Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5414–5418, August 1988
Biochemistry

Herpes simplex virus DNA replication: The UL9 gene encodes an origin-binding protein

(ori_S and ori_L/baculovirus expression)

PAUL D. OLIVO, NANCY J. NELSON, AND MARK D. CHALLBERG*

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, 9000 Rockville Pike, Bethesda, MD 20892

Communicated by Bernard Moss, April 4, 1988

ABSTRACT **Herpes simplex virus 1 contains seven genes that are necessary and sufficient for origin-dependent DNA synthesis in cultured cells. We have expressed the product of one of these genes, UL9, in insect cells by using a baculovirus expression vector. The apparent size of the UL9 protein, both in insect cells and in herpes simplex virus-infected Vero cells, is 82,000 Da. By using an immunoassay for protein–DNA interaction, we have shown that UL9 protein binds specifically to the herpes simplex virus origins of DNA replication, ori_S and ori_L. DNase I "footprint" analysis has shown that the UL9 protein interacts with two related sites on ori_S, located on each arm of a nearly perfect palindrome. Our data strongly suggest that the origin-binding activity described previously by Elias *et al.* [Elias, P., O'Donnell, M. E., Mocarski, E. S. & Lehman, I. R. (1986) *Proc. Natl. Acad. Sci. USA* 83, 6322–6326] is the product of the UL9 gene.**

Herpes simplex virus (HSV) contains a large double-stranded DNA genome that is replicated within the nucleus of a host cell. HSV provides an attractive model for probing the mechanisms of DNA replication in eukaryotic cells since many, if not all, of the proteins that are involved in viral DNA replication are virus-encoded. Thus, the process of HSV DNA replication can be studied using a combination of biochemical and genetic approaches.

The 153-kilobase HSV-1 genome contains 72 genes (21) and three cis-acting replication origins (1–5). By using a transient transfection assay, we have recently shown that 7 viral genes are both necessary and sufficient for origin-dependent DNA replication in cultured cells (6). Included in this set are the genes encoding a DNA polymerase (7), a single-stranded DNA-binding protein (ICP8; ref. 7), and an abundant protein of 51 kDa (UL42; refs. 6 and 8) that binds tightly to double-stranded DNA in a sequence-independent manner (ref. 9; C. Wu and M.D.C., unpublished results). The products of the remaining 4 genes (UL5, UL8, UL9, UL52; refs. 6 and 8) have not yet been characterized. The three origin sequences are presumably the sites at which DNA replication is initiated. Two of these sequences, named ori_S (1–4), are identical. The sequence of the third origin, ori_L, is closely related to that of ori_S (5), and there is evidence to suggest that the three origins are functionally identical and redundant (6, 10, 11).

By analogy with better characterized prokaryotic and eukaryotic DNA replication systems, it seems likely that the primary event in the initiation of HSV DNA replication involves the binding of one or more proteins to sequences within ori_S and/or ori_L. An origin-specific DNA-binding activity in fact has been identified by Elias *et al.* (12) in extracts of HSV-infected cells, but the involvement of this activity in HSV DNA replication has not been demonstrated. In this paper we show that the UL9 gene, one of the HSV-1 genes essential for DNA replication, encodes a protein that binds specifically to two related sites within ori_S. Our data strongly suggest that the origin-binding activity previously described by Elias *et al.* (12) is the product of the UL9 gene.

MATERIALS AND METHODS

Immunoprecipitation Assay for Origin Binding. Specific DNA binding was assayed by immunoprecipitation by a modification of the procedure of McKay (13). The plasmid pMC110 contains a 230-base-pair (bp) *Sma* I fragment including ori_S inserted into the *Sma* I site of pUC19 (7). pMC110 was digested with the restriction enzyme *Msp* I, and the resulting 16 fragments were end-labeled with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. Binding was carried out in a reaction mixture (50 μl) containing binding buffer (20 mM Hepes, pH 7.5/100 mM NaCl/0.5 mM dithiothreitol/1.0 mM EDTA), 25 ng of labeled DNA fragments, 5 μg of unlabeled sonicated calf thymus DNA, and 1–5 μl of the indicated source of protein. After incubation at room temperature for 20 min, protein–DNA complexes were immunoprecipitated with the indicated antiserum and protein A covalently coupled to Sepharose beads (Pharmacia). Precipitated DNA was analyzed by electrophoresis on a 7% polyacrylamide gel.

