The herpes simplex virus type 1 origin-binding protein carries out origin specific DNA unwinding and forms unwound stem–loop structures

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The UL9 protein of herpes simplex virus type 1 (HSV-1) binds specifically to the HSV-1 ori_S and ori_L origins of replication, and is a DNA helicase and DNA-dependent NTPase. In this study electron microscopy was used to investigate the binding of UL9 protein to DNA fragments containing ori_S. In the absence of ATP, UL9 protein was observed to bind specifically to ori_S as a dimer or pair of dimers, which bent the DNA by 35° ± 15° and 86° ± 38° respectively, and the DNA was deduced to make a straight line path through the protein complex. In the presence of 4 mM ATP, binding at ori_S was enhanced 2-fold, DNA loops or stem–loops were extruded from the UL9 protein complex at ori_S, and the DNA in them frequently appeared highly condensed into a tight rod. The stem–loops contained from a few hundred to over one thousand base pairs of DNA and in most, ori_S was located at their apex, although in some, ori_S was at a border. The DNA in the stem–loops could be stabilized by photocrosslinking, and when *Escherichia coli* SSB protein was added to the incubations, it bound the stem–loops strongly. Thus the DNA strands in the stem–loops exist in a partially paired, partially single-stranded state presumably making them available for ICP8 binding *in vivo*. These observations provide direct evidence for an origin specific unwinding by the HSV-1 UL9 protein and for the formation of a relatively stable four-stranded DNA in this process.
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Introduction

The earliest event in the replication of an organism's DNA involves recognition of specific origin sequences by an origin-binding protein followed by an alteration in the conformation of the DNA. Structural alterations at an origin frequently involve opening the two strands to allow entry of helicases, primases and other proteins required for the initiation of DNA synthesis. In studies of the enzymes and mechanics of DNA replication in eukaryotic cells, herpes simplex virus type 1 (HSV-1) has provided a valuable model. The HSV-1 genome encodes seven proteins that are required for origin-dependent DNA replication. They consist of a DNA polymerase and its accessory protein, a heterotrimeric helicase–primase, a single-strand (ss) DNA-binding protein and an origin-binding protein, termed UL9 (Elias *et al.*, 1986; McGeoch *et al.*, 1988; Wu *et al.*, 1988; Challberg and Kelly, 1989; Crute *et al.*, 1989). Key to understanding the early events in HSV-1 replication as in other systems is learning how UL9 protein interacts with the HSV-1 origins of replication and how it may alter the structure of the DNA at the origin.

HSV-1 contains three functional origins of DNA replication. One, ori_L, is in the long unique segment of the genome while the other highly homologous origin, ori_S, is present twice in the repeat region flanking the short unique segment. ori_L and ori_S consist of a 45 bp palindrome containing a central AT-rich element flanked on each side by two high affinity UL9 protein-binding sites designated box I and box II (Stow and McMonagle, 1983; Weller *et al.*, 1985; Elias and Lehman, 1988; Lockshon and Galloway; 1988). A third weaker UL9-binding site, box III, is located adjacent to box I (see Figure 2). The presence of inverted repeat elements and an AT-rich segment is a common theme in replication origins.

The binding of UL9 protein to ori_S has been examined in detail. Biochemical studies have revealed that UL9 protein binds as a dimer to a pair of the inverted penta-nucleotide repeats (Weir and Stow, 1990; Koff *et al.*, 1991; Elias *et al.*, 1992; Fierer and Challberg, 1995), and exhibits 5- to 10-fold greater affinity for box I over box II, with binding to box III being weak but detectable (Elias and Lehman, 1988; Koff and Tegtmeyer, 1988; Olivo *et al.*, 1988; Weir *et al.*, 1989). Binding of UL9 protein to ori_S is cooperative and mediated by the N-terminal domain, since a truncated form of UL9 protein consisting of the C-terminal 317 amino acids retains origin-binding activity but shows no cooperativity in ori_S binding (Weir *et al.*, 1989; Deb and Deb, 1991; Elias *et al.*, 1990, 1992; Hazuda *et al.*, 1992). Changes in the AT-rich segment alter the cooperative binding of UL9 protein to boxes I and II (Gustafsson *et al.*, 1994) and these alterations may also reduce the replication efficiency of virus containing such changes (Lockshon and Galloway, 1988). The AT-rich spacer likely serves as a site for initiation of DNA unwinding and it has been observed that the binding of UL9 protein to negatively supertwisted DNA containing ori_S renders the spacer sequence sensitive to digestion with micrococcal nuclease and to oxidation by potassium permanganate (Koff *et al.*, 1991).

