Visualization of the Unwinding of Long DNA Chains by the Herpes Simplex Virus Type 1 UL9 Protein and ICP8

Alexander M. Makhov†, Paul E. Boehmer, I. Robert Lehman and Jack D. Griffith*

1Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295, USA
2Department of Biochemistry, Beckman Center B-400, Stanford University, Stanford, CA 94305-5307, USA

UL9 protein and ICP8 encoded by the herpes simplex virus type 1 (HSV-1) were shown to catalyze a highly active, non-origin-dependent unwinding of DNA. UL9 protein, the HSV-1 origin binding protein, as a modest helicase activity that is greatly stimulated by the HSV-1 single strand (ss) binding protein, ICP8. Here, electron microscopy has been applied to examine the mechanics of this reaction. Negative staining of the proteins revealed particles consisting primarily of ICP8 monomers and UL9 protein dimers. When the binding of UL9 protein to double strand (ds) DNA containing ss tails was examined by shadowcasting methods, UL9 protein was seen bound to the ss tails or ss/ds junctions; addition of ATP led to its appearance internally along the ds segment. When UL9 protein and ICP8 were incubated together with the tailed dsDNA in the presence of ATP, a highly ordered unwinding of the DNA was observed by negative staining that appeared to progress through four distinct stages: (1) binding of ICP8 to the ss tail and progressive coverage of the ds portion by UL9 protein; (2) formation of highly condensed regular filaments; (3) relaxation of the condensed structures into coiled-coils; and (4) unwinding of the coils and release of ICP8-covered linear ssDNAs. This process represents a mechanism of unwinding that is very different from ones that proceed by a progressive unwinding at Y-shaped forks that move along the DNA.

Introduction

Replication of the herpes simplex virus type-1 (HSV-1) genome involves seven HSV-1 gene products (Challberg & Kelly, 1989; Crute et al., 1990; McGeoch et al., 1988; Wu et al., 1988). Two of the HSV-1 specific factors include ICP8 and UL9 protein. ICP8 is one of the most abundant early HSV-1 proteins and may play multiple roles as a ssDNA binding protein (Bayliss et al., 1975), transcription factor (Gao & Knipe, 1991; Godowski & Knipe, 1986), and organizer of nuclear replication sites (Quinlan et al., 1984; De Bruyn Kops & Knipe, 1988), and it exhibits strand transfer as well as strand annealing and strand displacement activities (Boehmer & Lehman, 1993a; Bortner et al., 1993; Dutch & Lehman, 1993). The origin-binding protein UL9 protein (Elias et al., 1986; Elias & Lehman, 1988; Olivo et al., 1988) specifically interacts with sequences present in the HSV-1 origins of replication and shares general similarities to the simian virus 40 (SV40) T antigen and the *Escherichia coli* DnaA protein. UL9 protein has been shown to act as a helicase on DNA that does not contain the HSV-1 origins, catalyzing the unwinding of DNA in the 3' to 5' direction (Boehmer et al., 1993; Bruckner et al., 1991; Fierer & Challberg, 1992), and this activity is greatly stimulated by ICP8 (Boehmer et al., 1993; Fierer & Challberg, 1992). The mechanics of the interaction between the two proteins, however, is poorly understood.

The helicase activity of UL9 protein is dependent on nucleoside triphosphate hydrolysis (Boehmer...
et al., 1993). Indeed, UL9 protein contains an ATP binding motif, the mutation of which inhibits both the helicase activity and its ability to stimulate origin-dependent replication (Stow et al., 1993). UL9 protein also contains several motifs characteristic of a superfamily of RNA and DNA helicases (Gorbalenya et al., 1988), and mutations within five of the six conserved motifs abolish the ability of the UL9 protein to catalyze viral DNA synthesis (Martinez et al., 1992). UL9 protein alone will unwind only short DNAs with a limit of ~200 bp, and this unwinding appears to be stoichiometric, requiring the assembly of a multimeric UL9 protein complex. The helicase activity, however, can be stimulated by ICP8 (but not other single strand binding (SSB) proteins), increasing both the rate and extent of unwinding (Boehmer et al., 1993; Fierer & Challberg, 1992). Under optimal conditions with both proteins, DNAs of at least 2 kb can be unwound (Boehmer et al., 1993) and addition of ICP8 decreases the amount of UL9 protein required for unwinding (Boehmer et al., 1993). Indeed, the stimulation of UL9 protein by ICP8 is not unique, as numerous helicases are stimulated specifically or non-specifically by SSBs (Poll et al., 1994; Seo & Hurwitz, 1993; Smith et al., 1989; Umezuz & Nakayama, 1993; Yancy-Wrona et al., 1992).

