

Origin-specific unwinding of herpes simplex virus 1 DNA by the viral UL9 and ICP8 proteins: Visualization of a specific preunwinding complex

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Herpes simplex virus 1 contains three origins of replication; two copies of oriS and one of a similar sequence, oriL. Here, the combined action of multiple factors known or thought to influence the opening of oriS are examined. These include the viral origin-binding protein, UL9, and single-strand binding protein ICP8, host cell topoisomerase I, and superhelicity of the DNA template. By using electron microscopy, it was observed that when ICP8 and UL9 proteins were added together to oriS-containing super-twisted DNA, a discrete preunwinding complex was formed at oriS on 40% of the molecules, which was shown by double immunolabeling electron microscopy to contain both proteins. This complex was relatively stable to extreme dilution. Addition of ATP led to the efficient unwinding of ~50% of the DNA templates. Unwinding proceeded until the acquisition of a high level of positive super-twists in the remaining duplex DNA inhibited further unwinding. Addition of topoisomerase I allowed further unwinding, opening >1 kb of DNA around oriS.

The initiation of DNA replication for most genomes begins with the recognition of the origin by specific origin-binding proteins. Binding frequently is accompanied by a structural alteration in the DNA that promotes unwinding and entry of the proteins required to initiate DNA synthesis (1, 2). Some origin-binding proteins also exhibit helicase activity, which facilitates origin unwinding, and several form hexamers or double hexamers on the DNA, a structure typical of many helicases; examples include simian virus 40 T antigen (3–5) and the E1 protein of the papillomaviruses (6, 7). Herpes simplex virus 1 (HSV-1) UL9 protein provides origin recognition function but binds as a double dimer to the HSV-1 origins rather than as a hexamer (8–10).

HSV-1 provides an excellent system for study of these early replication steps in a mammalian system. The HSV-1 genome encodes seven proteins required for origin-dependent DNA replication consisting of a DNA polymerase and its accessory protein, a heterotrimeric helicase-primase, a single-stranded (ss) DNA-binding protein, ICP8, and the origin-binding protein, UL9 protein (11–16). HSV-1 contains three functional origins of DNA replication. One, oriL, is present in the long unique segment of the genome, whereas the other highly homologous origin, oriS, is present twice in the repeat region flanking the short unique segment (17–20). The minimal functional oriS sequence (79 bp) consists of a 45-bp inverted repeat containing a central A/T-rich element flanked on each side by two high-affinity UL9 protein-binding sites designated box I and box II. A third weaker UL9 protein-binding site, box III, is located adjacent to box I.

ICP8 is the major ssDNA-binding protein coded by the HSV-1 genome. ICP8 stimulates the helicase activity of UL9 protein (21, 22) and binds to its C-terminal domain (23). The ICP8–UL9 protein interaction is stable in the presence of double-stranded DNA but not ssDNA likely due to the strong affinity of ICP8 for ssDNA (9, 24).

UL9 protein (8, 14, 25) is a DNA-dependent NTPase and helicase (22, 26, 27). When UL9 protein and ICP8 are incubated with linear, nonorigin-containing DNA possessing a 3' ss tail, UL9

is loaded onto the tails and, in the presence of ATP, translocates into the duplex segment unwinding it and providing a template for ICP8 binding (22, 28). UL9 protein is a homodimer in solution, and several studies have shown that two dimers are bound to oriS containing all three binding boxes (9, 10). Electron microscopy (EM) studies from this laboratory revealed a particle with a mass consistent with a double dimer assembled at oriS (29).

Several observations suggest that the binding of UL9 to oriS induces a conformational change in the DNA. DNase I footprint analysis revealed a UL9-dependent structural change of the A/T-rich spacer particularly when the phase or orientation of box I and II were different from the wild-type arrangement (30–32). However, the ability of oriS-containing plasmids to replicate remained most efficient with the natural spacing (33). The conformation of the DNA at oriS may also influence the strength of UL9 interaction. OriS contains an inverted repeat that, if placed under superhelical strain or partially melted, could rearrange, extruding a stem-loop cruciform (34), and Aslani *et al.* (35) showed that UL9 protein exhibits a stronger binding to oriS if the DNA is in a hairpin-loop arrangement.

UL9 protein can loop and distort oriS when it is present in a super-twisted plasmid even in the absence of ATP (36). In our EM studies, using linear plasmid DNAs containing the minimal oriS segment, a double dimer was visualized bound at oriS, which bent the DNA $\approx 90^\circ$ (29). After addition of ATP, stem-loop structures encompassing many hundreds of nucleotides were extruded from the UL9 complex, and the amount of DNA in the stem-loops increased with incubation time. Unwinding was bidirectional, centering about oriS, and the DNA in the stem-loops appeared partially ss and partially duplex. Only $\approx 5\%$ or less of the DNAs underwent unwinding, suggesting that other factors may be required to initiate unwinding *in vivo*.