The way in which UL9 protein may alter the conformation of DNA at ori_S is likely tied to its activity as a DNA helicase and DNA-dependent NTPase (Bruckner *et al.*, 1991; Fierer and Challberg, 1992; Boehmer *et al.*, 1993; Dodson and Lehman, 1993). While UL9 protein exhibits
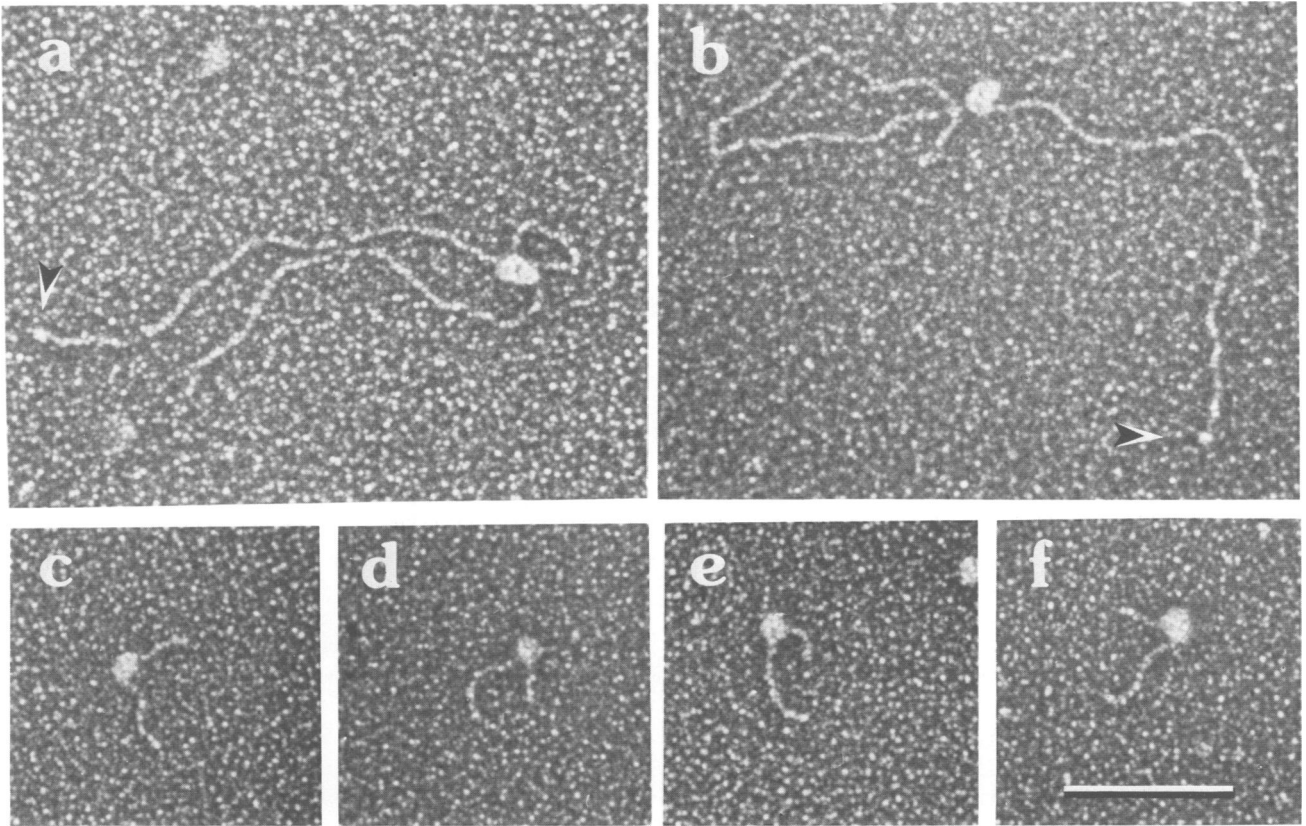
Fig. 1. Visualization of UL9 protein bound to the HSV-1 ori$_S$ replication origin in the absence of ATP. (**a** and **b**) UL9 protein (8 pmol) was incubated in the absence of ATP with a 2.6 kb DNA (1 pmol) containing ori$_S$ 40% from the ScaI end. The AvaII end was tagged with streptavidin (arrows). Incubation was for 15 min at 20°C after which the samples were fixed and prepared for EM by adsorbing to thin carbon supports, washing, air-drying and rotary shadowcasting with tungsten (Materials and methods). (**c–f**) UL9 protein was incubated as above using a 421 bp PvuII fragment containing ori$_S$ 40% from the nearest DNA end. Shown in reverse contrast. Bar represents 0.1 μm.

a weak helicase activity on non-origin-containing DNA, this activity is greatly stimulated by the HSV-1 ssDNA-binding protein, ICP8 (Fierer and Challberg, 1992; Boehmer et al., 1993).

Two of the earliest steps in the initiation of replication involve: (i) the recognition of the origin sequence and binding of multiple copies of a site-specific DNA-binding protein to generate an initiator complex and (ii) the binding of a helicase and its opening of the DNA to allow RNA priming and initiation of DNA polymerization. This sequential pathway has been clearly defined for replication of the Escherichia coli chromosome and for SV40 virus (Challberg and Kelly, 1989; Stillman, 1989; Kornberg and Baker, 1992).

Origin-binding proteins frequently induce a bending or looping of origin-containing DNA sequences when they assemble into an initiator complex (Ryder et al., 1986; Stenzel et al., 1987, 1991; Schnos et al., 1988, 1989; Bell and Stillman, 1992; Giraldo and Diaz, 1992; Mastrangelo et al., 1993). While some origin-binding proteins possess DNA-dependent ATPase and helicase activities and can unwind DNA at the origin (Dean and Hurwitz, 1991; Wessel et al., 1992), other DNA helicases involved in bidirectional DNA replication are unable to initiate DNA unwinding within an intact double-stranded (ds) DNA substrate. In the latter case, these helicases assemble onto a premelted region of DNA at the replication origin created by a corresponding initiator protein (Dodson et al., 1986,

1989; Sekimizu et al., 1987; Wyman et al., 1993). While UL9 protein has been shown to possess non-origin helicase activity and also binds to ori$_S$ sequences, little is known about its ability to specifically bind and unwind ori$_S$ in an ATP-dependent manner.

In this study we have used electron microscopy (EM) to analyze interactions of UL9 protein with ori$_S$ in the presence and absence of ATP. We found that UL9 protein unwinds DNA at ori$_S$ and forms looped or stem–looped structures containing four DNA strands. The DNA in the stem–loops could be photocrosslinked and also bound SSB protein, arguing that it was both unwound and partially base paired. These findings suggest a possible mechanism for the opening of the HSV-1 origin of DNA replication and initiation of the DNA synthesis.

