JOURNAL OF VIROLOGY, July 1992, p. 3986–3995
0022-538X/92/073986-10$02.00/0
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Vol. 66, No. 7

Purification and Characterization of UL9, the Herpes Simplex Virus Type 1 Origin-Binding Protein

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Received 30 December 1991/Accepted 25 March 1992

UL9, the origin-binding protein of herpes simplex virus type 1 (HSV-1), has been overexpressed in an insect cell overexpression system and purified to homogeneity. In this report, we confirm and extend recent findings on the physical properties, enzymatic activities, and binding properties of UL9. We demonstrate that UL9 exists primarily as a homodimer in solution and that these dimers associate to form a complex nucleoprotein structure when bound to the HSV origin of replication. We also show that UL9 is an ATP-dependent helicase, capable of unwinding partially duplex DNA in a sequence-independent manner. Although the helicase activity of UL9 is demonstrable on short duplex substrates in the absence of single-stranded DNA-binding proteins, the HSV single-stranded DNA-binding protein ICP8 (but not heterologous binding proteins) stimulates UL9 to unwind long DNA sequences of over 500 bases. We were not able to demonstrate unwinding of fully duplex DNA sequences containing the HSV origin of replication. However, in experiments designed to detect origin-dependent unwinding, we did find that UL9 wraps supercoiled DNA independent of sequence or ATP hydrolysis.

Herpes simplex virus type 1 (HSV-1) DNA is a linear double-stranded molecule of about 152 kb in size (3). Although viral DNA replication takes place in the nucleus of infected cells (3), it depends heavily on the activity of virus-encoded replication functions: of the over 72 genes contained on the HSV genome, seven encode proteins that are essential for viral DNA replication (33, 52). At least some of the functions of the products of each of these genes have been elucidated: the *pol* gene (UL30) (7, 8, 38) and the UL42 gene (17, 18, 21, 36) encode the subunits of DNA polymerase; UL5, UL8, and UL52 encode the subunits of a helicase-primase complex (9, 10); UL29 encodes a single-stranded-DNA-binding protein (ICP8) (37, 50); and UL9 encodes a protein that binds to the origins of replication (34, 48). Replication takes place predominantly by a rolling circle mechanism, generating long head-to-tail concatamers that are processed into unit-length genomes during encapsidation (40).

The HSV genome contains three origins of replication: two copies of a sequence named ori_S, and one copy of a closely related sequence, ori_L (42–44, 47, 51). The mechanism by which HSV DNA replication is initiated is not known, but it seems likely that a key event is the interaction of the origin-binding protein, UL9, with the origins of replication. The core origin sequence is composed of at least four domains: three binding sites for UL9, and an A+T-rich region between two of the sites. There are two high-affinity sites (14, 15, 34, 48), designated site I and site II (or box I and box II), and a site of much lower affinity, designated site III (or box III) (11, 14). The site I and site II sequences are inverted with respect to each other and comprise the arms of a 46-bp palindrome containing the A+T-rich domain at its center. Chemical modification and mutational analyses of the sequences within these sites have shown that UL9 specifically recognizes an 11-bp sequence, 5′-CGTTCG CACTT (14, 26). In ori_S (but not in ori_L), the 11-bp recognition sequence in site II differs from that in site I at a single position, leading to a 10-fold-reduced affinity for UL9 at site II relative to site I (14, 15). Site III is located just to the left of site I in inverted orientation and also differs from the site I sequence by a single base pair. This difference apparently leads to a greatly reduced affinity for UL9, since unlike the case with sites I and II, the binding of UL9 to site III has not been demonstrated by nuclease protection experiments; binding to a synthetic oligonucleotide duplex having the site III sequence has, however, been observed in a gel mobility shift assay (11).

Genetic experiments indicate that all four domains of the core origin sequence are necessary for optimal efficiency in promoting DNA replication in transient assays (23, 30, 31, 49). Mutations in site I or site II that abolished UL9 binding eliminated or greatly reduced DNA replication, and mutations in site III reduced replication efficiency by about a factor of 5. It is important to note, however, that there are as yet no data regarding the correlation of UL9 binding to site III with origin function. There is a report that mutation of site III to a high-affinity site identical in sequence to site I has a negative effect on replication efficiency (12a). The role of UL9 binding to site III therefore remains to be clarified. Finally, deletion or alteration of the length of the A+T-rich region at the center of the palindromic sequence also has an effect on replication efficiency (30) (see below).

The events following the binding of UL9 to the origin that lead to the initiation of DNA replication are not understood, but several lines of evidence suggest that UL9 may have functional features in common with other, better-characterized initiator proteins such as the simian virus 40 T antigen, the λ O protein, and the *Escherichia coli* DnaA protein (reviewed in reference 27). First, there are multiple binding sites for UL9 within the core origin sequence, and binding to at least two of these sites has been shown to be cooperative (14). These observations suggest that UL9, like other origin recognition proteins, may organize DNA into a specific higher-order structure. Second, examination of the predicted

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amino acid sequence of UL9 has revealed the existence of several short sequence motifs that are characteristic of a superfamily of DNA and RNA helicases (19, 20). Thus, it is possible that UL9 is a helicase and acts during initiation to unwind the two DNA strands at the origin. To gain more insight into the potential roles of UL9 in the initiation process, we have purified UL9 to homogeneity and begun a detailed study of the physical properties, enzymatic activities, and DNA-binding properties of the purified protein. In this report, we provide evidence that the predominate form of UL9 in solution is a homodimer and that these dimers direct the formation of a complex structure with the core origin sequence. In addition, we demonstrate that UL9 is a DNA helicase; unwinding takes place in the 3′-to-5′ direction and in the presence of the HSV-encoded single-stranded-DNA-binding protein is capable of unwinding long segments of duplex DNA.

MATERIALS AND METHODS

Reagents. All restriction enzymes and DNA modification enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim; the reaction conditions recommended by the manufacturer were followed. T4 gene 32 protein was purchased from United States Biochemical. *E. coli* single-stranded-DNA-binding protein was provided by N. Nossal (National Institutes of Health). Recombinant vaccinia virus topoisomerase I was provided by S. Shuman (National Institutes of Health) (41). HSV ICP8 was purified as described previously (21). Single-stranded M13 (ssM13) was purchased from Bethesda Research Laboratories. All radioisotopes were purchases from Amersham. All nucleoside triphosphates (NTPs) were purchased from Pharmacia.