Recent work from one of our laboratories (I.R.L.) using 25- to 30-bp model DNAs revealed that ICP8, a helix-destabilizing protein, may be an active participant in unwinding oriS. In the presence of ICP8, UL9 protein will open a DNA-containing box I and a 3'-ssDNA tail located on the side oriented toward the A/T-rich spacer (37). It will also open oriS constructs in which the A/T-rich spacer is replaced by a ss bubble formed from two ss oligo(dT) segments located between boxes I and II (38) or wild-type oriS (39). Together, these experiments suggest a pathway of origin activation that involves a specific interaction between UL9 and ICP8 proteins, leading to a conformational change in the origin that facilitates more extensive unwinding.

In this study, we have built on the model DNA studies and our previous EM work to examine the concerted action of ICP8 and UL9 on an oriS-containing plasmid DNA in the natural context of the HSV-1 flanking sequences, superhelicity, and the presence of topoisomerase I. We show that a preunwinding complex

Abbreviations: HSV-1, herpes simplex virus 1; EM, electron microscopy; ss, single-stranded.

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containing both proteins can be directly visualized, assembled with high efficiency at oriS, and that these complexes lead to a highly efficient unwinding reaction.

Materials and Methods

DNA and Proteins. UL9 protein and ICP8 were purified from nuclear extracts prepared from Sf21 cells infected with an *Autographa californica* nuclear polyhedrosis virus recombinant for the UL9 and ICP8 genes (40). Restriction endonucleases were purchased from New England Biolabs. Plasmid pGEM822 was the gift of P. E. Boehmer (University of Miami School of Medicine, Miami) and is described in Wong and Schaffer (41). Orientation of the insert was determined by restriction endonuclease analysis of the insert and flanking sequences.

Preparation of DNA-Protein Complexes for EM. In a typical reaction, supertwisted pGEM822 DNA (450 ng) was incubated with ICP8 protein (1.0 or 0.5 μg) in 20 μl of a buffer containing 20 mM Hepes, pH 7.5, 30 mM NaCl, and 1 mM DTT for 1 h at 37°C. The reaction was stopped by adding glutaraldehyde to 0.6% for 10 min at 20°C. After fixation, the reaction mixture was chromatographed over 2 ml of BioGel A5m equilibrated with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. The fractions containing DNA-protein complexes were collected and prepared for EM (below), or 180 μl of those fractions was mixed with 20 μl of 10 \times concentrated *ScaI* buffer (New England Biolabs) and 1 μl of *ScaI* enzyme (10 units/ μl). The mixture was incubated for 1 h at 37°C and then rechromatographed over BioGel A5m and prepared for EM. Variations including UL9 protein and topoisomerase I are described in the text.

EM. DNA-protein complexes were prepared for EM as described (42). In brief, the samples were adsorbed to thin, glow-charged carbon foils in the presence of a buffer containing 2 mM spermidine, washed in water and water-ethanol mixtures, air dried, and rotary shadowcast with tungsten. The samples were examined in a Philips CM12 instrument. Contour lengths were determined from measurements of micrographs projected onto a Summagraphics digitizer coupled to a Macintosh computer programmed with software developed by J.D.G. Images on sheet film were scanned with a Nikon 4500 film scanner, and the images were arranged into figures by using PHOTOSHOP (Adobe Systems, Mountain View, CA).

Immuno-electron Microscopy. The supertwisted plasmid pGEM822 (0.13 pmol) was incubated with ICP8 and UL9 protein (13 pmol each) for 1 h at 37°C in a buffer containing 20 mM Hepes, pH 7.4, 30 mM NaCl, 4 mM MgCl₂, and 1 mM DTT. The reactions were stopped by adding glutaraldehyde to 0.6% for 10 min at 20°C, followed by chromatography over BioGel A5m as described above. Concentrated *ScaI* buffer (10 \times) was added to 177 μl of the fractions containing the DNA-protein complexes followed by 1 μl of *ScaI* (10 units/ μl) (New England Biolabs), 1 μl of rabbit anti-UL9 protein IgG (dilutions from 1:1,000 to 1:5,000), and 1 μl of monoclonal anti-ICP8 IgG (the gift of W. T. Ruyechan; dilutions from 1:1,000 to 1:5,000). The mixture was incubated for 1 h at 37°C and fixed with glutaraldehyde as described above. After incubation, the reaction mixture was chromatographed over BioGel A5m again. The fractions containing DNA-protein complexes were collected, and after adding NaCl to 60 mM and dithiothreitol to 1 mM, complexes were incubated with goat anti-rabbit IgG and goat anti-mouse IgG conjugated with 5- and 10-nm gold particles (Amersham Pharmacia), respectively, for 1.5 h at 37°C. The samples then were fixed with 0.6% glutaraldehyde, chromatographed over BioGel A5m, and prepared for EM (above).