Results

Multiple copies of UL9 protein bind ori$_S$ in the absence of ATP and bend the DNA

The plasmid pCG5 contains the minimal 79 bp ori$_S$ origin of HSV-1 replication, and the orientation of the insert was determined by sequencing (data not shown). Following cleavage of pCG5 DNA with AvaII and ScaI restriction endonucleases to yield a 2.6 kb fragment, the AvaII end was labeled with biotin-derivitized dCTP (Materials and methods). UL9 protein (8–24 pmol) was incubated with the DNA (0.5–1.0 pmol) for 20 min in binding buffer at
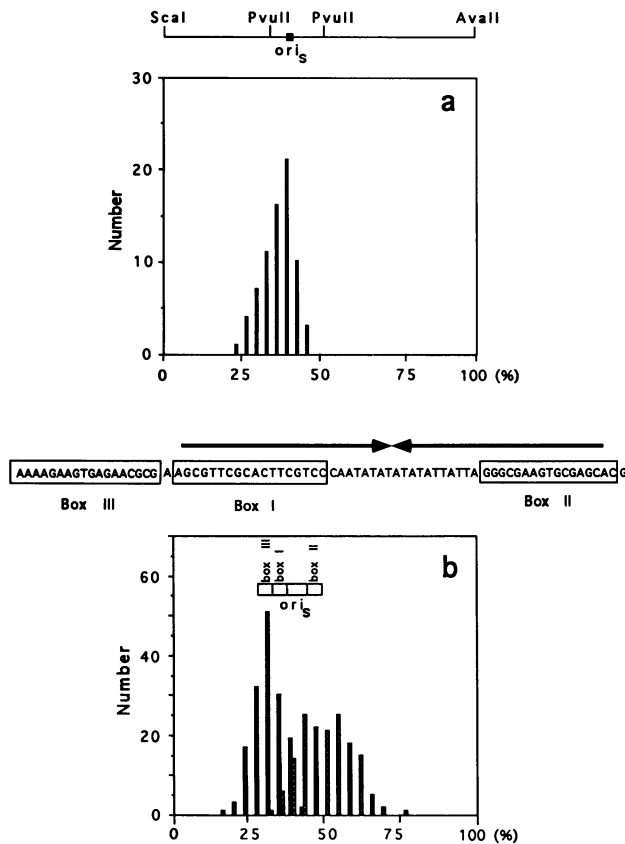
Fig. 2. Distribution of UL9 protein complexes bound to ori$_S$. (a) The position of UL9 protein complexes along the 2.6 kb DNA fragments labeled at the *Ava*II end (from the experiment described in Figure 1a and b) were measured from the *Sca*I end to the edge of the protein complex and are plotted as a fraction of the length of protein-free DNAs present in the same fields of molecules. (b) The position of UL9 protein complexes on the 421 bp fragments illustrated in Figure 1c–f was analyzed by measuring the short (solid bars) and long (hatched bars) arms from the DNA ends to the edges of the protein complex. The center of the protein complexes would be displaced to the right or left for the short and long arm measurements respectively.

Fig. 3. Distribution of UL9 particle sizes at ori$_S$ and their bending angles. (a) Diameter of the UL9 protein complexes on the 421 bp fragments. (b and c) The angle to which the 421 bp fragment was bent from the straight (0° = no bending) was determined for molecules such as those shown in Figure 1c–f where the diameter of the particles corresponded to peak 1 in (a) above (b) or peak 2 in (a) above (c). The angles were measured from micrographs by using the NIH IMAGE program and CCD camera coupled to a Macintosh computer.

20°C followed by addition of streptavidin (Life Technologies, Inc.) which bound to the biotin to uniquely tag one DNA end. The sample was then fixed with glutaraldehyde and prepared for EM (Materials and methods). Examination of fields of molecules revealed that ~10–15% of the DNAs contained a single large protein complex ~60% from the end labeled with streptavidin (Figure 1a and b). The protein complex was round or ellipsoidal and in some cases appeared to be composed of two subdomains.

From previous footprinting studies (Elias *et al.*, 1990; Hazuda *et al.*, 1992) it is known that UL9 protein binds preferentially to box I in ori$_S$ followed by box II, and much less well to box III (see Figure 2a). To localize the UL9 protein complexes on the DNA, the distance from the edge of the DNA–protein complex to the *Sca*I end was measured as well as the full length of protein-free DNA molecules. The latter value (average 0.76 μm) was taken as 100% and the position of the protein complexes from the *Sca*I end was plotted as a fraction of this value (Figure 2a) showing that the complexes were clustered about ori$_S$. A more precise measure of the directionality of UL9 protein binding over ori$_S$ was obtained by using
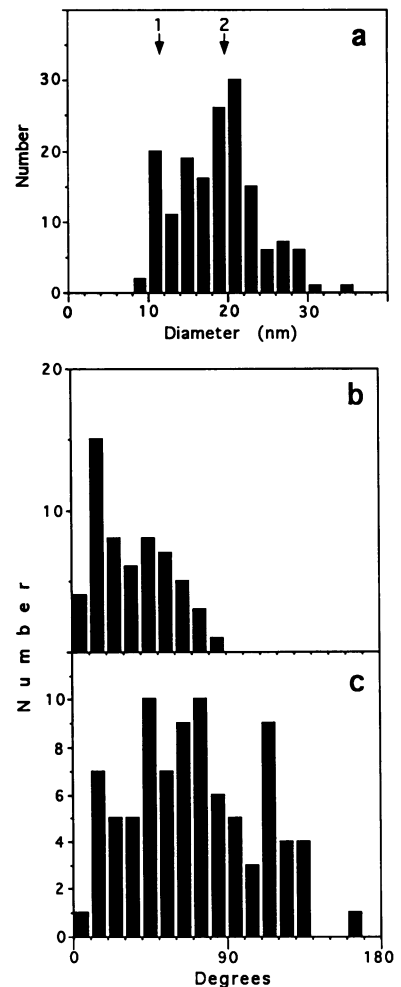
a 421 bp *Pvu*II fragment (Figure 2b). The fragment was incubated with UL9 protein as above and micrographs taken of fragments that were either protein-free or bound by UL9 protein (Figure 1c–f). ori$_S$ is located asymmetrically on the fragment and the lengths of the long and short arms from the edge of the protein complex to the DNA end were measured (Figure 2b). While the length distribution for the short arms was sharp, the distribution for the long arms was broader and possibly bimodal. Because measurement was made from the edge of the metal shadowcast particle, the position of the center of the protein complex would be to the right for the left arm measurement, and to the left for the right arm measurement. Taking this into account as well as the footprinting data, the distributions in Figure 2b suggest that UL9 protein binds first at box I and then proceeds in a cooperative manner rightwards into box II.