Cells and viruses. *Spodoptera frugiperda* (Sf9) cells were maintained as a monolayer with TMNFH medium (GIBCO) containing 10% fetal bovine serum. The recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus/UL9 (AcNPV/UL9) was generated and propagated as previously described (34, 45).

Purification of UL9. Sf9 cells were grown to near confluence in 225-cm^2 flasks and infected with recombinant AcNPV/UL9 at a multiplicity of infection of 2 to 10 PFU per cell. After 60 to 66 h, the flasks were shaken, and the cells were decanted from the flasks and washed with iced phosphate-buffered saline. Nuclear extracts of the cells in 1.7 M NaCl were made as described previously (16) and dialyzed into 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6)–1 mM EDTA–1 mM dithiothreitol–10% (vol/vol) glycerol (buffer C)–0.25 M NaCl. Insoluble protein was removed by centrifugation, and the soluble protein fraction was applied to a 50-ml phosphocellulose column (2.8 by 4.8 cm) previously equilibrated in the same buffer. Proteins were eluted from the column with a 500-ml linear gradient containing 0.25 to 1 M NaCl in buffer C. Fractions were assayed for origin-binding activity by using a filter binding assay and for DNA-dependent ATPase activity. Fractions containing peak enzymatic activity were pooled, dialyzed into buffer C–0.25 M NaCl, and applied to a 10-ml heparin agarose column (2.0 by 2.5 cm) equilibrated with the same buffer. Proteins were eluted with a 100-ml linear gradient containing 0.25 to 1 M NaCl in buffer C. Fractions with peak origin-binding and ATPase activity were pooled, dialyzed into buffer C–0.25 M NaCl, applied to a 1-ml phosphocellulose column (1.3 by 0.5 cm) equilibrated with the same buffer, and eluted with 5 ml of buffer C–0.5 M NaCl. Fractions with peak origin-binding and ATPase activity were pooled, concentrated to 1.0 ml in a Centricon 30 concentrator (Amicon), loaded onto two 12.4-ml 15 to 30% glycerol gradients in buffer C–0.25 M NaCl–0.01% Nonidet P-40, and centrifuged in an SW40Ti rotor for 63 h at 37,600 rpm. Fractions were collected from the bottom of the centrifuge tubes in 0.3-ml increments by using positive pressure, and the fractions with peak origin-binding and ATPase activity were pooled. Protein fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (28).

Determination of hydrodynamic properties. The sedimentation coefficient of UL9 was determined by glycerol gradient centrifugation by using the conditions described above. Parallel gradients containing gel filtration-grade molecular weight standards (Sigma) of known sedimentation coefficients ($s_{20,w}$) (1 mg each of either β-amylase [8.9S] and carbonic anhydrase [2.8S] or alcohol dehydrogenase [7.6S] and carbonic anhydrase) were run. The Stokes radius of UL9 was determined by gel filtration chromatography on a Superose 12 10/30 column (Pharmacia) calibrated with proteins of known Stokes radii (thyroglobulin [8.10×10^{-7} cm], β-amylase [5.14×10^{-7} cm], and alcohol dehydrogenase [4.52×10^{-7} cm]; all from Sigma) and using the calculations of Ackers (1).

Assay conditions. (i) UL9 origin binding assay. The reaction mixture (50 μl) contained 2 to 4 ng of ^{32}P-ori$_S$, 100 μg of sonicated calf thymus DNA per ml, 5 mM $MgCl_2$, and 100 mM NaCl in buffer C. Three to 5 pmol of unlabeled site I of ori$_S$ (see below) and 1 to 5 μl of the protein fraction were added, and the mixture was incubated for 10 min at room temperature; 1 ml of ice-cold buffer C–0.1 M NaCl was added, the mixture was filtered through a 0.45-mm-pore-size nitrocellulose filter (Schleicher & Schuell) that had been wetted in the same buffer, and the filter was washed with 1 ml of ice-cold buffer C–0.1 M NaCl. Filters were immersed in scintillation fluid (Beckman) and analyzed for retained ^{32}P-labeled DNA in a liquid scintillation counter.

(ii) ATPase assay. The ATPase assay was performed as described previously (29). Standard curves were constructed by incubating the reaction mixtures with 1, 5, and 10 nmol of KH_2PO_4 and plotting the optical density at 660 nm readings of these samples against the known amount of P_i in the reaction mixtures.

(iii) Helicase assay. The standard reaction mixture for helicase activity contained 1 mM dithiothreitol, 100 μg of bovine serum albumin (BSA) per ml, 20 mM Tris HCl (pH 8.0), 2 mM ATP, 3 mM $MgCl_2$, 80 mM NaCl, and 20 fmol of ^{32}P-labeled substrate in a volume of 20 μl. UL9 was added as indicated, the mixture was incubated for 1 h at 37°C, and the reactions were terminated by addition of 1/10 volume of a stop solution to give final concentrations of 5 mM EDTA, 1% SDS, and 0.05% bromophenol blue. Six microliters of the reaction mixture was loaded onto a continuous 10% polyacrylamide gel and run at 15 to 20 V/cm for 1 h in 45 mM Tris-borate–1.25 mM EDTA (1/2× TBE). Total heat-denaturable counts were determined by heating one reaction mix for 5 min to 100°C immediately before the sample was loaded onto the gel. The gels were dried at 80°C under vacuum and exposed to Kodak XAR X-ray film. Radioactivity was quantitated on a Betagen Betascope 603 blot analyzer.

(iv) DNase I footprinting. The DNase I footprint reaction mixture (20 μl) contained 50 mM HEPES (pH 7.6), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of BSA per ml, ~0.1 pmol of labeled DNA fragment, and 20 to 50 ng of UL9. DNase I was added to a final concentration of 5 μg/ml,

and the mixture was incubated at room temperature for 1 min. The reactions were terminated by phenol extraction; the DNA was precipitated with ethanol and analyzed on an 8% polyacrylamide sequencing gel.

DNA substrates. (i) Origin-binding assay. The origin-containing fragment used was the *Hin*dIII-to-*Eco*RI sequence from pORI100-2 (derived from pMC110 [34]), labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by using the Klenow fragment of *E. coli* DNA polymerase I (Klenow).