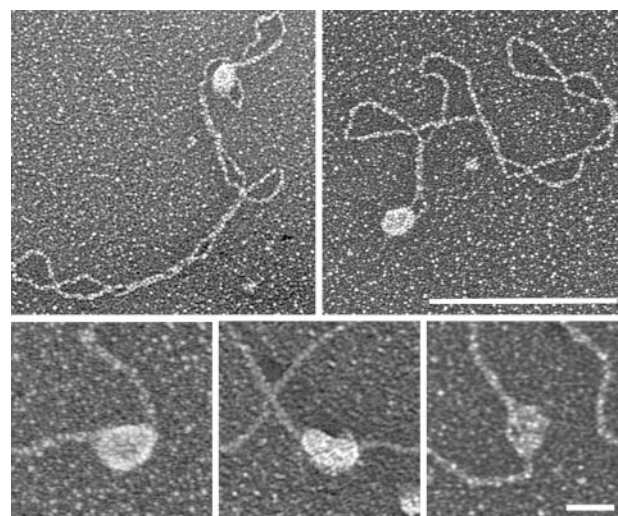


Fig. 1. Visualization of preunwinding complexes containing UL9 protein and ICP8 assembled on a supertwisted plasmid containing oriS. UL9 protein and ICP8 were incubated with a supertwisted plasmid containing oriS in the presence of magnesium. The samples were prepared for EM by fixing the proteins in place, removing free protein by gel filtration, adsorption to carbon foils, and rotary shadowcasting with tungsten. (Lower) Enlargements of individual preunwinding complexes (see *Materials and Methods*). Images are shown in reverse contrast. (Scale bars: Upper, 250 nm; Lower, 25 nm.)

Results

Assembly of a Preunwinding Complex of UL9 and ICP8 Proteins on Supercoiled DNA. To investigate the influence of negative superhelicity on the assembly of ICP8 and UL9 at oriS, these proteins were incubated with a supertwisted plasmid. pGEM822 includes an 822-bp HSV-1 *Bam*HI fragment containing oriS located 20–22% from the nearest end of the fragment and the G/C-rich HSV-1 segments to the left of the origin. The DNA (0.13 pmol) was incubated with UL9 and ICP8 proteins (13 pmol each) in binding buffer (20 mM Hepes, pH 7.5/30 mM NaCl/1 mM dithiothreitol/4 mM magnesium). The proteins were then fixed in place, the free protein removed by gel filtration, and the samples prepared for EM. Examination of the DNAs revealed supertwisted DNA with a single protein complex bound (Fig. 1). The majority of the protein complexes were similar in size. Scoring fields of DNAs revealed that 47% ($n = 212$) and 58% ($n = 292$) of the DNA had one protein complex (termed preunwinding complexes) bound after 15 and 60 min of incubation, respectively.

To localize the preunwinding complexes on the supertwisted DNA, the samples were fixed with glutaraldehyde, chromatographed over BioGel A5m, and cleaved with *ScaI*. EM analysis revealed that 68% ($n = 365$) were located at or near oriS, whereas 32% were bound elsewhere on the plasmid. The latter proteins likely represent the background of ICP8 binding to the supertwisted vector sequences. When the pGEM822 DNA was linearized with *ScaI* and then incubated with ICP8 and UL9 as described above for 30 min, only 14% of the DNA showed a protein complex bound. Visual inspection indicated that most of these complexes were located $\approx 40\%$ from the nearest end, consistent with binding at oriS. Thus, 58% (at 60 min) of the supertwisted DNAs showed protein complexes bound when ICP8 and UL9 were present, and of these, 68% were at oriS. Hence, 40% of the supercoiled DNA contained a specific complex at oriS. This is 3-fold greater than what was observed for linear DNA, arguing for an important role of negative superhelicity in assembly of the preunwinding complex.