Measurement of the diameter of the shadowcast UL9 protein complexes on the DNA (Figure 3a) showed a heterodisperse distribution suggestive of a series of com-

plexes of increasing size bound at ori_S. There were at least two major peaks, one centered at 10–12 nm and the second at 20–22 nm. In another study employing negative staining (Makhov *et al.*, 1996) UL9 protein dimers were visualized bound to DNA and they appeared as ellipsoidal particles 12–13 nm long and 8–9 nm in width. Subtraction of ~1 nm from the values determined from shadowcasting

Table I. The influence of ATP on the formation the UL9 protein-ori_S complexes and the induction of stem–loop structures in the region of ori_S

	No. of counted molecules	No. with UL9 protein bound	No. with stem–loops
Control	876	135 (15%)	25 (3%)
5 min with ATP	550	149 (27%)	65 (12%)
10 min with ATP	597	140 (23%)	78 (13%)
15 min with ATP	292	87 (30%)	38 (13%)

to account for the amount of metal deposited suggests that the 10–12 nm particles are single dimers and the 20–22 nm particles are pairs of dimers. Thus the first peak would correspond to a dimer likely binding at box I and the second peak would reflect a pair of dimers bound over boxes I and II.

The length of the protein-free 421 bp DNA fragment (133 ± 9 nm; $n = 111$) was compared with the length of the DNA containing a 20–22 nm particle. For this comparison the two arms of each fragment were added to the straight line distance across the protein particle. This value (127 ± 10 nm; $n = 118$) argues that the DNA must make a nearly straight line trajectory through the protein and is not wrapped about its exterior as had been previously suggested (Rabkin and Hanlon, 1991).

Micrographs of UL9 protein particles bound to the 421 bp fragment showed marked bending of the DNA (Figure 1c–f). The bending angle was measured for the
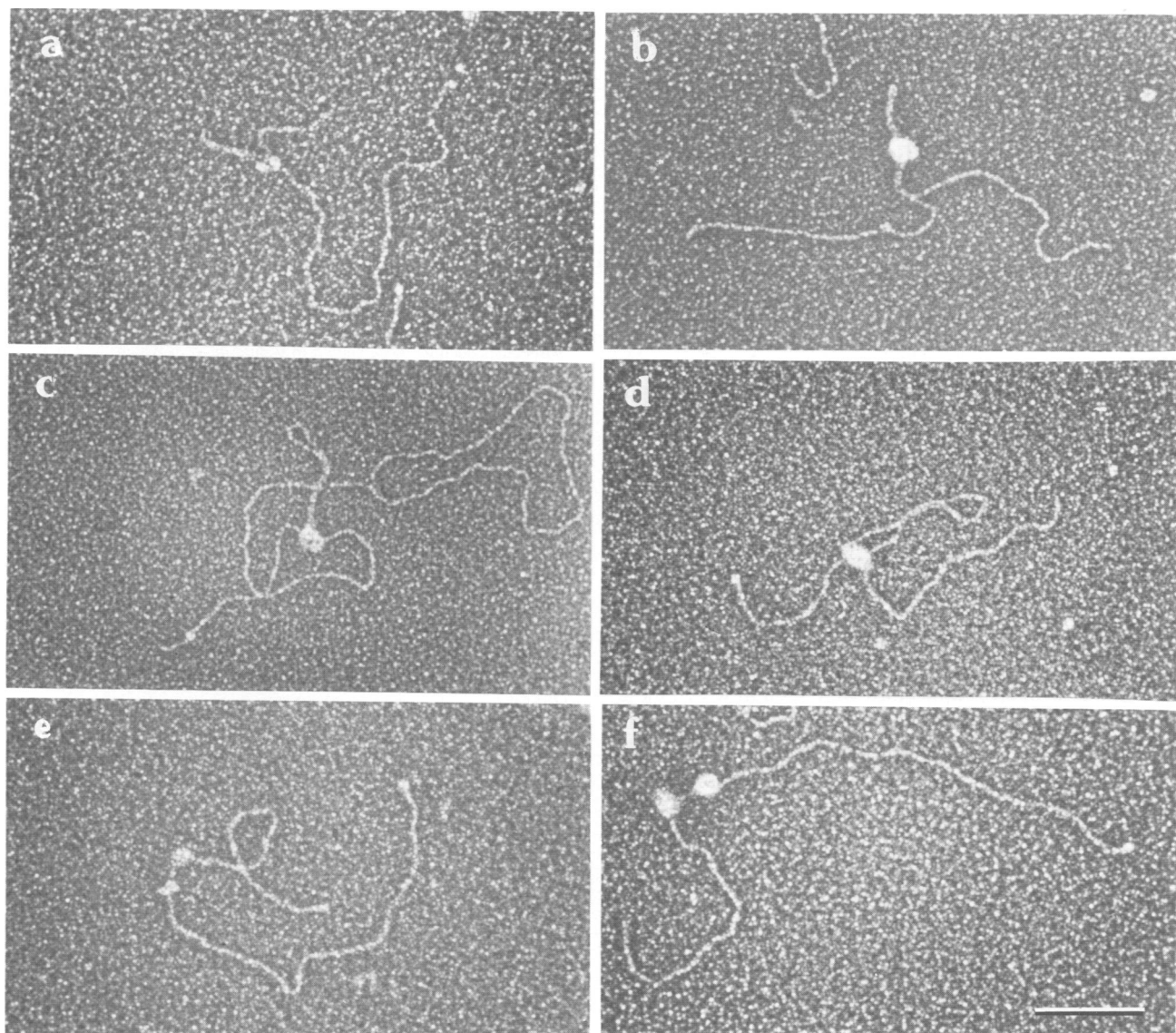

Fig. 4. Visualization of UL9 protein induced stem–loop structures formed at ori_S in the presence of ATP. UL9 protein (8 pmol) was incubated with the 2.6 kb ori_S-containing DNA (1 pmol) in binding buffer with 4 mM ATP for 15 min at 20°C (Materials and methods). The samples were then fixed and prepared for EM as described in Figure 1. Roughly 50% of the DNA containing a UL9 protein complex also showed a loop or stem–loop extruded from the UL9 protein complex (a–d), and less frequently two UL9 protein complexes separated by a short DNA segment (e and f). Shown in reverse contrast. Bar represents 0.1 μm.