Further studies revealed that the two proteins interact in solution to form a stable co-complex (Boehmer & Lehman, 1993b). The 27 amino acid residues at the C terminus of UL9 protein are required for ICP8 binding (Boehmer et al., 1994), and deletion of these 27 amino acid residues generated a protein whose helicase activity was eightfold greater than that of the wild-type protein but which had a diminished ability to replicate origin-containing plasmids in vivo. These observations argue that ICP8 binds to and modulates the activity of UL9 protein. During the HSV-1 replication process it is thus likely that ICP8 and UL9 protein interact with each other, one function of which must be to unwind the HSV-1 origins of replication. Understanding how this occurs is important not only for a detailed understanding of HSV-1 replication but possibly for other reactions in which these proteins may function, such as general recombination. To this end, electron microscopic (EM) studies of these complexes should provide valuable information.

EM studies of UL9 protein and ICP8 individually have been reported. Ruyechan and colleagues (Ruyechan, 1983; Ruyechan & Weir, 1984) used negative staining methods and reported that ICP8 binds rapidly and cooperatively to ssDNA to form regular DNA–protein filaments. Bortner et al. (1993) also observed condensed ICP8–ssDNA filaments by shadowcasting methods, and O’Donnell et al. (1987) noted that at low temperature in the absence of DNA, ICP8 forms highly regular helical protein filaments. UL9 protein was examined in one EM study (Rabkin & Hanlon, 1991), and was seen to bind to HSV-1 origin-containing DNA as compact protein balls.

In the study reported here, negative staining and shadowcasting methods were used to examine ICP8 and UL9 protein alone and bound to DNA lacking HSV-1 origins. ICP8 monomers and UL9 protein dimers were detected by negative staining, and an ATP-dependent appearance of UL9 protein internally along duplex DNA containing ssDNA tails was observed. The unwinding of long DNA segments by UL9 protein and ICP8 was initiated by the binding of ICP8 to the tails and appearance of UL9 protein internally on the dsDNA. Intermediate nucleoprotein complexes in the form of highly condensed coils and coiled-coils were observed in the process of the DNA unwinding. The unwinding process described here differs greatly from mechanisms in which a Y-shaped fork is generated at the ends of the DNA followed by a progressive movement of the fork along the DNA (Wessel et al., 1990a,b, 1992).

Results

Visualization of UL9 protein and ICP8, and ICP8 rings formed on short ssDNA by negative staining

Negative staining provides a classic means of visualizing individual proteins and their macromolecular complexes without exposing them to chemical fixation. In separate experiments, ICP8 and UL9 protein were diluted to 20 μg/ml in 20 mM Hepes (pH 7.5), 40 mM NaCl buffer, applied to thin glow-treated carbon supports, and stained with 2% (w/v) aqueous uranyl acetate. ICP8 is a large protein (128 kDa), and visualization of ICP8 molecules revealed distinct, relatively uniform round or ellipsoid globular particles (Figure 1A). In some molecules, an acentric 1 to 2 nm hole could be seen (arrows, Figure 1A). While the particles had a relatively similar size (9 to 10 nm diameter), their shape was irregular. This may reflect a complex three-dimensional shape of ICP8 and the imaging of different projections on the EM support. It could also be due to multiple conformations of ICP8, or an inherent flexibility in the molecule combined with distortions in the native shape due to the EM preparation.

Evaluation of the molecular mass of the ICP8 particles was carried out using streptavidin (66 kDa) as an internal standard (Griffith et al., 1995). Measurement of the projected areas of 30 ICP8 particles and comparison with the projected areas of streptavidin monomers, followed by conversion to mass provided a value of 170(±30) kDa, suggesting that the particles represent ICP8 monomers.

The molecular mass of UL9 protein is 92 kDa. Visualization of UL9 protein revealed a strong tendency for it to aggregate; however, individual molecules were present (Figure 1B), and the predominant species consisted of ellipsoidal particles measuring 12 to 13 nm along the long axis and 8 to 9 nm across the width. These particles were slightly larger than the ICP8 monomers (Figure 1A),
suggesting that they might be dimers. Estimation of their molecular mass using streptavidin as an internal size marker provided a value of 220(±30) kDa ($n = 30$) supporting this conclusion. Here as with ICP8, the mass estimate appeared to be on the high side, suggesting that extrapolation based on a significantly smaller standard (streptavidin) is limited but, for this purpose nonetheless argues clearly for ICP8 monomers and UL9 protein dimers as major species.

Observations of ICP8–ssDNA filaments indicated that the filaments tend to coil on themselves. When flattened on the EM support, this resulted in a sinusoidal curving of the filaments when long ssDNA was used. This suggested that if the ssDNA was short, it might close on itself to form a ring, and

![Figure 1](image)

*Figure 1.* Visualization of ICP8 and UL9 protein by negative staining. ICP8 (A) and UL9 protein (B) were diluted to a concentration of 20 µg/ml in buffer containing of 20 mM Hepes (pH 7.5), 40 mM NaCl and applied to thin glow-discharged carbon films. The samples were negatively stained with 2% aqueous uranyl acetate. Arrows in A indicate 1 to 2 nm holes in the ICP8 particles. The bar represents 20 nm.
Helicase Action of HSV-1 UL9 Protein and ICP8

Figure 2. ICP8 rings formed on a 158 nucleotide ssDNA fragment. A 158 bp HaeIII fragment of M13 dsDNA purified on 1% (w/v) agarose gels was denatured by boiling and chilling on ice. ICP8 (40 μg/ml) was mixed with the denatured DNA (0.2 μg/ml) for ten minutes at 20°C followed by negative staining with 2% uranyl acetate as for Figure 1. The bar represents 30 nm.