Incubation of 0.13 pmol of supertwisted pGEM822 DNA with 13.0 pmol of UL9 in binding buffer resulted in 10% of the DNA

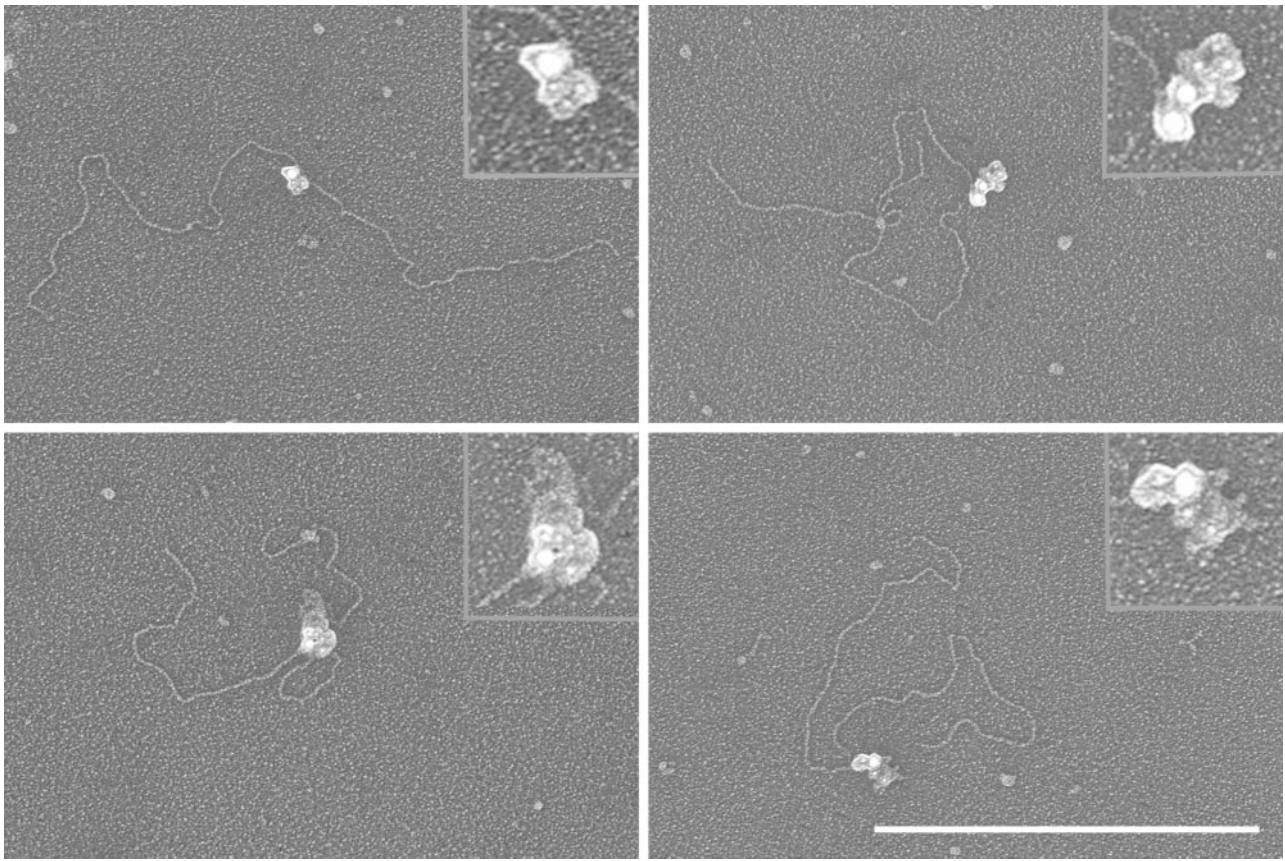


Fig. 2. Immunoelectron microscopic demonstration that the preunwinding complex contains both ICP8 and UL9 protein. Preunwinding complexes were prepared as described for Fig. 1 and then labeled with rabbit polyclonal IgG against UL9 protein and mouse monoclonal IgG against ICP8. This was followed with addition of goat anti-rabbit IgG and goat anti-mouse IgG conjugated with 5- and 10-nm gold particles, respectively (*Materials and Methods*). Hence, the large gold particles denote the presence of ICP8 and the small ones denote the presence of UL9. (*Insets*) Complexes at higher magnification. Images are shown in reverse contrast. (Scale bar, 500 nm.)

($n = 179$) showing protein particles bound. We previously observed (29) with linear oriS-containing DNA that in the presence of 4 mM magnesium, between 5% and 10% of the DNAs showed UL9 dimers assembled at oriS, and mapping suggested that most were located near oriS. When linearized pGEM822 DNA (0.13 pmol) was incubated with 13 pmol of ICP8 in binding buffer, <4% ($n = 284$) of the DNA had ICP8 bound, and there was no indication of preferential localization to oriS. Incubation of supertwisted pGEM822 DNA with ICP8 under these conditions resulted in $\approx 7\%$ of the DNA showing a protein particle bound. In both cases when only one protein was present in the incubation, the protein complex was smaller than the preunwinding complex, which we estimated to be ≈ 1.5 - to 2.5-fold larger in mass than the ICP8 or UL9 complexes.