10–12 nm particles (peak 1 in Figure 3a; Figure 3b) and the 20–22 nm particles (peak 2 in Figure 3a; Figure 3c). The average angle to which the DNA was bent from the straight (a value of 0° indicates no bending) for the 10–12 nm particles was 35° ± 15° ($n = 57$) and for the 20–22 nm particles was 86° ± 38° ($n = 86$). Thus, in the latter case $oris$ is bent by nearly 90° when UL9 protein binds presumably as a pair of dimers over $oris$.

In the presence of ATP, UL9 protein extrudes a four-stranded stem–loop structure centered about $oris$

UL9 protein is a helicase and DNA-dependent NTPase (Bruckner *et al.*, 1991; Fierer and Challberg, 1992; Boehmer *et al.*, 1993; Dodson and Lehman, 1993) and ATP or its non-hydrolyzable analogs have been found to promote UL9 protein binding to $oris$ (Gustafsson *et al.*, 1994). To examine the effect of ATP on binding, UL9 protein was incubated with the 2.6 kb $oris$ fragment as above for 5–15 min but with 4 mM ATP included, and the samples prepared for EM. Scoring fields of molecules, the fraction of DNA containing a UL9 protein complex over $oris$ increased from 10–15% with no ATP, to 23–30% with ATP (Table I). The most striking difference, however, was the appearance of DNA loops or stem–loops extruded from the UL9 protein complex in ~50% of the molecules with UL9 protein bound (Figure 4a–d; Table I). The DNA in the stem–loop structures frequently appeared highly condensed and rod-like (Figure 4a–c) suggesting that it was either tightly supertwisted about itself, or that the four DNA strands were held together by some form of cross pairing. Open loops at the site of the UL9 complexes on $oris$ were much less common in the samples without ATP (Table I), and highly condensed stem–loop structures were never seen in the absence of ATP. In some molecules (not shown) stem–loops were present but no protein could be detected at the base of the stem–loop structures, most likely having dissociated during the incubation or preparation for EM analysis. Since local supertwisting in a linear molecule would be lost immediately once the proteins pinching off a twisted domain were released, this observation argues against the DNA in the stems being supertwisted. Occasionally in the incubations with ATP, molecules were seen in which there were two UL9 complexes near $oris$ but which were separated by a short segment of DNA (Figure 4e and f). The average diameter of the UL9 complexes formed in the presence of ATP was 22 ± 5 nm ($n = 33$) indicating that it consists of a pair of UL9 dimers.

The region of the DNA in the 2.6 kb $oris$ fragment that was taken up within the UL9 protein-induced stem–loop structure was determined by using the end tagged DNA as in Figure 1 above. For 88 such molecules containing a stem–loop, the distance from each DNA end to the beginning of the stem–loop was measured and plotted (Figure 5). In some cases the stem–loop structures formed preferentially in one direction from $oris$, but in most, the DNA within the stem–loop was centered about $oris$. No cases were observed in which the entire 2.9 kb DNA appeared to have been extruded into a stem–loop nor were collapsed linear single strands seen in the background.
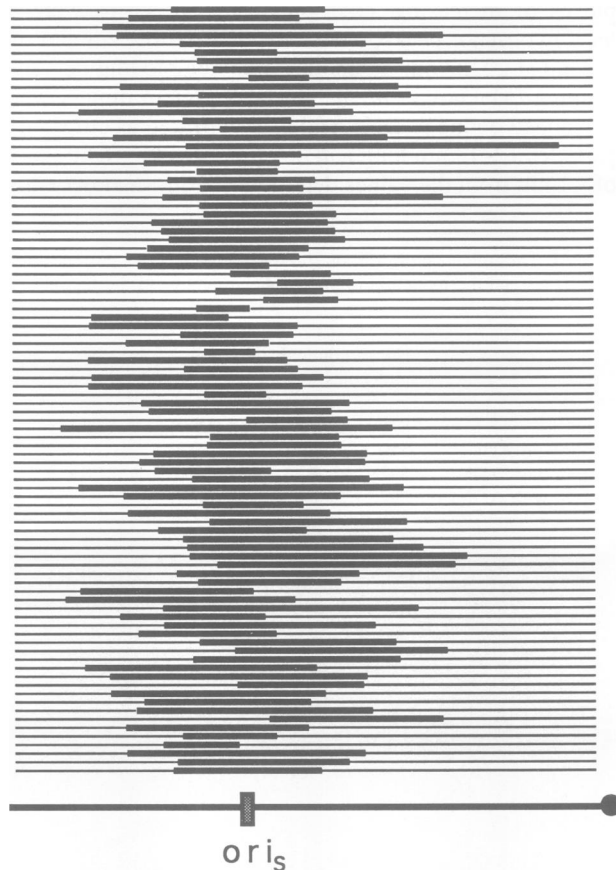

Fig. 5. Position of loops and stem–loops extruded by UL9 protein. Molecules such as those shown in Figure 4a–d in which the DNA was labeled at the *Ava*II end with streptavidin (see Figure 1) were analyzed by measuring the distance from each DNA end to the beginning of the loop or stem–loop. Heavy bar shows the position of the loop or stem–loop. Lengths were plotted relative to the average length of protein-free DNA fragments.