(ii) Helicase assay. Partially duplex DNA molecules were formed by the annealing of a 5′-^{32}P-labeled synthetic 30-base oligonucleotide to M13mp19 (5′-CAGTCACGACGTTGT AAAACGACGGCCAGT). The oligomer was 5′ end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase. The annealing reaction mixture (100 μl), containing 10 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 4.2 pmol of M13mp19, and 21 pmol of oligomer, was heated to 90°C and allowed to cool to room temperature over 2 to 3 h. EDTA was added to 10 mM, and excess oligonucleotide was removed by gel filtration through a 10-ml Bio-Gel A15m column (26 by 0.7 cm) (Bio-Rad) equilibrated with 10 mM Tris HCl (pH 7.5)–1 mM EDTA (TE). The variable-strand-length substrate was made by using the U.S. Biochemical Sequenase 2.0 kit and the reaction conditions supplied by the manufacturer. All reaction mixes were scaled proportionally to the amount of M13mp19 DNA used. The labeled 30-mer oligonucleotide was annealed to M13mp19 as described above, the reaction mix was ethanol precipitated, and the DNA was resuspended in the reaction buffer supplied in the kit. The DNA was divided into three aliquots and processed by using three standard reaction conditions: (i) 1:5 diluted labeling mix, no termination mix, (ii) undiluted labeling mix, no termination mix, and (iii) undiluted labeling mix, 1:1.5-diluted termination mix. The three reaction mixes were then pooled, EDTA was added to 10 mM, and the unannealed oligonucleotide population was separated on the Bio-Gel column as described above.

(iii) Helicase polarity. The linear partially duplex substrate for testing the polarity of the UL9 helicase was prepared as described previously (32). The 341-bp *Hae*III restriction fragment of M13mp18 was isolated by gel electrophoresis and annealed to ssM13mp18 DNA. The resulting duplex was cleaved with *Cla*I to generate a linear DNA molecule with double-stranded ends. The three available 3′-OH termini were then labeled with [α-^{32}P]dCTP by using Klenow.

(iv) DNase I footprinting. DNase I footprint substrates containing the wild-type origin fragment (201) or insertional derivatives (AT6, AT18, and AT23) were obtained from plasmids pS201, pS201AT6, pS201AT18, and pS201AT23 (30), respectively. Plasmid DNAs were digested with *Hin*dIII, 3′ end labeled by using [α-^{32}P]dCTP and Klenow, and further digested with *Pvu*I. The labeled origin-containing fragments were subsequently purified by agarose gel electrophoresis. The substrate containing site I was constructed by the ligation of an annealed pair of complementary synthetic oligonucleotides comprising site I (5′-GGGCGAAGCGT TCGCACTTCGTCCCAA) into the *Sma*I site of pUC19. The DNA was cleaved with *Hin*dIII and *Eco*RI and end labeled with [α-^{32}P]dCTP by using Klenow, and the 79-bp labeled fragment was purified by agarose gel electrophoresis.

RESULTS

Purification and physical properties of recombinant UL9. UL9 was purified to homogeneity from Sf9 insect cells infected with a recombinant baculovirus expressing the full-length UL9 coding sequence. The purification scheme involved (i) column chromatography on phosphocellulose and heparin agarose and (ii) glycerol gradient centrifugation (Table 1). Throughout the course of purification, UL9 was assayed by means of a filter binding assay for site-specific binding to the HSV origin sequence, by DNA-dependent ATPase activity (see below), and by an immunoblot analysis using specific antipeptide antibody. Analysis of the most highly purified fraction (fraction V; Table 1) by SDS-PAGE followed by staining with Coomassie brilliant blue revealed a single polypeptide of 88 kDa, greater than 95% free from contaminants (Fig. 1). With this protocol, 4×10^9 Sf9 cells yielded about 2 mg of purified protein, with an overall recovery of about 10%.

TABLE 1. Summary of UL9 purification

Fraction	Total protein (mg)	Total binding activity (pmol)	Specific act (pmol/mg)
I. Nuclear extract	780	49,100	60
II. Phosphocellulose no. 1	140	30,800	220
III. Heparin agarose	9.5	8,480	890
IV. Phosphocellulose no. 2	5.9	4,830	820
V. Glycerol gradient	1.8	4,540	2,500

As has been noted previously, there is a small discrepancy between the size of UL9 predicted from the sequence of the UL9 gene (94 kDa) and that observed by SDS-PAGE (82 to 88 kDa) (5, 15, 34, 35; this report). The availability of large amounts of purified protein offered the opportunity to determine whether this discrepancy is due to proteolytic processing of UL9. The amino-terminal amino acid sequence of the purified protein was determined by standard microsequencing methods. The sequence obtained (12 of 14 residues) was

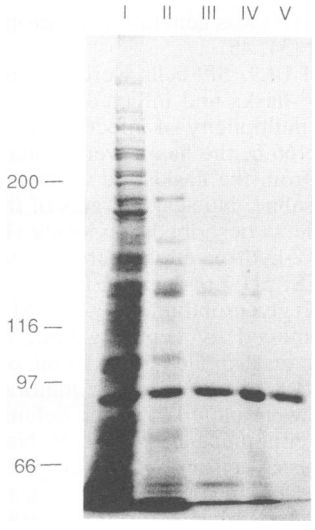

FIG. 1. SDS-PAGE analysis of fractions from UL9 purification. Electrophoresis was performed by using a resolving gel of 5% acrylamide–0.13% bisacrylamide (28). Molecular size markers used (molecular sizes are indicated in kilodaltons): myosin (200), β-galactosidase (116), phosphorylase B (97), and BSA (66). Each lane contains equal amounts (10 pmol) of UL9-binding activity, as measured by filter binding assay. Following electrophoresis, the gel was stained with Coomassie brilliant blue. Fractions I to V are as listed in Table 1.