Construction of Recombinant Baculovirus Expressing UL9. Wild-type and recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) were propagated in *Spodoptera frugiperda* (SF9) cells as described by Summers and colleagues (14–16). The complete UL9 open reading frame (predicted size of 94 kDa) was excised from the plasmid pMC160 (6) by digestion with *Nar* I and *Eco*RV. The *Nar* I site is located 279 bp upstream of the amino terminus of the UL9 open reading frame, and the *Eco*RV site is located 245 bp downstream of the carboxyl terminus (8). This fragment was inserted by blunt-end ligation into the unique *Bam*HI site of the plasmid pAc373 (14), and the recombinant plasmid was used to generate the recombinant baculovirus AcNPV/UL9 as described (15, 16).

Antisera. Decapeptides corresponding to the predicted carboxyl-terminal amino acid sequence of HSV-1 genes UL5, UL8, UL9, and UL52 were purchased from Biosearch (San Rafael, CA) and were covalently coupled to keyhole limpet hemocyanin. Approximately 0.5 mg of coupled peptide was used to immunize rabbits biweekly for a total of three injections. Sera were collected at biweekly intervals beginning one month after the first injection. A complete characterization of these sera will be published elsewhere.

Preparation of Baculovirus-Infected Cell Extract. Fifteen 150-cm^2 flasks of nearly confluent SF9 cells were infected with the recombinant virus AcNPV/UL9 at a multiplicity of infection of 10–20 plaque-forming units per cell. After 54 hr at 28°C, the cells were dislodged from the flasks by shaking and were washed with phosphate-buffered saline. Nuclei

Abbreviations: HSV, herpes simplex virus; AcNPV, *Autographa californica* nuclear polyhedrosis virus.

*To whom reprint requests should be addressed.

were isolated and extracted with 1.7 M NaCl as described by Elias et al. (12). The protein concentration of the nuclear extract following dialysis was ≈2 mg/ml.

DNase I "Footprint" Analysis. The 100-bp *Msp* I fragment of pMC110 containing ori_S was isolated by gel electrophoresis and was cloned by blunt-end ligation into the *Sma* I site of pUC19. Clones containing the inserted fragment in each of the two possible orientations were identified by DNA sequencing. The 150-bp fragment produced by digestion of each of these two plasmids with *Eco*RI and *Hin*dIII was isolated by gel electrophoresis and was 3′ end-labeled at the *Hin*dIII site with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. Approximately 1 ng of labeled DNA fragment was incubated in 20 μl of binding buffer containing 2 μg of sonicated calf thymus DNA with the indicated amount of nuclear extract from SF9 cells infected with AcNPV/UL9. After 20 min at room temperature, $MgCl_2$ and pancreatic DNase I were added to concentrations of 5 mM and 7 μg/ml, respectively. After incubation at room temperature for 1 min, digestion was interrupted by the addition of EDTA to 10 mM and extraction with phenol. The resulting DNA fragments were then analyzed on an 8% polyacrylamide sequencing gel. The positions of DNase I cleavage sites were identified by comparison with Maxam–Gilbert G+A cleavage patterns (17) of the same DNA fragments.