Photocrosslinking and binding by SSB confirm the presence of four unwound strands in the stem–loops

Photocrosslinking should provide a means of determining whether the condensed rod-like nature of the DNA in the stem–loops is due to supertwisting or pair-wise pairing among the four strands in the stem–loop. When supercoiled DNAs are treated with UV light in the presence of a psoralen, the two individual DNA duplexes are not crosslinked to each other. If, however, two or more single strands lie close together and are partially paired, they can be crosslinked. Thus inside the M13 phage filament, the ssDNA circle is stretched into a rod with the two strands lying in close proximity and sharing occasional pairing provided by random sequence alignments, and treatment of phage filaments with UV light and psoralen will crosslink the two strands (Griffith *et al.*, 1982). Here the 2.6 kb $oris$ fragment was incubated with UL9 protein for 15 min at 4°C. ATP was then added to 4 mM for 5 min more at 20°C and a psoralen derivative, HMT (4′-hydroxymethyl-4, 5′, 8-trimethylpsoralen), was added to 60 mM for 5 min on ice followed by UV irradiation for 20 min (Materials and methods). The samples were deproteinized and prepared for EM. Examination of fields of molecules (Figure 6a–d) revealed linear DNA molecules
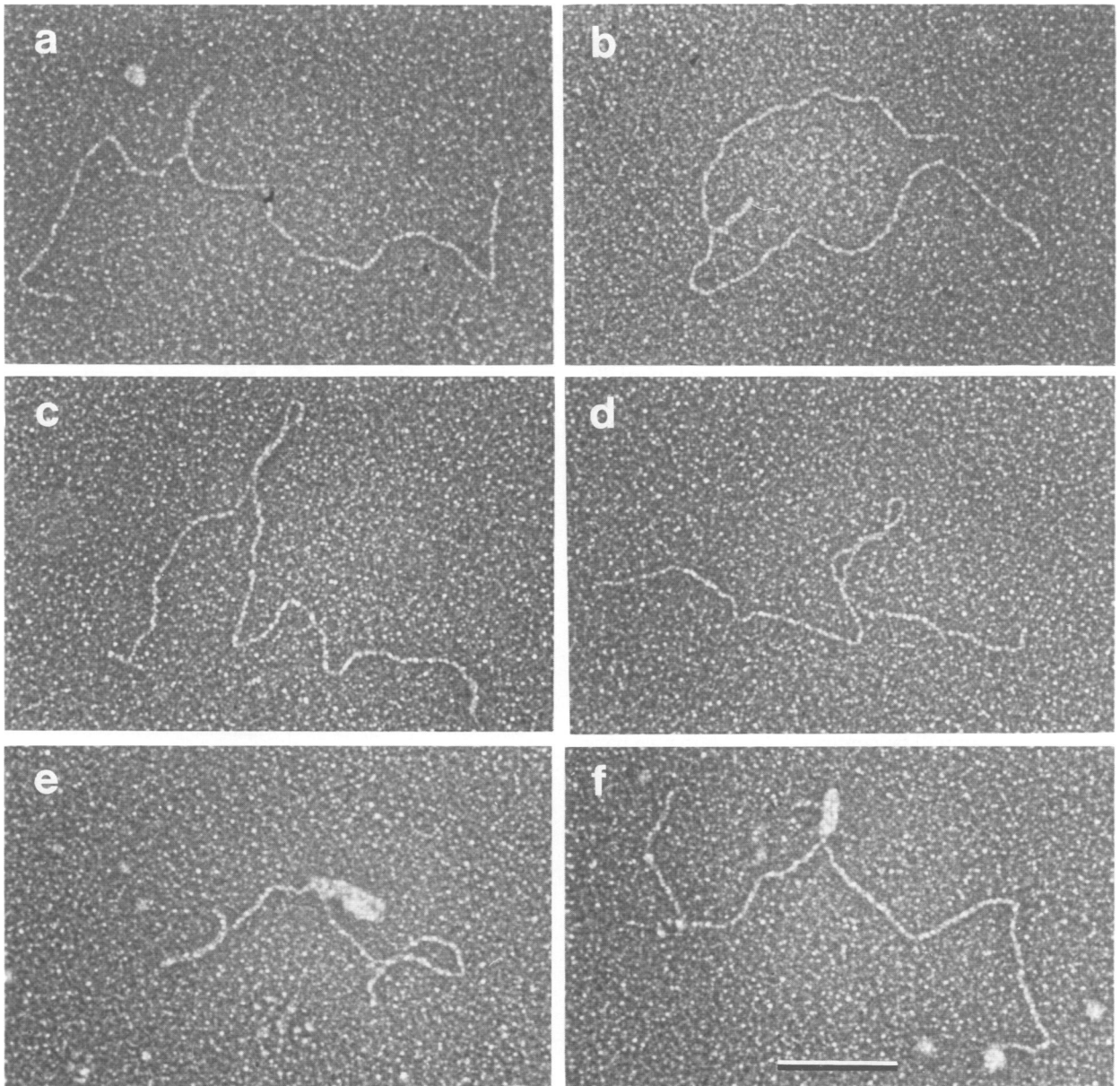
Fig. 6. Probing the stem–loop structures by photocrosslinking and SSB binding. (a–d) UL9 protein was incubated with the 2.6 kb DNA in the presence of ATP as described in Figure 4 for 5 min at 20°C followed by the addition of HMT, a psoralen derivative and irradiation with UV light. The samples were deproteinized and prepared for EM as described in Figure 1 (Materials and methods). (e and f) UL9 protein was incubated with the 2.6 kb DNA for 5 min in the presence of ATP at 20°C followed by the addition of *E.coli* SSB protein for an additional 5 min. Samples were then fixed and prepared for EM as in Figure 1. Shown in reverse contrast. Bar represents 0.1 μm.

with prominent loops and stem–loops like those observed in the presence of UL9 protein, but here no protein was present at the base of the stem–loops. Counting fields of molecules, 40% ($n = 446$) of the DNA contained loops, or stem–loops that included ori_S within them. Most of the structures were relatively open loops (Figure 6c and d) as contrasted to highly condensed stems (e.g. Figure 6a and b) but this may reflect the degree of photocrosslinking. No such structures were present when the DNA alone was treated with HMT and UV light (data not shown).