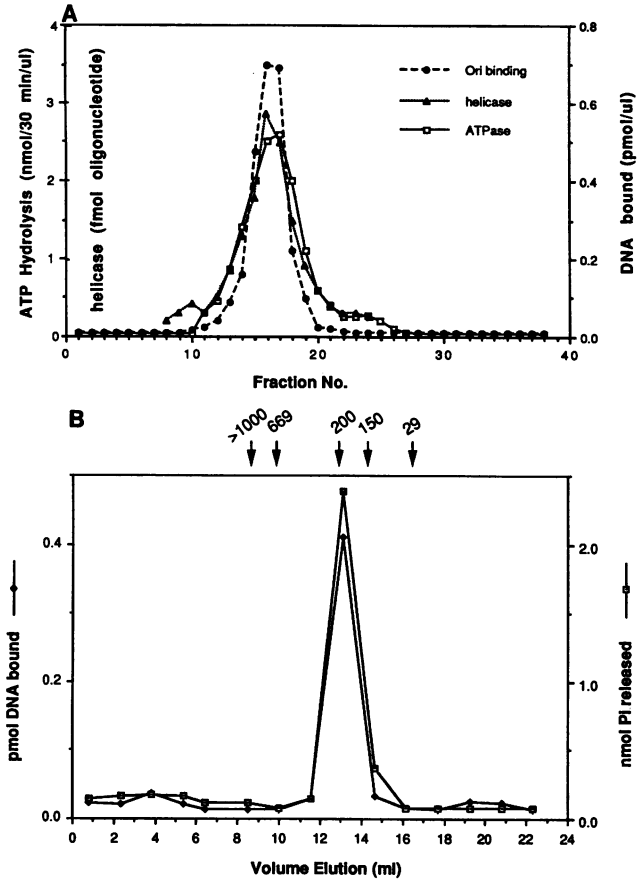

FIG. 2. (A) Glycerol gradient sedimentation of UL9. Fraction IV of a UL9 preparation was sedimented through a glycerol gradient, and the fractions were collected and assayed for DNA-binding, DNA-dependent ATPase, and helicase activities as described in Materials and Methods. (B) Gel filtration analysis of UL9. Fraction V of a UL9 preparation was subjected to gel permeation chromatography, and the fractions were assayed for DNA-binding and DNA-dependent ATPase activity as described in Materials and Methods.

identical to the amino-terminal sequence predicted by the nucleic acid sequence. This fact, in conjunction with the fact that the purified protein reacts with rabbit antiserum raised against a peptide corresponding to the carboxy-terminal 10 amino acids of the predicted UL9 sequence, argues strongly that protein purified after synthesis in the recombinant baculovirus overexpression system is a full-length copy of the UL9 gene.

The molecular weight of native UL9 was determined from its hydrodynamic properties. The sedimentation coefficient of the purified protein was 8.3S, as determined by glycerol gradient centrifugation (Fig. 2A), and the Stokes radius was 5.0 nm, as determined by gel filtration (Fig. 2B). Using these values, we calculated a native molecular weight of 1.9×10^5. This molecular weight is in close agreement with the value predicted for a homodimer of subunits of 94 kDa. We therefore conclude that UL9 exists in solution predominantly as a dimer. To gain some insight into the stability of the UL9 dimer and to determine whether UL9 dimers associate into higher-order structures, the sedimentation behavior of the purified protein was analyzed over a wide range of protein concentrations (3.4×10^{-1} to 1×10^{-4} mg/ml). At all concentrations tested, we observed a single peak of protein sedimenting at 8.3S (data not shown). From the lowest concentration tested, we can estimate that the dissociation constant of the UL9 dimer is less than 10^{-10} M; from the highest concentration tested, we estimate that the dissociation constant of higher-order associations of UL9 is greater than 10^{-7} M.

UL9 forms a complex nucleoprotein structure with the origin. Although sedimentation experiments did not provide convincing evidence for higher-order associations of UL9 dimers in solution, it has been reported that UL9 binds cooperatively to the two binding sites present in ori_S (14). The existence of a cooperative binding interaction suggests that UL9 dimers may associate when bound to DNA. To gain additional evidence for higher-order nucleoprotein structures, we carried out DNase I footprint experiments using derivatives of ori_S in which the spacing between the two UL9-binding sites was increased by insertion of additional (AT) dinucleotides into the alternating AT sequence at the center of the ori_S dyad (30). Three ori_S derivatives were used: pS201AT6, containing an insertion of 12 bp; pS201AT18, containing an insertion of 36 bp; and pS201AT23, containing an insertion of 46 bp. In each case, when UL9 was added in amounts sufficient to completely protect the two UL9-binding sites from DNase I digestion, a regular, repeating pattern of DNase I hypersensitivity and insensitivity was also observed within the alternating AT sequence separating the two binding sites (Fig. 3). The periodicity of the repeating pattern was 10 bp, and the length of the repeating pattern was dependent on the length of the inserted AT segment: two repeats were observed with pS201AT6, four repeats were observed pS201AT18, and five repeats were observed with pS201AT23. As shown previously, such a periodicity in sensitivity to DNase digestion is an indication that the DNA is held in a fixed conformation in which one side of the DNA helix is more susceptible to nuclease attack (22, 24). A plausible interpretation of these results is that the DNA between the two UL9-binding sites is organized into a fixed loop structure by protein-protein interactions between the UL9 molecules bound at the two sites. This conclusion, however, must be viewed with some caution; other data suggest that the regular DNase hypersensitivity pattern may not necessarily depend on interactions between UL9 protein bound at two sites. For example, Fig. 4 shows a DNase I footprint experiment on a DNA fragment containing a single UL9-binding site in which no loops formed by protein-protein interaction are possible. As in the case of the insertion mutants described above, a pattern of hypersensitive sites spaced approximately 10 bp apart was observed on one side of the sequence block protected by UL9. We therefore suggest that UL9 interacts with the DNA sequences adjacent to a binding site, perhaps by wrapping or bending the DNA around a central core of protein.

UL9 is a helicase. Because the predicted amino acid sequence of UL9 contains several motifs characteristic of DNA helicases (19, 20), it seemed likely that the purified protein would exhibit some form of helicase activity. As a first step in characterizing such an activity, fractions containing UL9 were assayed for DNA-dependent ATPase activity throughout the course of purification. A DNA-dependent ATPase activity, in fact, cofractionated with UL9 at each step in the purification up to and including the final glycerol gradient sedimentation step, during which the ATPase activity cosedimented with DNA-binding activity (Fig. 2A). The ATPase activity associated with UL9 was stimu-