RESULTS

Immunoprecipitation of ori_S-Binding Activity from Partially Purified Extracts of HSV-Infected Cells. To determine if any of the HSV genes that are essential for DNA replication encode a protein that participates in origin-specific binding, we have used the immunoassay for protein–DNA interaction originally developed by McKay (13). ori_S-binding activity was first partially purified from a high-salt nuclear extract of HSV-infected Vero cells by using a nitrocellulose filter assay to detect binding exactly as described by Elias et al. (12). The peak fraction of binding activity was incubated with a mixture of ^{32}P-labeled DNA fragments produced by digestion of the plasmid pMC110 with the restriction enzyme *Msp* I; this mixture of 16 fragments includes a 100-bp fragment containing ori_S. Protein–DNA complexes were then immunoprecipitated with antisera against the products of the four uncharacterized HSV genes required for DNA replication (UL5, UL8, UL9, and UL52) (8). DNA fragments contained in the immunoprecipitates were eluted and analyzed by polyacrylamide gel electrophoresis. The 100-bp fragment containing ori_S was preferentially precipitated by antisera directed against the UL9 protein (Fig. 1, lane 5). No specific precipitation of any DNA fragment was observed with preimmune serum from the rabbit injected with the UL9 peptide (Fig. 1, lane 3) nor with the UL9 immune serum in the absence of added ori_S-binding activity (Fig. 1, lane 1). A small amount of the 100-bp ori_S fragment was also specifically precipitated by the anti-UL8 serum (Fig. 1, lane 4). Since similar results were obtained by using an extract of insect cells containing the UL9 protein but no other HSV gene product (Fig. 1, lane 10; see below), we favor the view that the small amount of ori_S precipitated by the anti-UL8 serum represents cross-reactivity between that serum and the UL9–ori_S complex; we cannot rule out the possibility, however, that the UL8 protein is also present in ori_S–protein complexes. We conclude that the UL9 protein plays a role in the specific recognition of the HSV origin of replication.

Expression of the UL9 Gene Product in Insect Cells by a Recombinant Baculovirus. To facilitate further analysis of the interaction between the UL9 protein and the HSV origins, we have constructed a recombinant baculovirus in which the UL9 gene is expressed at high levels. The recombinant virus, AcNPV/UL9, contains the complete HSV-1 UL9 coding

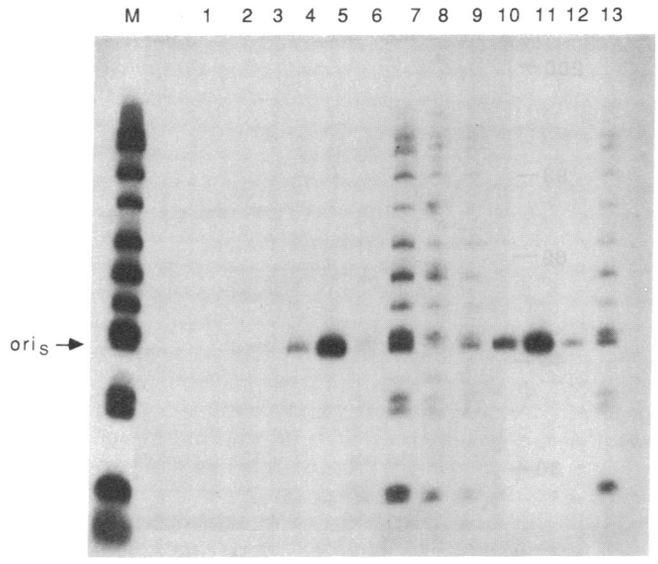

FIG. 1. Immunoprecipitation of ori_S–protein complexes. ^{32}P-labeled, *Msp* I-digested pMC110 was incubated either with partially purified ori_S-binding activity from HSV-infected Vero cells (lanes 2–8) or with nuclear extract from AcNPV/UL9-infected SF9 cells (lanes 9–13) and was immunoprecipitated with different rabbit antisera. Lanes: M, *Msp* I-digested pMC110 DNA prior to immunoprecipitation; 1, anti-UL9 serum in the absence of added binding protein; 2 and 8, no serum; 3 and 9, preimmune serum; 4 and 10, anti-UL8 serum; 5 and 11, anti-UL9 serum; 6 and 12, anti-UL5 serum; 7 and 13, anti-UL52 serum. ori$_S$, position of the 100-bp fragment that contains the ori_S sequence.

sequences positioned so that they should be expressed under the control of the polyhedrin promoter. The proteins synthesized in AcNPV/UL9-infected insect cells were analyzed by [^{35}S]methionine labeling followed by $NaDodSO_4$/polyacrylamide gel electrophoresis. AcNPV/UL9 directed the synthesis of a major ^{35}S-labeled protein with an apparent molecular mass of 82 kDa that was not present in cells infected with the wild-type parent, AcNPV (Fig. 2). This protein was specifically immunoprecipitated with the anti-UL9 serum described above (Fig. 3, lane 3). By using the same serum, a protein of identical size was observed in immunoprecipitates of [^{35}S]methionine-labeled proteins synthesized in HSV-1-infected Vero cells (Fig. 3, lane 2). The predicted size of the UL9 open reading frame is 94 kDa. We cannot at present rule out the possibility that the discrepancy between the predicted and observed values results from proteolysis or other posttranslational modification of the UL9 protein. If such modifications occur, however, then our data suggest that similar modifications occur in baculovirus-infected insect cells and HSV-1-infected Vero cells. We conclude that the 82-kDa protein synthesized by AcNPV/UL9-infected insect cells is the authentic product of the UL9 gene.