The photocrosslinking results suggest a model in which short stretches of homology are created at random, pairwise, between segments of two of the four strands in the stem–loop and that this pairing retains the four strands in a semi-stable condensed structure. The DNA, however,

could also contain many unpaired regions. To examine this possibility, *E.coli* SSB protein, a potent ssDNA-binding protein was used as an additional probe. The 2.6 kb fragment was first incubated with UL9 protein in the presence of ATP for 5 min at 20°C, and then SSB added to 3 μg/ml for an additional 5 min. The complexes were fixed and prepared for EM as in Figure 1. As shown (Figure 6c and d), thick SSB-covered stems were observed along the DNA at the same relative position as the stem–loops seen previously. Thus, from these cumulative observations, the stem–loop structures induced by UL9 protein must contain four strands in a partially paired, partially single-stranded state, presumably resulting from the extrusion and unwinding of the DNA by UL9 protein. A model of how this may occur is discussed below.

Discussion

In this study, EM has been used to investigate the binding of UL9 protein to DNA fragments containing ori_S. In the absence of ATP, UL9 protein bound specifically to ori_S as a dimer or pair of dimers, the latter bending the DNA by nearly 90°. The DNA appeared to take a relatively straight path through the protein complex. In the presence of 4 mM ATP, binding at ori_S was enhanced. Further, roughly half of the DNA–protein complexes exhibited DNA loops or highly condensed stem–loops of up to 1 kb size extruded from the UL9 protein complex. The four DNA strands in the stem–loops could be photocrosslinked together but also bound SSB protein avidly. Thus the four strands in the stem–loops must exist in a partially paired, partially single-stranded state, presumably resulting from the extrusion and unwinding of the DNA by UL9 protein. These observations provide direct evidence for an origin-specific unwinding by the HSV-1 UL9 protein and for the formation of a relatively stable four-stranded DNA.

In this study we employed the medium resolution rotary shadowcasting method. In another study (A.M.Makhov, P.E.Boehmer, I.R.Lehman and J.D.Griffith, submitted) we used higher resolution negative staining to examine the binding of UL9 protein to non-origin containing DNA, and dimers of UL9 protein were clearly resolved bound to DNA. Here, efforts to elucidate the structure of nucleoprotein complexes bound to ori_S by negative staining were not satisfactory but will be continued. In addition we have not described studies of the binding of the HSV-1 ssDNA-binding protein ICP8 to the stem–loop structures. While avid binding was observed, a variety of structures were formed that will require analysis beyond the scope of this study. It was not clear why extrusion of the stem–loops did not progress past ~1 kb of DNA, leading to full denaturation of the 2.9 kb DNA. Possibly longer incubations, the use of an ATP regenerating system, or inclusion of an SSB might have allowed further progress of the unwinding.

In an earlier EM study, Rabkin and Hanlon (1991) reported that UL9 protein specifically interacts with ori_S sequences and forms large nucleoprotein complexes frequently showing intermolecular interactions between DNA–protein complexes. We observed similar large nucleoprotein complexes at ori_S and intermolecular interactions. Our results are in disagreement with their suggestion of the DNA being greatly foreshortened since direct measurements indicated that the DNA takes a straight line path through the protein complex. This is in agreement with our negative staining results (Makhov et al., 1996) in which the path that the strands took through the UL9 protein dimers could occasionally be traced.

The EM studies are in good agreement with previous biochemical work on UL9 protein–ori_S interactions. EM analysis of UL9 protein binding to ori_S was suggestive of a cooperative binding that began at box I and moved into box II in agreement with the work of Elias et al. (1990) and Hasuda et al. (1992). Further, we noted a 2-fold higher binding to ori_S in the presence of ATP, in accord with the observations of Gustafsson et al. (1994). The EM results provide direct evidence for the suggestion of Koff et al. (1991) that UL9 protein binds to both box I and box II and that this interaction induces a bending of the
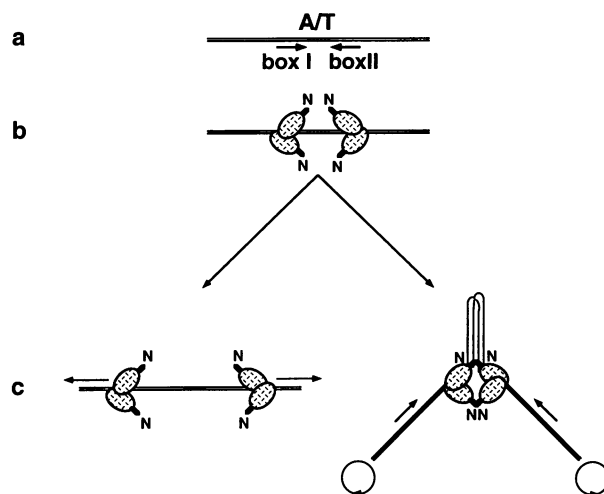

Fig. 7. Model of the unwinding of ori_S by a pair of UL9 protein dimers. (a) The arrangement of DNA sequence elements in HSV-1 ori_S involves two elements known to bind UL9 protein that face in opposite directions spaced by an AT-rich segment. (b) The binding of two UL9 protein dimers to ori_S will orient the dimers in opposite directions, shown here with their N-termini toward the inside. (c) Upon the addition of ATP, two possible outcomes are illustrated. On the left, the dimers have not associated with each other and are shown translocating in opposite directions. On the right, the two dimers have associated through their N-termini resulting in a bending of the DNA by ~90° about the AT-rich element followed by a translocation of the DNA rather than the UL9 protein dimers. This translocation reels the DNA in towards the protein complex which extrudes a partially unwound four-stranded structure.

intervening AT-rich sequence. Here we showed that when UL9 protein binds to both boxes as a pair of dimers, the DNA is bent by nearly 90°.