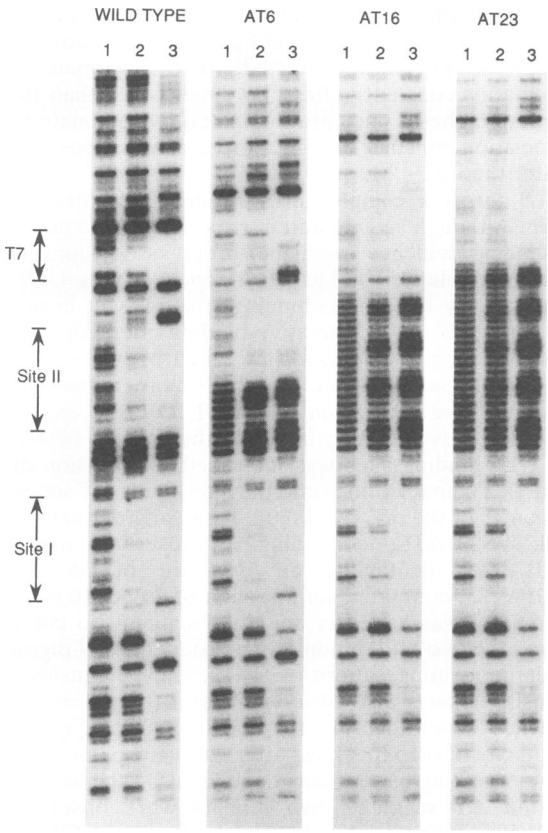

FIG. 3. DNase I footprint analysis of $(AT)_n$ insertion mutants of ori_S. The isolation of end-labeled DNA substrates and the reaction conditions are described in Materials and Methods. Lanes: 1, no UL9; 2, 25 ng of UL9; 3, 40 ng of UL9. The headings above the lane numbers refer to the fragments derived from pS201, pS201AT6, pS201AT18, and pS201AT23, respectively (30). Site I and Site II refer to those sites in ori_S (see text). T7 refers to a sequence (5′-ATTCGCCCT) formed by chance at the junction of the cloned origin sequences and the multiple cloning site of pBluescript that is similar to the UL9 recognition sequence.

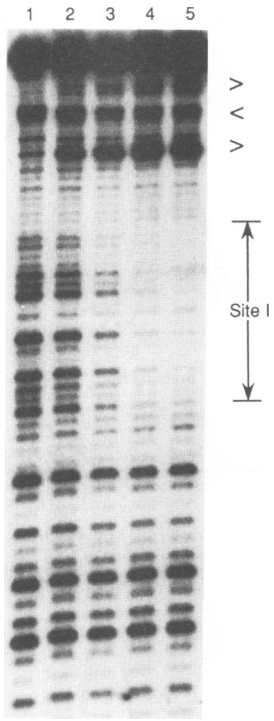

FIG. 4. DNase I footprint analysis of site I. The isolation of end-labeled DNA substrate and the reaction conditions are described in Materials and Methods. Lanes: 1, no UL9; 2, 4 ng of UL9; 3, 8 ng of UL9; 4, 16 ng of UL9; 5, 32 ng of UL9. Hypersensitive cleavage sites are designated >, and an insensitive site between the two hypersensitive sites is designated <.

lated approximately ninefold by single-stranded DNAs and approximately two- to threefold by fully double-stranded DNAs. The presence of the HSV origin of replication (ori_S) had no effect on the degree of stimulation of ATPase by double-stranded DNA (Table 2). As shown in Table 3, dATP, CTP, and dCTP were hydrolyzed at rates approaching that of ATP, while GTP, dGTP, UTP, and TTP were hydrolyzed at substantially lower rates.

DNA helicase activity of purified UL9 was assayed on a substrate consisting of ssM13 DNA annealed to a 5′-^{32}P-labeled synthetic oligonucleotide 30 bases in length. As shown in Fig. 5A, UL9 efficiently catalyzed the displacement of the oligonucleotide. Helicase activity was not affected by the presence of an unpaired tail at either the 5′ or 3′ end of the oligonucleotide (not shown); helicase activity did, however, require that one of the two strands of the substrate be partially unpaired, since no unwinding of fully duplex DNA molecules of any size was observed (not shown). Similarly, no unwinding of double-stranded DNA fragments containing ori_S was detected (not shown).

The conditions for optimal helicase activity of UL9 were determined by systematically varying the components of the reaction. UL9 helicase activity required the presence of a hydrolyzable 5′ NTP: neither of the two nonhydrolyzable analogs of ATP, ATPγS or AMP-P-N-P (not shown), supported unwinding at levels above background. As expected from the pattern of NTP hydrolysis seen with UL9, CTP, dATP, and dCTP all catalyzed levels of helicase activity comparable to the level catalyzed by ATP; GTP, dGTP, UTP, and TTP were substantially less effective (Fig. 5B). Maximal helicase activity catalyzed by ATP hydrolysis occurred at an ATP concentration of 2 mM. The addition of phosphocreatine with creatine phosphokinase as an ATP regeneration system did not enhance helicase activity compared with the addition of phosphocreatine alone (not shown). No activity was observed in the absence of divalent cation: both magnesium and manganese could satisfy the divalent cation requirement, with the optimal magnesium

TABLE 2. Stimulation of ATPase activity by DNA[a]

DNA	nmol of P_i/μg of UL9
None	1.6
Calf thymus	5.9
Calf thymus, heat denatured	14
pUC19	4.8
pMC110 (ori$^+$)	3.5
Poly(dI-dC)	4.5
ssM13	14

[a] ATPase assays were done by using standard reaction conditions listed in Materials and Methods. All DNAs were added to a final concentration of 100 μg/ml, and 0.6 μg of UL9 was added to each reaction mixture.

TABLE 3. Effects of NTPs on ATPase activity[a]

NTP	P_i released (nmol)
ATP	8.9
CTP	6.0
GTP	2.0
UTP	4.2
dATP	7.3
dCTP	7.2
dGTP	1.4
dTTP	1.4
ATPγS	<0.2

[a] ATPase assays were done by using optimal helicase reaction conditions as described in Materials and Methods. Each reaction mixture contained 1.3 μg of UL9; the NTPs were added to a concentration of 2 mM.

concentration at 3 mM and the optimal manganese concentration at 45 to 50 mM. Optimal helicase activity also required the presence of a monovalent salt (sodium chloride or potassium chloride) at concentrations of 60 to 80 mM. In the absence of added salt, activity was reduced by about 80%. The pH optimum for the reaction was 8.0 to 9.0. The optimal assay conditions described above resulted in linear accumulation of released oligomer for 1 h, and 77% of total hybridized oligomer was displaced within 2.5 h. Under the conditions that were optimal for helicase activity, the turnover number for UL9 was 1 molecule P_i per molecule of UL9 per s.