The Preunwinding Complexes Contain Both UL9 and ICP8. To directly determine whether the preunwinding complexes contain both proteins, we used immunoelectron microscopy. Complexes were formed on supertwisted DNA and fixed, and the DNA was cleaved with *ScaI*. The samples were then reacted with polyclonal rabbit IgG prepared against UL9 protein and a monoclonal IgG prepared against ICP8. The samples were then further incubated with goat anti-rabbit and goat anti-mouse IgG conjugated with 5- and 10-nm gold particles, respectively, which can be distinguished one from the other by EM. In this way, a 5-nm gold particle bound at the complex would denote the presence of UL9 protein, whereas a 10-nm gold particle would indicate the presence of ICP8.

By EM, individual particles were scored as protein-free DNA, DNA with a single protein complex bound (labeled or not), and aggregates of two to seven DNAs held together by a large protein mass. The latter forms were the result of the multiple steps in the procedure. In this experiment, scoring 131 molecules, 22% consisted of single DNAs with a protein complex bound that was labeled with both gold particles (Fig. 2), whereas 12% and 8% consisted of single DNAs with a protein complex that was labeled with only a 5- or 10-nm gold particle, respectively. Of the nonaggregated DNAs containing a protein complex, 21% showed no labeling, 16% of the molecules consisted of protein-free DNA, and finally the remainder (21%) were scored as aggregates. The aggregates in most cases contained both 5- and 10-nm gold particles. As a control, the complexes were formed in the same way but without the primary antibodies. In this case, the level of labeling (nonspecific) was very low, not exceeding 2–3% of the total. Thus, the secondary antibodies do not have a significant nonspecific affinity for the UL9 protein–ICP8 complexes. Further, when one of the primary antibodies was excluded in the incubations, then labeling was observed only with one of the two gold particles, and that corresponded to the one in which the primary antibody was present.

These results provide direct evidence for the formation of a discrete preunwinding complex containing both ICP8 and UL9 protein at oriS. Formation of the preunwinding complex requires magnesium and is greatly stimulated by superhelicity of the DNA template. Studies of how this complex initiates origin unwinding after addition of ATP follow below.