Our observations support a model of how UL9 protein generates stem–loop structures. As shown in Figure 7, a pair of UL9 protein dimers binds to boxes I and II, and with an orientation dictated by the DNA sequence of the boxes. This orientation arranges the dimers so that they face in opposite directions with their N-termini toward the inside. This, we suggest, would allow the left dimer to translocate leftwards on the DNA in the presence of ATP and the right dimer rightwards except for the fact that the two dimers are held together through their N-termini (Perry et al., 1993; Stow et al., 1993). Upon the addition of ATP, the net result is that the DNA is translocated rather than the UL9 protein dimers. This results in the DNA being reeled in toward the UL9 protein complex at ori_S and as the DNA passes through each dimer it is unwound and extruded upwards between the dimers. The four unwound strands would then undergo adventitious pairing to stabilize the stem–loop. The bending of the DNA about the AT-rich segment by the two dimers would initiate the kinking of the DNA and unwinding process.

This model is supported by numerous observations. First, we observed occasional pairs of UL9 protein dimers that appeared to have lost contact with each other and had begun moving apart. In our study of UL9 protein binding to non-origin DNA (A.M.Makhov, P.E.Boehmer, I.R.Lehman and J.D.Griffith, submitted) we were able to observe an ATP-dependent translocation of UL9 protein along duplex DNA. Moreover here the protein complex at the base of the stem–loop was found to have a size appropriate for

that of two dimers, and the ori$_S$ element was most often found at the apex of the stem–loop. When it was not, it was generally at a border as if the two dimers remained attached but one had not initiated translocation and unwinding. The origin-specific DNA unwinding induced by UL9 protein and T antigen (Wessel *et al.*, 1992) share strong similarities. In both cases, DNA unwinding is induced by a binary complex. The unwound DNA is extruded upwards between the dimers (UL9 protein) or double hexamers (T antigen) and in either case can interact with a single-strand DNA-binding protein.

In further studies it would be of interest to test this model by examining the interactions of UL9 protein with ori$_S$ constructs containing different orientations of boxes I and II, so that the two dimers might move along the DNA in the same direction or by using mutants of UL9 protein missing N terminal sequences in which case they might move away from each other, but in neither case forming stem–loops. While the four-stranded DNA formed in these reactions is of inherent interest, *in vivo* it is likely that ICP8 binds to the DNA strands as they are extruded from the UL9 protein complex operating at ori$_S$. Further studies will be required to determine the structure of such complexes and how ICP8 may enhance, alter, or regulate the activity of UL9 at the HSV-1 origins.

Materials and methods

Proteins and DNA
UL9 protein was purified from nuclear extracts prepared from Sf9 cells infected with a *Autographa californica* nuclear polyhedrosis virus recombinant for the UL9 gene as described (Boehmer and Lehman, 1993). *Escherichia coli* SSB protein was purified as described (Chase *et al.*, 1980). Restriction endonucleases were purchased from New England Biolabs.

Plasmid pCG5 (2.9 kb) was constructed by cloning a 79 bp DNA fragment containing minimal HSV-1 ori$_S$ into the *Bam*HI site of plasmid pTZ18R, thus destroying this site (Hernandez *et al.*, 1991). The orientation of the insert was determined by sequencing the region containing the insert and flanking sequences. A 421 bp fragment of pCG5 was generated by treatment of the DNA with *Pvu*II restriction endonuclease followed by electrophoresis on 1.0% agarose gels and isolation of the fragment from gel slices using mini-columns (Wizard, Promega). Restriction of pCG5 with *Sca*I and *Ava*II restriction endonucleases generated a 2.6 kb DNA fragment which was purified by electrophoresis on 1% agarose gels. The 2.6 kb DNA was labeled at the *Ava*II end using biotinylated deoxycytosine triphosphate (ENZO Diagnostics, Inc.) in a fill-in reaction with the large fragment of DNA polymerase I (New England Biolabs) as described (Bortner *et al.*, 1993).

UL9 protein–ori$_S$ DNA-binding reactions
DNA–UL9 protein incubations were carried out by diluting 0.5–1 pmol of DNA into 100 µl of binding buffer (50 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 0.1 mM EDTA, 0.5 mM DDT, 10% glycerol, 100 mM NaCl). UL9 protein (8, 16 or 24 pmol) was then added and incubation continued for 5–20 min at 20°C. For incubations with ATP, ATP was added to 4 mM following a 15 min incubation in its absence. The buffer described by Boehmer *et al.* (1993) containing 20 mM Tris–HCl, pH 7.5, 30 mM NaCl, 3.5 mM MgCl$_2$, 1 mM DDT and 10% glycerol (helicase buffer) was also utilized as indicated above.

Psoralen photocrosslinking
UL9 protein was incubated with the 2.6 kb fragment as described above for 15 min at 4°C. ATP was added to 4 mM for an additional 5 min followed by the addition of HMT (the gift of Dr J.Hearst, UC Berkeley, CA) to a concentration of 60 mM and the samples were incubated for 5 min at 0°C. The samples were then irradiated using a Spectronics Corp. ENF-240C UV lamp (365 nm) for 20 min at 0°C. The samples were deproteinized by adding proteinase K, EDTA and SDS to 100 µg/ml, 5 mM and 0.5% respectively, followed by incubation for 60 min at

55°C. Samples were then extracted with 1:1 mixture of phenol/chloroform and precipitated with ethanol.

Electron microscopy
Samples to be examined by EM were fixed with 0.6% glutaraldehyde for 10 min at 20°C and then chromatographed over Biogel A5m (Bio-Rad Lab. Inc.) equilibrated in 10 mM Tris–HCl pH 7.5, 1 mM EDTA. Peak fractions were mixed with spermidine hydrochloride to 2 mM and applied to thin glow discharged carbon foils, washed, dehydrated and rotary shadowcast with tungsten (Griffith and Christiansen, 1978). Micrographs were taken on a Philips CM12 electron microscope at 40 kV. Images recorded on sheet or 35 mm film were digitized using a CoHu CCD camera and the NIH IMAGE program was applied to adjust the contrast in the images, which were transferred to 35 mm film using a GCC film recorder. Contour lengths were measured using a Summagraphics digitizing tablet coupled to Macintosh computer programmed with software developed by J.D.G.

Acknowledgements

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