNA-dependent ATPase activity were further purified by chromatography on DEAE-Sepharose. Again, two peaks of DNA primase activity were observed (Fig. 1*B*). The majority of the primase activity was eluted ahead of the DNA-dependent

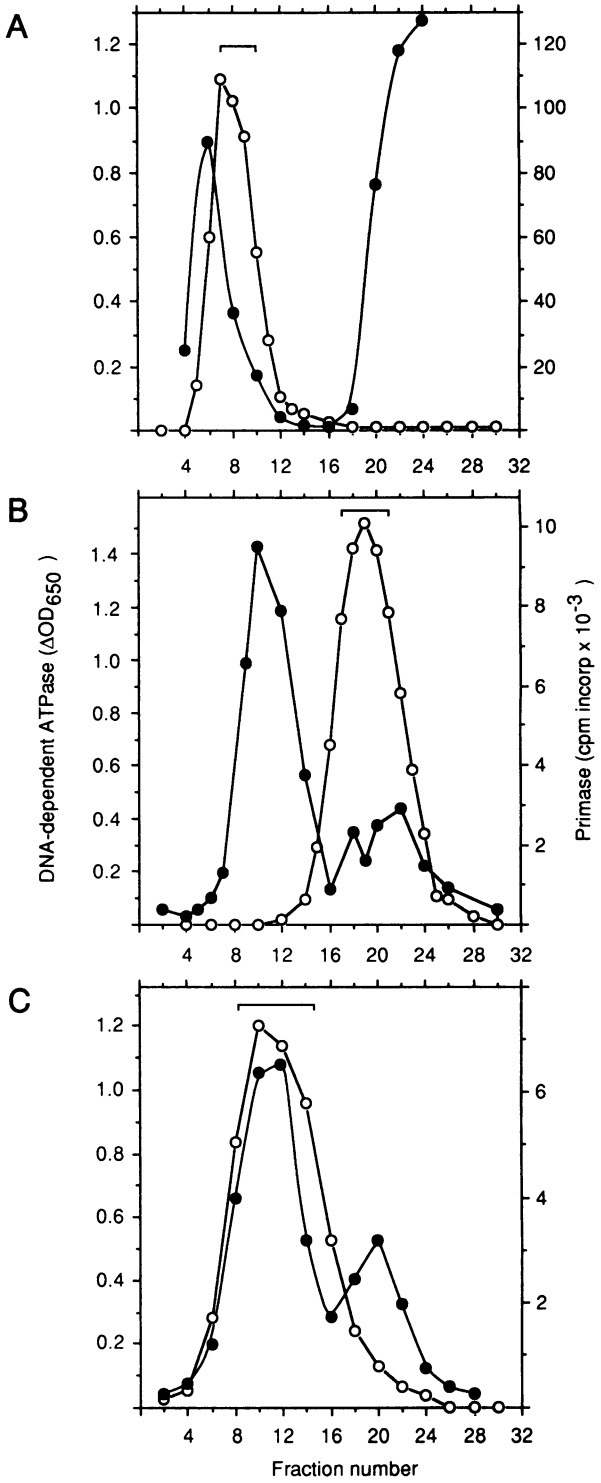

FIG. 1. Chromatography of clarified cytosolic extract from insect cells coexpressing UL5 and UL52 proteins. The indicated fractions were assayed for DNA-dependent ATPase (7.5 μl; ○) and primase activity (4 μl; ●). Active fractions that were pooled for subsequent chromatographic steps are indicated by the bracket above the peaks. Serial chromatography of the extract was performed using heparin-agarose (*A*), DEAE-Sepharose (*B*), and phosphocellulose (*C*) as described in *Materials and Methods*.