UL9 can unwind long regions of duplex DNA. To determine whether UL9 can unwind long regions of duplex DNA, the 30-base 5′-^{32}P-labeled oligonucleotide on the standard helicase substrate was extended with T7 DNA polymerase and limiting dNTPs, thereby generating a new substrate consisting of M13 DNA annealed to a random population of labeled DNA strands ranging in size from 30 to about 500 bases. UL9 quantitatively displaced strands of up to about 100 bases in length; strands longer than 100 bases were displaced at decreasing efficiency with increasing length, so that strands longer than about 250 bases were not displaced at appreciable levels. To determine whether the failure of UL9 to displace long DNA strands might be due to the reannealing of the unwound region of the substrate before the enzyme translocates to the end of the duplex region, the HSV-encoded single-stranded-DNA-binding protein (ICP8) was added to the reaction mixture. ICP8 markedly stimulated the displacement of longer DNA strands while having little or no effect on the displacement of short DNA strands (Fig. 6). Since ICP8 did not stimulate the DNA-dependent ATPase activity of UL9 (not shown), it seems likely that its role in stimulating helicase activity on long DNA strands relates to its ability to prevent reassociation of partially unwound DNA, but we cannot conclusively rule out a more specific interaction between ICP8 and UL9.

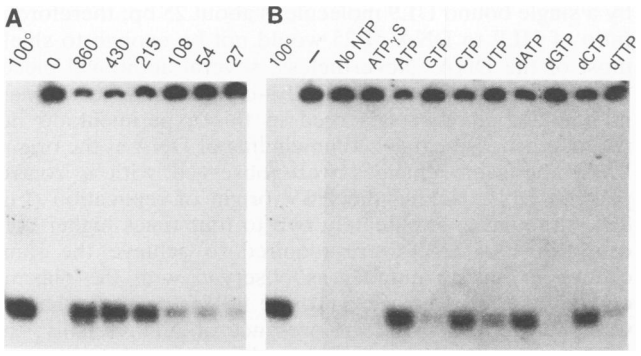

FIG. 5. Helicase activity of purified UL9. Reactions were carried out as described in Materials and Methods. (A) The numbers above each lane indicate the amount of UL9 (in nanograms) added to each reaction mixture. In the reactions corresponding to the leftmost two lanes, no UL9 was added; the first (100°) was heated to 100°C for 5 min before loading onto the gel, and the second (0) was incubated at 37°C for 1 h before loading onto the gel. (B) Each reaction contained 430 ng of UL9 and the indicated NTP at a concentration of 2 mM.

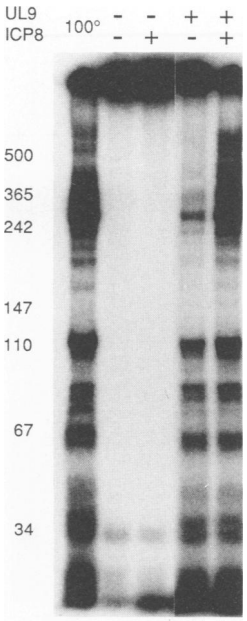

FIG. 6. Unwinding by UL9 of long regions of duplex DNA in the presence of ICP8. The DNA substrate was composed of random lengths of ^{32}P-labeled single strands of 30 to about 500 bases annealed to ssM13 as described in Materials and Methods. ICP8 (40 ng) and UL9 (215 ng) were added to reaction mixtures as indicated (+). The reaction mixture loaded onto the leftmost lane was heated to 100°C for 5 min immediately prior to electrophoresis. The reactions were carried out as described in Materials and Methods but were fractionated by electrophoresis on a 5% polyacrylamide gel. Numbers at the left indicate the base positions of single-stranded DNA size standards (denatured *Msp*I-digested pUC19 DNA) run on the same gel.

UL9 unwinds DNA 3′ to 5′. To test the polarity of the UL9 helicase, a substrate was constructed as described in Materials and Methods. The result is a long 3′-labeled single-stranded region with a 3′-labeled 143-base fragment annealed at the 5′ end and a 3′-labeled 202-base fragment annealed at the 3′ end (Fig. 7A). In the presence of ICP8, the 143-base fragment annealed at the 5′ end of the substrate was released by UL9 in significant excess over the 202-base fragment annealed at the 3′ end, demonstrating that the UL9 helicase translocated 3′ to 5′ along the single-stranded DNA segment (Fig. 7B). A small percentage of the annealed 202-base 3′ fragment was released, possibly due to minor fraying of the termini of the duplex regions of the substrate, allowing UL9 access to this 3′ end.

Interaction of UL9 with duplex DNA. The fact that UL9 has helicase activity suggests that this protein may act to initiate replication after binding to the origin by catalyzing the

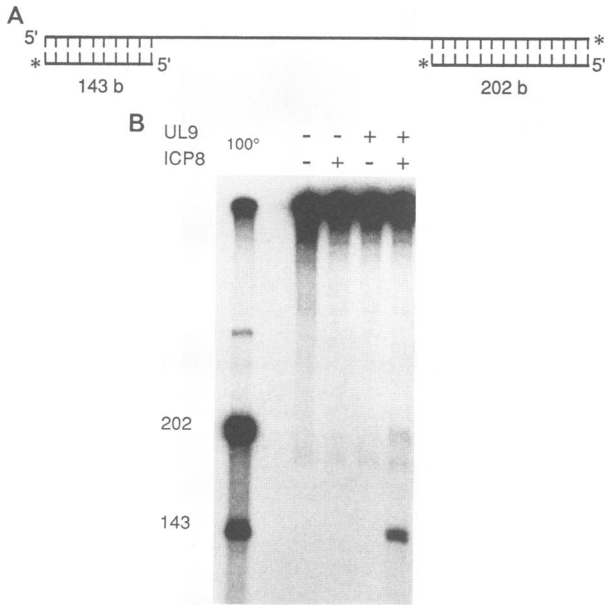

FIG. 7. Unwinding of DNA 3′ to 5′ by UL9. (A) Diagrammatic representation (not drawn to scale) of partially duplex DNA substrate used to determine helicase polarity (32). *, ^{32}P-labeled 3′ termini. b, bases. (B) Helicase assays. ICP8 (0.6 μg) and UL9 (125 ng) were added to reaction mixtures as indicated (+). The reaction mixture loaded onto the leftmost lane was heated to 100°C for 5 min immediately prior to electrophoresis. Numbers at the left refer to the sizes (in bases) of the labeled strands shown in panel A.