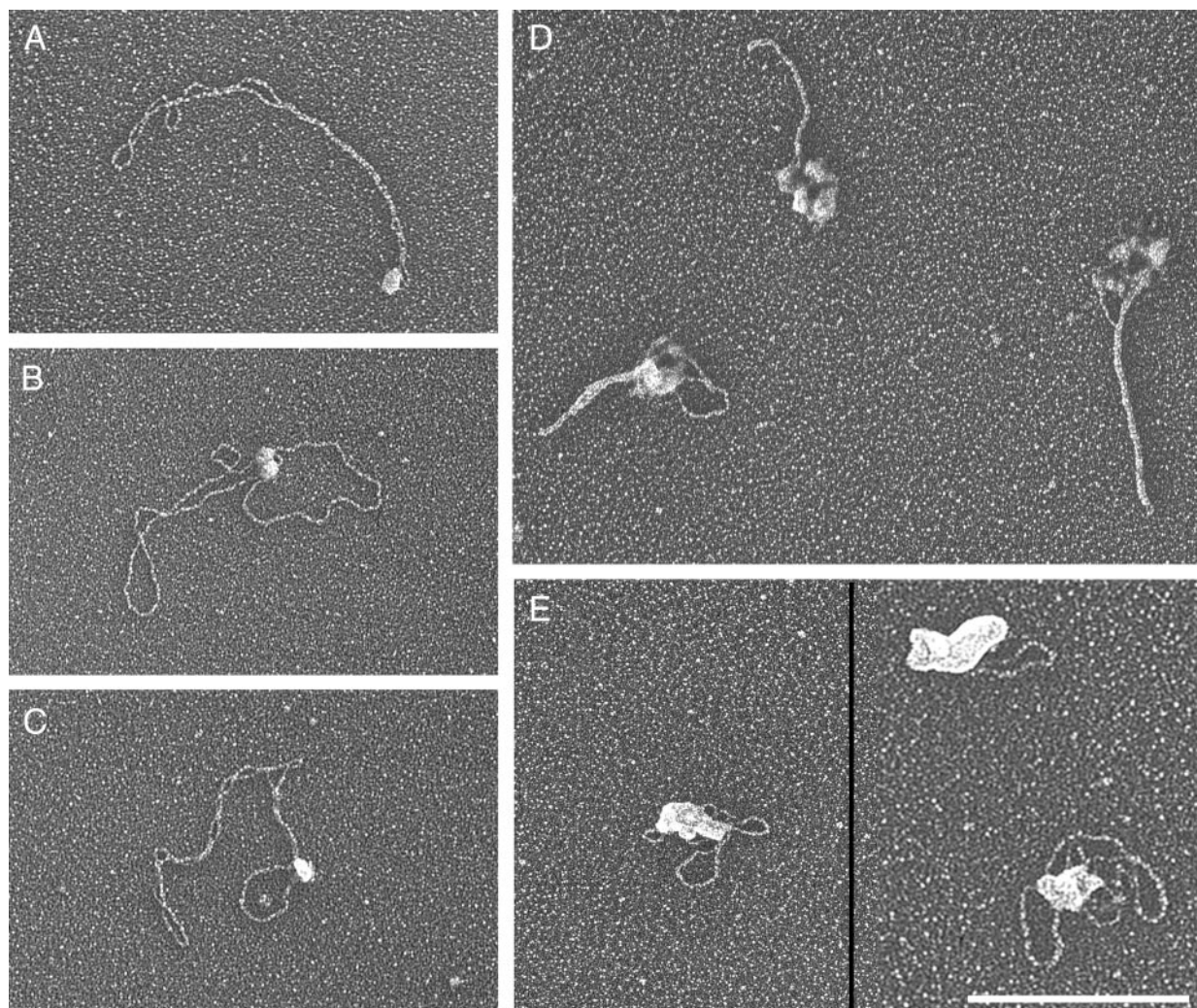


Fig. 3. Visualization of the unwinding of supercoiled DNA by UL9 protein and ICP8. Preunwinding complexes were assembled on supercoiled plasmid containing oriS for 15 min (see text) followed by the addition of 5 mM ATP for 2 min (A), 6 min (B), 10 min (C), and 30 min (D). (E) Incubation as described for D was followed by the addition of topoisomerase I for 30 min. The samples were prepared for EM as described for Fig. 1. Images are shown in reverse contrast. (Scale bar, 250 nm.) (F) In fields of molecules from an unwinding time course as described for A–D, the number of supercoils (strand crossovers) in each DNA containing an unwinding complex was scored by EM. The maximum that can be reliably counted by this method is 10–15 crossovers for this DNA.

UL9 and ICP8 Unwind oriS in an ATP-Dependent Manner. To examine the ability of the preunwinding complexes to initiate unwinding at oriS, supercoiled pGEM822 DNA (0.13 pmol) was incubated with UL9 protein and ICP8 (13.0 pmol each) in binding buffer for 15 min at 20°C followed by addition of 4 mM ATP for an

additional 1–60 min. After 2 min of incubation, 24% ($n = 476$) of the DNAs contained a typical preunwinding complex. The DNA appeared highly twisted, with >10 supercoils per DNA as determined by EM (Fig. 3 A and F). After 6 min of incubation, the majority of the DNAs containing a protein complex (28%,

$n = 507$) appeared topologically open with only a few twists remaining (Fig. 3 *B* and *F*). After 10 min of incubation, the number of supertwists in the DNAs that contained a protein complex had now increased to an average of 10 or more per DNA (Fig. 3 *C* and *F*), the complex on the DNA was distinctly larger than a single preunwinding complex, and $\approx 30\%$ ($n = 583$) of the total DNA molecules exhibited such a structure. Finally, prolonged incubation (30–60 min) led to the appearance of a structure (Fig. 3*D*) of a very tightly twisted DNA rod 150–250 nm long with a large DNA–protein bush at one end. In three independent experiments from 44% to 56% of the total input, DNA was present in these structures. As a control, pUC18 plasmid DNA (0.13 pmol) was incubated with 13.0 pmol of ICP8 and UL9 proteins for 20 min at 20°C in the absence of ATP followed by the addition of ATP for 60 min at 37°C. The samples were prepared for EM. Inspection revealed 12% ($n = 303$ molecules) with protein bound somewhere along the DNA and $<1\%$ in which some unwinding may have occurred; however, none of these resembled the structures shown in Fig. 3*D*.

To address the lifetime of the preunwinding complexes, supertwisted pGEM822 DNA was incubated for 20 min at 20°C with ICP8 and UL9 proteins in the absence of ATP. The sample was then chromatographed over a 2-ml column of BioGel A5m equilibrated in binding buffer, and the excluded fractions (DNA with bound protein but no free protein as verified by EM) were collected. The presence of active preunwinding complexes was assayed by adding ICP8 back to 13.0 pmol and ATP to 4 mM and incubating for 60 min at 37°C followed by chromatography to remove the excess ICP8 and preparation for EM. Analysis of 232 molecules showed that 108 (46%) had a protein complex bound somewhere along the supertwisted rod of the DNA and 38 (16%) appeared identical to the structures shown in Fig. 3*D*. This is to be compared with 40–50% of the DNA in the structures when both proteins were present throughout the incubation. Given the extremely low concentration of ICP8 and UL9 proteins after the first chromatographic step, we presume that the DNA which showed active unwinding resulted from preunwinding complexes that had not dissociated during the ≈ 10 -min chromatographic step or subsequent 60-min incubation in which only ICP8 was added back. This would argue that the preunwinding complexes are highly stable once formed.