The UL9 Protein Expressed in Insect Cells Binds to ori_S. The UL9 protein expressed in AcNPV/UL9-infected insect cells was also assayed for origin-specific DNA binding by immunoprecipitation. The 100-bp fragment of pMC110 containing ori_S was preferentially precipitated by a combination of anti-UL9 serum and an extract prepared from AcNPV/UL9-infected SF9 cells (Fig. 1, lane 11). No specific precipitation of DNA was observed in the absence of cellular extract or with combinations of anti-UL9 serum and extracts of SF9 cells infected with baculovirus recombinants expressing any one of three other HSV replication genes (UL5, UL8, and UL52; data not shown). Thus, specific precipitation of the origin-containing fragment by SF9 cell extracts was dependent upon the expression of the UL9 gene. As was the

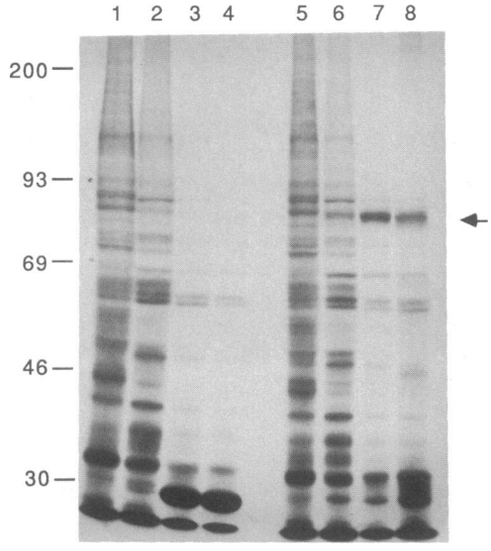

FIG. 2. [^{35}S]Methionine labeling of proteins synthesized in baculovirus-infected insect cells. SF9 cells were infected with wild-type AcNPV (lanes 1–4) or with AcNPV/UL9 (lanes 5–8) at a multiplicity of infection of 10–20 plaque-forming units per cell and were pulse-labeled with [^{35}S]methionine (0.1 μM; specific activity of $\approx$1000 Ci/mmol; 1 Ci = 37 GBq) for 2 hr at 12 hr (lanes 1 and 4), 24 hr (lanes 2 and 5), 36 hr (lanes 3 and 7), or 48 hr (lanes 4 and 8) after infection. Labeled proteins were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (18) and fluorography. The numbers at the left indicate the position and size (in kDa) of ^{14}C-labeled marker proteins run on the same gel. The arrow to the right indicates the position of the 82-kDa protein unique to AcNPV/UL9-infected cells.

case with *ori*$_S$-binding activity from HSV-infected cells, a small amount of specific precipitation of the 100-bp *ori*$_S$ fragment was also observed with other sera, particularly anti-UL8 serum (Fig. 1, lane 10). Since the HSV-1 UL8 gene product is unlikely to be present in AcNPV/UL9-infected SF9 cells, the low level of specific *ori*$_S$ precipitation is likely due to cross-reactivity between the *ori*$_S$–UL9 complex and the anti-UL8 serum. Extracts containing other HSV replication proteins (UL5, UL8, and UL52) were also tested for *ori*$_S$-binding activity with antisera specific for the expressed protein (data not shown). In no case was any specific DNA binding observed. As expected from the similarities in the sequences of *ori*$_S$ and *ori*$_L$, *ori*$_L$-containing DNA fragments were also preferentially precipitated by a combination of nuclear extract from AcNPV-infected SF9 cells and anti-UL9 antibody (data not shown). Taken together, our data strongly suggest that the UL9 protein binds to *ori*$_S$ and *ori*$_L$.