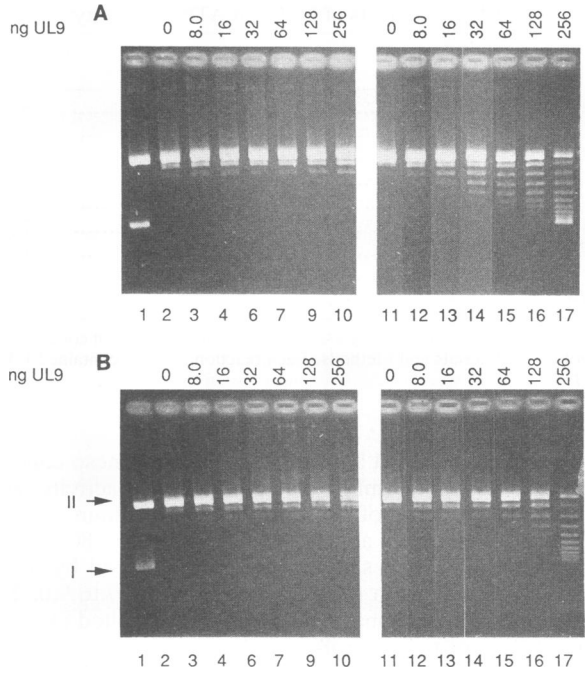

FIG. 8. Interaction of UL9 with duplex DNA. For lanes 1 to 10, plasmid DNA was incubated with 3 ng of recombinant vaccinia virus topoisomerase I (41) for 15 min by using standard helicase conditions, then the indicated amounts of UL9 were added, and the mixture was incubated for 1 h at 37°C. For lanes 11 to 17, plasmid DNA was incubated with the indicated amounts of UL9 for 1 h at 37°C by using standard helicase conditions, then topoisomerase was added, and the mixture was incubated for 15 min at 37°C. The reactions were then stopped with 1% SDS–10 mM EDTA–0.05% bromophenol blue, and one half of the reaction volume was loaded onto a 1.4% agarose gel–TBE and run in the same buffer at 100 V for 5 h. The gel was stained with 0.5 μg of ethidium bromide per ml in TBE for 1 h and photographed under reflected short-wave UV light. Plasmid DNA was pMC110 (A) or pUC19 (B). I and II at the left show the positions of supercoiled and relaxed DNAs, respectively.

unwinding of the two strands of the parental duplex. As noted above, the complete unwinding of fully duplex linear DNA fragments containing the origin was not observed, even in the presence of sufficient ICP8 to prevent the reannealing of complementary strands following unwinding. To test the possibility that UL9 might cause a more limited local unwinding event at the origin, we carried out an experiment in which UL9 was incubated with covalently closed plasmid DNA in the presence of topoisomerase I. Unwinding of the duplex will result in the introduction of positive supercoils in the plasmid. Consequent relaxation of such supercoils by the topoisomerase will result in a net change in plasmid linking number, which can be assayed by agarose gel electrophoresis following deproteinization of the DNA. Examples of such experiments are displayed in Fig. 8. When the DNA was relaxed by incubation with topoisomerase prior to the addition of UL9 (Fig. 8A and B, lanes 2 to 10), no UL9-induced changes in linking number were observed under any of the experimental conditions tested. In contrast, addition of UL9 to the DNA prior to the addition of topoisomerase (lanes 11 to 17) resulted in the accumulation of plasmid DNA with increasing numbers of superhelical turns. Identical results were observed with up to 100 times the concentration of topoisomerase in the reaction mixture (not shown), so we think it unlikely that this apparent linking number change is due to a simple inhibition of the topoisomerase by UL9. It is also unlikely that the plasmid linking number changes are the result of an indirect inhibition of topoisomerase activity due to shielding of the DNA from topoisomerase action by bound UL9: the molar ratio of UL9 to plasmid DNA in the experiment shown in Fig. 8 ranged from 5 to 25. DNase footprint experiments have shown that the extent of DNA protected by a single bound UL9 molecule is about 25 bp; therefore, a ratio of UL9 to DNA of 25 would not be enough to shield most of the DNA. Nevertheless, several additional pieces of evidence suggest that the UL9-induced changes in plasmid linking number observed in this experiment do not reflect a helicase-catalyzed unwinding of DNA at the origin. First, the same changes were observed with a control plasmid DNA lacking the HSV origin of replication (Fig. 8B), although approximately two to four times higher concentrations of UL9 were required to achieve the same change in linking number as observed with the plasmid containing the origin. Second, the linking number changes were not dependent on the presence of ATP. Finally, the magnitude of the linking number change was dependent on the amount of UL9 added, and the average number of supercoils remaining in the DNA corresponded roughly to the molar ratio of UL9 to DNA. The interpretation of this experiment that we favor is that UL9 wraps any DNA to which it is bound into some nucleosomelike structure that isolates at least one superhelical turn. Whether this nonspecific wrapping reflects any biologically important function remains to be determined.

DISCUSSION

We have examined the properties of highly purified HSV origin-binding protein (UL9) in an attempt to gain some insight into the mechanism by which this protein initiates viral DNA replication. Our results indicate that UL9 shares some properties that are reminiscent of other virus- and cell-encoded initiator proteins. In particular, we have demonstrated that (i) UL9 has an intrinsic 3′-to-5′ helicase activity and (ii) it forms a complex higher-order structure when bound at an origin of replication. These results both confirm and extend two recent reports from other laboratories (5, 25).