The pattern of progressive relaxation of DNA supertwists followed by the acquisition of new twists follows the classic unwinding and rewinding observed when ethidium bromide is titrated with negatively supertwisted DNA or when recA protein binds and progressively unwinds supercoiled DNA (43). The rod-with-a-bush structures represent endpoints in which the unwinding has generated such a high degree of positive twisting in the protein-free segment of the DNA circle that further unwinding is inhibited. Hence, it would be expected that addition of topoisomerase I to these structures would allow unwinding to proceed further.

When incubations as described above were carried out for 10 min and then calf thymus topoisomerase I was added for 30–60 min, large DNA–protein complexes with a few associated DNA loops were observed (Fig. 3*E*). Approximately 35% ($n = 146$) of the total input DNA was in this form. In these complexes, the average length of the remaining protein-free duplex DNA measured only 50% of the full plasmid length, and in some complexes it was as small as 10%, suggesting that half or more of the DNA was present in the dense complex, apparently representing ssDNA bound predominantly by ICP8. These results suggest that in the presence of topoisomerase I, UL9 protein and ICP8 can facilitate extensive unwinding of the circular DNA.

Unwinding of Linear oriS DNA Fragments by UL9 Protein and ICP8. To determine whether superhelicity was absolutely required for the preunwinding complexes to initiate extensive unwinding, pGEM822 DNA was cleaved with *ScaI* to generate a linear DNA

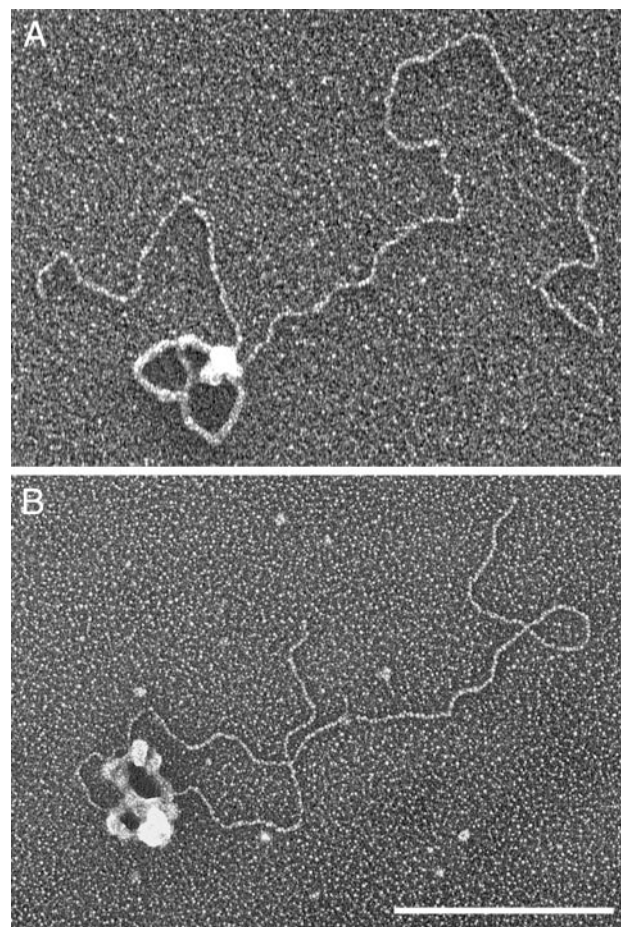


Fig. 4. Visualization of UL9 protein and ICP8 unwinding linear oriS-containing DNA. Linear pGEM822 DNA was incubated with UL9 protein and ICP8 in the presence of ATP for 15 min. The samples were then prepared for EM as described for Fig. 1. The two thick loops in *A* are typical of ssDNA bound by ICP8. The location of the large complexes is consistent with the location of oriS, which is $\approx 2,000$ bp from one end of the DNA and $\approx 2,700$ bp from the other. Images are shown in reverse contrast. (Scale bar, 100 nm.)

with oriS located 44% (2,140 bp) from the nearest end. This DNA (0.05 pmol) was incubated with UL9 protein (1.3 pmol) and ICP8 (11.8 pmol) for 15 min at 20°C followed by addition of 4 mM ATP for 10–60 min more. The samples were processed for EM as described above. Examination of the samples revealed that 15% of the total DNA ($n = 240$) had a large, compact protein complex assembled $\approx 40\%$ from the nearest end, corresponding to the location of oriS (Fig. 4). A few of the complexes contained DNA–protein loops; in the example shown (Fig. 4*A*), the loops appeared indistinguishable from filaments formed by incubation of purified ICP8 with ssDNA (24). In most cases, the ssDNA–ICP8 domains were condensed, likely a result of glutaraldehyde fixation. From this we conclude that superhelicity facilitates the assembly of preinitiation complexes by 2- to 3-fold, but once formed they are able to initiate extensive unwinding equally well on linear or supertwisted DNA.