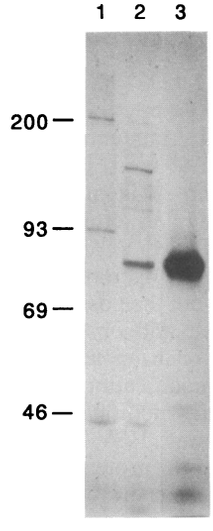

FIG. 3. Immunoprecipitation of UL9 protein synthesized in AcNPV/UL9-infected SF9 cells and in HSV-1-infected Vero cells. HSV-1 (KOS)-infected Vero cells were labeled with [^{35}S]methionine (0.1 μM; specific activity of $\approx$1000 Ci/mmol) for 2 hr beginning at 6 hr postinfection. AcNPV/UL9-infected SF9 cells were labeled as described in the legend to Fig. 2 for 2 hr at 35 hr postinfection. After labeling, the cells were disrupted in 20 mM Tris·HCl, pH 7.4/100 mM NaCl/0.5% sodium deoxycholate/1% Triton X-100/0.1% NaDodSO$_4$/1 mM phenylmethylsulfonyl fluoride and were immunoprecipitated with anti-UL9 serum as described (19). Immunoprecipitated proteins were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (18). Lanes: 1, mock-infected Vero cells; 2, HSV-1-infected Vero cells; 3, AcNPV/UL9-infected SF9 cells. The numbers at the left indicate the position and size (in kDa) of ^{14}C-labeled marker proteins run on the same gel.

Analysis of the Sites to Which the UL9 Protein Binds. The sequences with which the UL9 protein interacts were determined by DNase I footprint analysis by using a nuclear extract of AcNPV/UL9-infected SF9 cells as the source of UL9 protein. At the highest concentrations of extract tested, two regions on both strands of the origin fragment were preferentially protected from DNase I cleavage (Fig. 4). One of these regions, labeled site I in Figs. 4 and 5, corresponds closely to the 18-bp binding site of the *ori*$_S$-binding protein reported by Elias *et al.* (12). As noted previously, site I

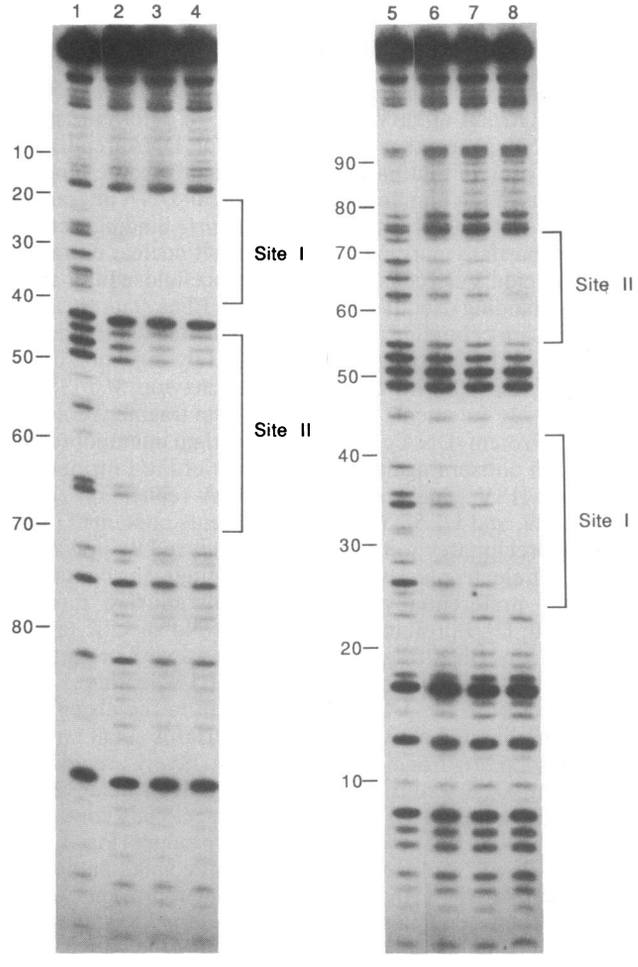

FIG. 4. DNase I footprint analysis of UL9 protein binding to *ori*$_S$. DNase I footprint analysis was carried out with increasing amounts of nuclear extract from AcNPV/UL9-infected SF9 cells and DNA fragments in which either the top strand (see Fig. 5; lanes 1–4) or the bottom strand (lanes 5–8) was 3′ end-labeled with ^{32}P. Lanes: 1 and 5, no added UL9 protein; 2 and 6, 2 μg of nuclear extract; 3 and 7, 4 μg of nuclear extract; 4 and 8, 6 μg of nuclear extract. Nucleotide positions corresponding to the numbering system in Fig. 5 are indicated at the left.