ATPase, while the second, smaller peak was coeluted with the peak of DNA-dependent ATPase but was not exactly co-incident (Fig. 1*B*). Peak DEAE-Sepharose fractions with DNA-dependent ATPase activity were next subjected to chromatography on phosphocellulose. Again, two peaks of DNA primase activity were apparent (Fig. 1*C*). However, the

major peak of primase activity was now coincident with the peak of DNA-dependent ATPase activity, while the second primase peak was eluted with the trailing edges of the preceding DNA-dependent ATPase and primase activities. The phosphocellulose fractions containing the coincident peaks of DNA-dependent ATPase and primase activities were purified to near homogeneity by Superose 12 gel filtration (Fig. 2). The peaks of DNA-dependent ATPase, DNA-dependent GTPase, and primase activities coincided at a position corresponding to a M_r of ≈320,000. The absorbance profile at 280 nm showed a single peak of protein coincident with the peak of enzymatic activity. The silver-stained polyacrylamide gel showed two distinct bands that had intensities coincident with enzymatic activity and migrated with the expected apparent M_r (120,000 and 97,000) for the UL52 and UL5 gene products.

Comparison of Enzymatic Activities of the UL5/UL52 Subassembly with the Three-Subunit Helicase–Primase Holoenzyme. The DNA-dependent ATPase, DNA-dependent GTPase, and DNA primase activities of the two-subunit subassembly were compared with those of the three-subunit holoenzyme (Table 1). The molar specific activity of the DNA-dependent nucleoside triphosphatase and DNA primase for the two enzymes was similar. The ratio of DNA-dependent ATPase to DNA-dependent GTPase for the subassembly was essentially the same as that for the holoenzyme, indicating that, as for the DNA-dependent ATPase, UL8 is not required for DNA-dependent GTPase activity. However, the ratio of DNA-dependent ATPase to primase was 2-fold higher for the subassembly than it was for the holoenzyme.

The DNA helicase activity of the two enzymes was compared by incubating equimolar quantities of each enzyme with an oligonucleotide having a heterologous 3′ tail, and annealed to M13 single-stranded DNA, in the presence or absence of ATP (2). In the presence of ATP the holoenzyme