Visual inspection of the complexes in Fig. 4 indicated that the amount of protein-free DNA was less in the complexes with the greatest apparent mass, suggesting that ssDNA was being generated and bound by ICP8 at the expense of the duplex segments flanking oriS. To quantify this, the length of protein-free DNA present in these complexes (the sum of the two arms exiting the large complex) was measured for complexes incubated with ATP for 15, 30, and 60 min. This length was subtracted from the total

length to provide an estimate of the amount of unwound DNA (based on the assumption that the DNA in the large complex is in an unwound ICP8-bound state). The greatest change occurred in the first 15 min, with the average amount of unwound DNA calculated to be 300, 450, and 600 bp at 15, 30, and 60 min, respectively. A few molecules, however, showed a degree of unwinding corresponding to at least 1,200 bp.

Discussion

We present a study of the activation of the HSV-1 oriS origin that extends previous studies from our two groups and others. First, although it had been suggested that UL9 and ICP8 proteins cooperate to unwind the DNA at oriS, there has been no evidence for a highly stable, discrete preunwinding complex assembled at oriS. Here, we present such data. We directly visualized this complex assembled at oriS and provide immunoelectron microscopic evidence that the complex contains both proteins bound simultaneously. Up to 50% of the supertwisted DNA was found to contain a preunwinding complex at oriS when the DNA was negatively supercoiled, 3-fold greater than when the DNA was linear. The preunwinding complex was relatively stable to extreme dilution, and when assembled on supertwisted DNA, nearly all lead to extensive unwinding of the plasmid DNA. The progress of unwinding was limited only by the build up of positive supercoiling, which could be relieved by addition of topoisomerase I leading to further unwinding. Whereas our previous EM study detected extensive unwinding of an oriS containing plasmid by UL9 alone, the efficiency was nearly 10-fold lower than what we have been able to achieve here.

The first stage of origin unwinding involves the assembly of a stable preunwinding complex at oriS. Assembly will occur on linear DNA, but at a 3-fold lower frequency than on negatively supercoiled DNA. One explanation is that negative supertwisting facilitates opening of the A/T-rich spacer in oriS, allowing the binding of ICP8, which then recruits UL9. Driven by the energy of negative supercoiling, oriS might adopt an extruded

cruciform configuration (35), a structure shown to favor the assembly of the preunwinding complex.

The recent work of Lee and Lehman (10, 37) and He and Lehman (38, 39) with model DNAs further supports the model in which supertwisting drives the melting of the A/T-rich spacer. In Lee and Lehman (10, 37), it was found that ICP8 would promote the unwinding of a short oriS-containing duplex if it either contained a ss tail or if the A/T-rich spacer was unpaired. In He and Lehman (39), the 18-bp A/T-rich spacer between boxes I and II was shown by hyperchromicity assays to be bound and opened by the addition of ICP8 and UL9 in an ATP-independent manner. Unwinding was optimal near 37°C and was not observed if the A/T spacer was replaced with a base-paired G/C-rich spacer. Furthermore, in our earlier EM study, we observed that the assembly of a pair of UL9 dimers at oriS bent the DNA by at least 90° (29), an event that would clearly be facilitated by melting of the A/T-rich spacer. Although it is not likely that HSV-1 DNA globally is under significant superhelical strain *in vivo* as it was in our *in vitro* studies, transcription through oriS could induce local negative superhelicity behind the moving transcription complex (44). Indeed, activation of oriS has been shown to be promoted by transcription factors (41, 45). Such local coiling could either induce transient cruciform or open the A/T-rich spacer allowing assembly of the preunwinding complex.

In vivo, numerous host and viral factors could promote a structural change in oriS that would lead to the formation of the preunwinding complex. UL9 protein interacts with ICP8 (23), UL8 protein, a component of the trimeric helicase/primase (46), and UL42 protein, the DNA polymerase accessory protein (47). The G/C-rich flanking sequences contain multiple binding sites for transcriptional factors, and their presence has been shown to stimulate replication significantly (41, 45).

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