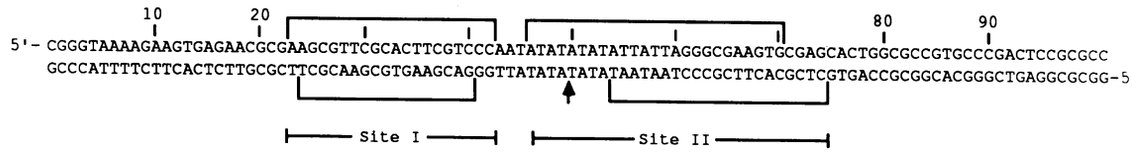

FIG. 5. Sequence of ori_S. The brackets indicate nucleotides protected from DNase I cleavage in Fig. 4. The arrow indicates the center of a hyphenated palindrome extending from nucleotide 22 to 74.

occurs within a region of hyphenated dyad symmetry (3, 12). The other region of UL9 protein binding, labeled site II, is located in a region homologous to site I on the other side of the dyad axis. Although site I and site II contain sequences that are similar (see Fig. 5), the extent of protection from DNase I cleavage at site II is somewhat greater than at site I. In particular, site II appears to extend further in towards the A+T-rich region at the center of the palindrome. The significance of the different patterns of protection from DNase I cleavage at site I and site II is not clear. The difference may simply be a consequence of the fact that the sequence with which the UL9 protein interacts is not precisely the same at the two sites, or it may reflect an altered conformation of the DNA induced by the binding of the UL9 protein at both sites.

DISCUSSION

Two separate lines of evidence indicate that the product of the HSV-1 UL9 gene is an origin recognition protein. First, protein–DNA complexes containing ori_S and an origin-specific binding activity from HSV-infected cells were immunoprecipitated with anti-UL9 serum but not with serum directed against other HSV DNA replication proteins. Second, extracts from insect cells infected with a recombinant baculovirus expressing the UL9 open reading frame also contain an HSV origin-specific binding activity, and again, this binding activity was preferentially immunoprecipitated with anti-UL9 serum. Taken together, these results strongly suggest that origin recognition is an intrinsic property of the UL9 protein. Since baculovirus-infected insect cells are unlikely to contain any other HSV gene product, we can rule out the obligatory participation of other HSV-encoded proteins in origin recognition. Our data cannot, however, exclude the possibility that origin recognition occurs only in conjunction with one or more cellular proteins present both in mammalian cells and in insect cells, but we regard this possibility as unlikely. Purification of the recombinant UL9 protein will be required to resolve this issue completely.

DNase I footprint analysis revealed that the UL9 protein interacts with ori_S at two related sites that are situated on the two arms of a nearly perfect palindrome. One of these sites (site I) corresponds closely to the 18-bp binding site of the ori_S-binding protein reported by Elias *et al.* (12). Recently, Elias and Lehman (I. R. Lehman, personal communication) have purified an ori_S-binding activity to homogeneity and have shown that it consists of a single polypeptide with an apparent molecular mass of 83 kDa; this protein also binds to both arms of the ori_S palindrome. As indicated above, the immunoassay experiments reported here show that anti-UL9 serum recognizes ori_S-binding activity partially purified from HSV-infected Vero cells according to the procedure of Elias *et al.* (12). These results all strongly suggest that the origin-binding activity observed by Elias *et al.* (12) is in fact the product of the UL9 gene.