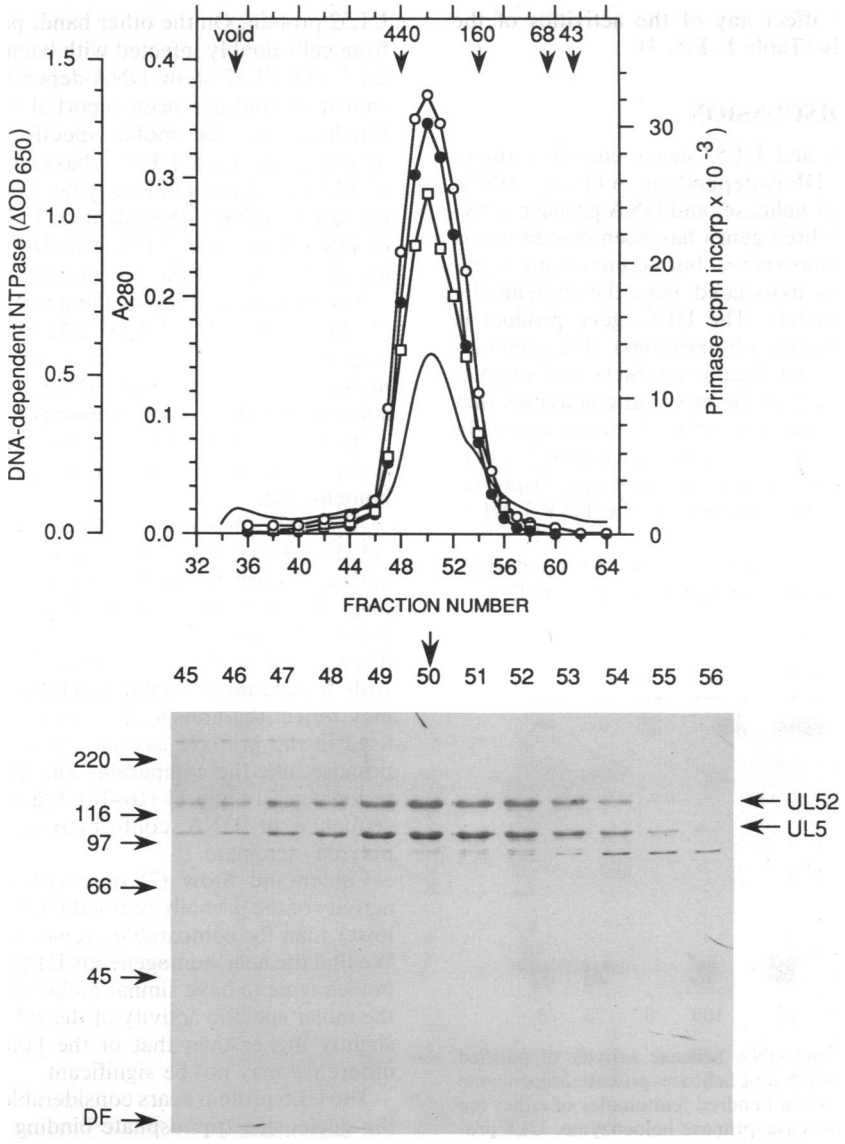

FIG. 2. Superose 12 gel filtration of the UL5/UL52 subassembly. (*Upper*) Superose 12 gel filtration of the phosphocellulose fraction containing coincident DNA-dependent ATPase and primase activities. The indicated fractions were assayed for DNA-dependent ATPase (2 μl; ○), DNA-dependent GTPase (2 μl; ●), and primase (3 μl; □). The absorbance at 280 nm is also shown (—). The position of elution of known molecular weight standards ($M_r \times 10^{-3}$) is indicated: ferritin (440), aldolase (160), bovine serum albumin (67), and ovalbumin (43). (*Lower*) SDS/polyacrylamide gel electrophoresis of Superose 12 fractions. Following electrophoresis of each fraction (10 μl), the polypeptides were visualized by silver staining. The position of each molecular weight standard ($M_r \times 10^{-3}$) is indicated. DF, dye front. The vertical arrow denotes the fraction containing the peak of DNA-dependent ATPase, DNA-dependent GTPase, and primase activities. NTPase, nucleoside triphosphatase.

Table 1. Comparison of enzymatic activities of UL5/UL52 subassembly, UL5/UL52/UL8 holoenzyme, and UL8 protein

Enzyme	Activity, units × 10^{-13} per mol			Ratio	
	DNA-dependent ATPase	DNA-dependent GTPase	DNA primase	DNA-dependent ATPase/ DNA-dependent GTPase	DNA-dependent ATPase/ primase
Holoenzyme	1.2	0.67	0.43	1.8	2.8
UL5/UL52	1.9	1.2	0.33	1.6	5.8
UL8	0	0	0	—	—
UL8 + UL5/UL52	1.6	1.0	0.33	1.6	4.8

Seven hundred femtomoles of UL5/UL52 subassembly, helicase–primase holoenzyme, UL8 protein, or UL5/UL52 subassembly plus UL8 protein was assayed for DNA-dependent ATPase, DNA-dependent GTPase, and primase as described under *Materials and Methods*.

unwound 57% of the substrate; the UL5/UL52 subassembly unwound 76% of the substrate (Fig. 3).

UL8 Protein Has No Detectable Enzymatic Activity. The purified UL8 protein did not exhibit DNA-dependent nucleoside triphosphatase, primase, or DNA helicase activities; nor did it significantly affect any of the activities of the UL5/UL52 subassembly (Table 1, Fig. 3).

DISCUSSION

The HSV-1 UL5, UL8, and UL52 genes encode a three-subunit enzyme with DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase, and DNA primase activities (2, 3). Each of the three genes has been cloned into a baculovirus overexpression vector, but no enzymatic activities have thus far been associated with the individually overexpressed gene products. The UL52 gene product is largely insoluble (unpublished observations). The individually expressed UL5 and UL8 gene products are soluble; however, they exhibit none of the enzymatic activities that are associated with the helicase–primase holoenzyme. In contrast, the fully active three-subunit holoenzyme is overexpressed and properly assembled in insect cells triply infected with baculovirus recombinant for the HSV-1 UL5, UL8, and UL52 genes (6).

The successful overexpression of the holoenzyme in triply infected cells suggested that subassemblies of the helicase–primase might be generated from doubly infected cells. Using this approach we find that partially purified extracts from the UL8/UL5 or UL8/UL52 double infections are lacking in DNA-dependent ATPase and helicase activities and that the UL8 protein does not associate stably with either the UL5 or UL52 protein. On the other hand, partially purified extracts from cells doubly infected with baculovirus recombinant for UL5 and UL52 show DNA-dependent ATPase activity. A similar finding has been reported by Calder and Stow (7). Furthermore, the molar specific activity of the near-homogeneous UL5/UL52 subassembly is very similar to that of the three-subunit holoenzyme. The UL5/UL52 subassembly also has DNA-dependent GTPase activity, and the ratio of DNA-dependent ATPase to DNA-dependent GTPase is identical to that of the holoenzyme.