There are two high-affinity binding sites for UL9 in the core origin sequence (14, 15, 34, 48). The form of UL9 that binds to a single site has not yet been rigorously established. The DNA-binding domain of the protein has been localized to the carboxy-terminal third of the polypeptide chain (12, 48), and it has been suggested that this region contains a coiled-coil sequence motif that directs dimerization (12), although dimerization of the DNA-binding domain has not been directly demonstrated. The gel filtration and sedimentation analyses reported here show conclusively that purified full-length UL9 exists predominantly as a dimer in solution. This result is in basic agreement with the gel filtration analysis of Bruckner et al. (5). By analyses of the sedimentation behavior of UL9 at varying concentrations of protein, we estimated that the dimer-monomer equilibrium dissociation constant is no less than 10^{-10} M. Although cooperativity in binding to sites I and II has been observed (14), the magnitude of this cooperative effect is considerably smaller than would be predicted if the monomer-dimer equilibrium contributed the binding energy responsible for cooperativity (2). Our results therefore suggest that it is the dimeric form of UL9 that binds to a single 11-bp recognition site. The recognition site itself, however, does not have an obvious twofold axis of symmetry. Although the recognition of an asymmetric sequence by a homodimeric protein is somewhat unusual, there is a well-documented precedent. The bacteriophage P1 repressor protein cI is a homodimer of 30-kDa subunits, and it has been shown to bind to an asymmetric 17-bp recognition sequence (13, 46). On the other hand, it has been proposed (26) that the sequence recognized by UL9 monomer units is 5′-GT(T/G)CG, which is contained twice within the central 8 bp of the recognition sequence as inverted repeats that share a two-base overlap. There are as yet, however, no reports of mutational analyses of the binding site that support this proposal. Clearly, more work will be required to understand the interaction of UL9 with its recognition site in detail.

Regardless of the form of UL9 that binds to a single recognition site, there is a good deal of evidence suggesting that binding of UL9 to the core origin results in the formation of a complex, higher-order nucleoprotein structure (14, 25, 39). As already mentioned, the binding of UL9 to sites I and II is cooperative (14), suggesting some form of interaction between the proteins bound at the two sites. In this study, we probed the structure of the DNA between the two sites by footprinting experiments using derivatives of the origin in which the intersite distance was lengthened. Our results, which both confirm and extend those of Koff et al. (25), revealed that the binding of UL9 resulted in the formation of a periodic pattern of DNase hypersensitivity in the DNA between to two binding sites, with an interval between hypersensitive sites of 10 bp. This pattern of hypersensitivity is a clear indication that the DNA between the two UL9-binding sites must be held in some sort of fixed conformation, and the most straightforward interpretation of this result is that protein-protein interaction between UL9 molecules bound at the two sites holds the DNA between the sites in a loop (25). Two results suggest that this interpretation is an oversimplification of the actual nucleoprotein structure, however. First, we showed that footprinting experiments on DNAs containing a single UL9-binding site display similar, although not as extensive, hypersensitive sites with a periodicity of 10 bp. Thus, UL9 appears to interact with DNA sequences adjacent to a binding site, perhaps by wrapping or bending the DNA around a central core of protein. Moreover, UL9 may also wrap DNA even when it is bound to that DNA nonspecifically: as shown in Fig. 8, covalently closed plasmid DNA that was incubated with UL9 retained some superhelicity after it was relaxed by the addition of topoisomerase I. Second, the periodic DNase hypersensitivity pattern observed with the insertion derivatives was not sensitive to the length of the inserted sequence. The same pattern of hypersensitivity was observed with DNA fragments having 12, 36, and 46 bp inserted between the two binding sites. The fact that the formation of the structure giving rise to the periodic hypersensitivity pattern occurs on DNA fragments in which the intersite lengths differ by a nonintegral number of helical turns suggests that the structure does not require a specific spatial relationship between the UL9 bound at the two sites. Such a requirement is characteristic of loops formed by specific protein-protein contacts, at least over the range of intersite distances used in our experiments. We suggest, therefore, that the structure of the nucleoprotein complex formed between UL9 and the origin is more complicated than a simple loop formed by a specific interaction between UL9 protein dimers bound at the two high-affinity binding sites. Thus, the molecular interactions that lead to the observed cooperativity in binding of UL9 to the origin are not clear, and further work will be required to characterize the nucleoprotein structure in detail.

The events following the binding of UL9 to the core origin sequence that lead to the actual initiation of DNA synthesis are even less well understood than the structure of the UL9-DNA complex itself. Clearly, the two parental strands must be unwound as a prelude to the initiation of daughter strand synthesis. By analogy with other prokaryotic and eukaryotic replication origin recognition proteins, the binding of UL9 to ori_S and ori_L may initiate the assembly of a multiprotein replication complex that carries out the primary unwinding event, or UL9 may unwind the DNA prior to the recruitment of replicative enzymes. Consistent with this latter possibility is the finding, reported here and by Bruckner et al. (5), that UL9 has an intrinsic helicase activity. The UL9 helicase activity can be assayed in vitro on substrates that are partially single stranded, such as M13 DNA annealed to a short complementary oligonucleotide, and is capable of unwinding duplex segments of over 500 bases in the presence of the HSV-encoded single-stranded-DNA-binding protein. Genetic evidence supports the idea that the helicase activity of UL9 is essential for viral DNA replication: nonconservative amino acid substitutions in six conserved helicase motifs in the amino-terminal third of the polypeptide sequence render the molecule inactive for DNA replication (49a).

The helicase activity of UL9 has several properties that are at least superficially similar to those of the simian virus 40 T antigen, another virus-encoded initiator protein that is known to act by catalyzing a localized unwinding event at

the origin of replication (4, 6). Helicase activity on nonspecific (i.e., non-origin-containing) substrates requires that one, but not both, of the two strands be partially single stranded. Unwinding occurs in the 3′-to-5′ direction on the single strand to which the enzyme is bound and is stimulated by the cognate single-stranded-DNA-binding protein. On the other hand, origin-dependent unwinding by T antigen is readily demonstrated, but attempts reported both here and by other investigators to demonstrate origin-specific unwinding by UL9 have proven unsuccessful (4, 6). UL9-induced distortions of the DNA in the vicinity of the origin were detected by permanganate footprinting experiments (25), but these distortions were not energy requiring, as might be expected if they were the result of unwinding catalyzed by the helicase activity of UL9. It seems likely that these distortions reflect the formation of a specific nucleoprotein structure, rather than local unwinding at a site within the origin. Nevertheless, despite the lack of any experimental confirmation to date, the idea that the helicase activity of UL9 plays an essential role in initiation by unwinding DNA at the origin is an attractive hypothesis. It is possible that the experimental conditions necessary to detect such an unwinding event in vitro have not yet been discovered, or it is possible that there is another component required for the reaction, a cellular protein perhaps, that has not yet been identified. Future work on UL9 will have to be directed toward understanding the exact role both of its helicase activity and of the complex nucleoprotein structure that is formed upon binding of the protein to the origins.

ACKNOWLEDGMENTS

We are grateful to Denise A. Galloway and Daniel Lockshon for providing the pS201 series plasmids.

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