Two lines of evidence suggest that the interaction between the UL9 protein and ori_S and/or ori_L plays a key role in HSV DNA replication. First, genetic experiments clearly indicate that the product of the UL9 gene is required for viral DNA replication. UL9 is one of seven viral genes necessary for origin-dependent plasmid replication using a transient transfection assay (6), and conditionally lethal HSV-1 mutants with lesions in the UL9 gene synthesize normal amounts of the known early gene products but fail to replicate DNA under restrictive conditions (20). Second, there is a correlation between the DNA sequences in ori_S that are necessary for origin function and the sites of UL9 binding, particularly at site II. D. Lockshon and D. Galloway (personal communication) have shown that the right-hand boundary of the minimal ori_S sequence is between nucleotides 74 and 77 in the ori_S sequence shown in Fig. 5. This corresponds closely to the right-hand boundary of UL9 binding at site II. The left-hand boundary of ori_S has been shown to be located between nucleotides 5 and 11 (D. Lockshon and D. Galloway, personal communication). This is well outside the left-hand boundary of UL9 binding at site I (nucleotide 23). It seems reasonable to assume that the sequences between nucleotide 23 and the left-hand boundary of the minimal origin sequence constitute the binding site for a protein, although there is no evidence to date concerning the identity of such a protein. It will be of considerable interest to extend the functional analysis of the origin to include a correlation with UL9 binding.

The role of UL9 binding in HSV DNA replication is not known. 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 complex capable of carrying out efficient semiconservative DNA synthesis. Alternatively, or additionally, UL9 may be involved in unwinding the two parental strands at the origin as a prelude to the initiation of daughter-strand synthesis. The ability to produce large amounts of apparently functional UL9 protein free from contamination by other HSV proteins may provide the means to answer some of the questions remaining concerning its function.

We thank Max Summers for baculovirus stocks, plasmid vectors, and detailed instructions on baculovirus technology; Kathy Coelingh for SF9 cells and useful hints on propagating insect cells; and I. R. Lehman and also Dan Lockshon and Denise Galloway for communicating their results prior to publication.

1. Spaete, R. R. & Frenkel, N. (1982) *Cell* **30,** 295–304.
2. Stow, N. D. (1982) *EMBO J.* **1,** 863–867.
3. Stow, N. D. & McMonagle, E. C. (1983) *Virology* **130,** 427–438.
4. Vlasny, D. A. & Frenkel, N. (1982) *Proc. Natl. Acad. Sci. USA* **78,** 742–746.
5. Weller, S. K., Spadaro, A., Schaffer, J. E., Murray, A. W., Maxam, A. M. & Schaffer, P. A. (1985) *Mol. Cell. Biol.* **5,** 930–942.
6. Wu, C. A., Nelson, N. J., McGeoch, D. J. & Challberg, M. D. (1988) *J. Virol.* **62,** 435–443.
7. Challberg, M. D. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9094–9098.
8. McGeoch, D. J., Dalrymple, M. A., Dolan, A., McNab, D., Perry, L. J., Taylor, P. & Challberg, M. D. (1988) *J. Virol.* **62,** 444–453.
9. Marsden, H. S., Campbell, M. E. M., Haarr, L., Frame, M. C., Parris, D. S., Murphy, M., Hope, R. G., Muller, M. T. & Preston, C. M. (1987) *J. Virol.* **61,** 2428–2437.
10. Polvino-Bodnar, M., Orberg, P. D. & Schaffer, P. A. (1987) *J. Virol.* **61,** 3528–3535.
11. Longnecker, R. & Roizman, B. (1986) *J. Virol.* **58,** 583–591.

12. Elias, P., O'Donnell, M. E., Mocarski, E. S. & Lehman, I. R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6322–6326.
13. McKay, R. D. G. (1981) *J. Mol. Biol.* **145,** 471–488.
14. Smith, G. E., Summers, M. D. & Fraser, M. J. (1983) *Mol. Cell. Biol.* **3,** 2156–2165.
15. Smith, G. E., Fraser, M. J. & Summers, M. D. (1983) *J. Virol.* **46,** 584–593.
16. Summers, M. D. & Smith, G. E. (1987) *Tex. Agric. Exp. Stn. Bull.* 1555.
17. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
18. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
19. Weir, J. P. & Moss, B. (1985) *J. Virol.* **56,** 534–540.
20. Carmichael, E. P., Kosovsky, M. J. & Weller, S. K. (1988) *J. Virol.* **62,** 91–99.
21. McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E. & Taylor, P. (1988) *J. Gen. Virol.* **69,** 1531–1574.