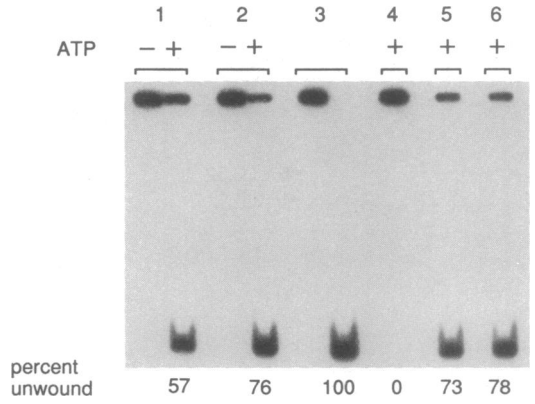

FIG. 3. Comparison of the DNA helicase activity of purified UL5/UL52 subassembly with that of helicase–primase holoenzyme and purified UL8 protein. Seven hundred femtomoles of either the UL5/UL52 subassembly, helicase–primase holoenzyme, UL8 protein, or UL5/UL52 subassembly mixed with UL8 was incubated with 80 pmol of M13mp18 single-stranded DNA annealed to a 3′-tailed 45-residue oligonucleotide labeled at its 5′ end, and DNA helicase activity was determined as described in *Materials and Methods*. ATP (3.0 mM) was added as indicated. Lanes 1, holoenzyme; lanes 2, UL5/UL52 subassembly; lane 4, UL8; lane 5, UL5/UL52 subassembly; lane 6, UL5/UL52 plus UL8; lane 3, fully annealed substrate (left) and substrate denatured in 50 mM NaOH at 70°C for 2 min (right).

The primase activity of the holoenzyme itself is very low (3, 6). To test the UL5/UL52 subassembly for primase activity, it was therefore necessary to eliminate all contaminating insect primase and RNA polymerase. The purified subassembly does have primase activity. However, we have consistently observed that the ratio of DNA-dependent ATPase to primase is greater for the subassembly than for the holoenzyme.

Although absolutely required for viral DNA replication (13, 14), the UL8 protein is clearly not required for primase activity; however, the UL8 protein may in some way modulate the primase activity of the holoenzyme. As noted previously, the primase activity associated with the HSV-1 helicase–primase is considerably lower than that associated with, for example, mammalian DNA polymerase α (15). This may reflect the nonspecific nature of the poly(dT) template used in the primase assays. Possibly the HSV-1 helicase–primase, like the comparable enzymes encoded by the bacteriophages T4 and T7 (16–18), requires a specific nucleotide sequence or DNA conformation that is lacking in the poly(dT) template.

Calder and Stow (7) observed that the DNA helicase activity of the partially purified UL5/UL52 subassembly was lower than for comparable preparations of the holoenzyme. We find the near-homogeneous UL5/UL52 subassembly and holoenzyme to have similar molar specific activities. In fact, the molar specific activity of the subassembly appears to be slightly higher than that of the holoenzyme, although the difference may not be significant.

The UL5 protein bears considerable sequence homology to the nucleoside triphosphate binding domain and conserved sequence motifs characteristic of numerous DNA helicases (19–21) and would be expected to exhibit DNA-dependent ATPase and helicase activity, but it does not (7). Possibly, expression of these activities requires that the UL5 protein be associated with the UL52 protein. Alternatively, these activities may be intrinsic to the UL52 protein. The resolution of this question awaits the availability of soluble UL52 protein.

We do not know which of the two subunits has the primase activity. The UL5/UL52 subassembly may be analogous to the 41/61 primosome assembly encoded by bacteriophage T4 (16, 17). Gene *41* encodes the DNA helicase activity, and primase activity is associated with the gene *61* product. However, the enzymatic activities of these two proteins are substantially diminished if dissociated from one another (16, 17). Thus, UL5 protein may be the helicase, and UL52 protein the primase, but these activities appear only upon proper association of the two polypeptides.

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