The work described below indicated that this might occur every ~150 nucleotides. To test this, a 158 bp restriction fragment was thermally denatured and quick-cooled to generate 158 nucleotide ssDNA fragments. ICP8 was incubated with the DNA (see details in the legend to Figure 2) and the complexes negatively stained with 2% uranyl acetate. As shown in Figure 2, closed rings 26 nm in diameter and containing nine to ten ICP8 monomers were observed.

UL9 protein binds to ss tails on dsDNA and appears internally in the presence of ATP

Most helicases initiate the unwinding of duplex DNA from a single strand region that serves as a high affinity binding site facilitating the formation of an initiation complex (reviewed by Lohman & Bjornson, 1996). To examine the binding of UL9 protein to a dsDNA containing ss tails, a ~3 kb linear DNA with 150 to 200 nucleotide 3’ extended ss tails was prepared (see Materials and Methods).

The 3’-tailed DNA was incubated with UL9 protein (12:1 protein/DNA molar ratio) for 15 minutes at 37°C in binding buffer with 10% (v/v) glycerol (see Materials and Methods). Aliquots were removed from the mixture, fixed with 0.6% (v/v) glutaraldehyde, and chromatographed through Biogel A5M to remove unbound protein and fixatives. The DNA–protein complexes were then visualized by rotary shadowcasting (see Materials and Methods). When Mg2+ was deleted from the binding buffer, 22% of the DNA molecules (n = 332) examined had a protein ball bound at one or both ends, and it appeared that the protein was localized to the junction of the ss and ds segments. When Mg2+ was included, 52% of the DNA molecules examined (n = 286) had a protein complex at one or both ends and frequently the protein complex appeared larger in size than in the absence of Mg2+ (Figure 3a and b). Little or no protein was seen bound internally along the duplex portion of the DNA. The inclusion of Mg2+ also resulted in a significant fraction (13%, n = 286) of the DNA being sequestered into aggregates of two or more molecules, or being arranged into monomer length circles (24%, n = 286) in which the ends of the DNA were held together by the protein complex(es) at the end (data not shown).
EM was used to examine the influence of ATP on the distribution of UL9 protein along the DNA. Here, limiting UL9 protein was employed (1:1 DNA/protein molar ratio). Following a ten minute incubation at 20°C as described above, nearly all of the UL9 protein was DNA bound since ~50% of the DNA ends had UL9 protein attached and few if any protein particles were seen internally in the duplex region. When ATP was added to 4 mM and the incubation continued for an additional five minutes, in 60 to 70% of the DNA with UL9 protein bound, the UL9 particles were present internally along the duplex portion (Figure 3c and d) and this appeared to occur at the expense of protein bound to the DNA ends. When blunt-ended duplex DNA was incubated as above with UL9 protein and 4 mM ATP no binding internally along the DNA was seen unless 10 to 20-fold higher concentrations of UL9 protein were used (data not shown). No evidence of extended ss bubbles induced by the translocation of UL9 protein was observed, suggesting that the protein induces only transient unwinding of DNA under these conditions.

ICP8 and UL9 protein cooperate to generate large nucleoprotein structures that catalyze DNA unwinding

It was shown (Boehmer et al., 1993) that ICP8 and UL9 protein interact with each other to efficiently unwind DNAs of at least 2 kb in an ATP-dependent manner. For this study, helicase reactions were carried out as described (Boehmer et al., 1993; see Materials and Methods) using the 3'-tailed DNA. ICP8 and UL9 protein (1:1 protein/DNA molar ratio) were incubated with the DNA in binding buffer for ten minutes at 37°C. ATP was then added to 4 mM and incubation continued. Aliquots were removed over an additional one to 30 minutes and the samples prepared for EM by negative staining. This procedure provided a non-selective way of following these reactions since no chromatography was involved that might have excluded certain complexes.

Examination of fields of stained complexes over a course of incubation from one to 30 minutes revealed four distinct species termed complexes I through IV. Complex I molecules consisted of the 3 kb 3'-tailed DNA in which the ssDNA tails were complexed with protein, but no protein particles were present internally along the duplex segments (not shown). The protein-covered tails were very similar in appearance to the ICP8–ssDNA filaments first described by Ruyechan & Weir (1984). Over 90% and 70% of the complexes examined in the first one and five minutes of incubation, respectively, consisted of complex I molecules (Table 1). Complex II molecules (Figure 4A to C) appeared similar to complex I molecules but also contained protein particles alone and in short clusters along the

Figure 3. Visualization of UL9 protein on duplex DNA. a and b, UL9 protein was incubated with a ~3 kb dsDNA containing 150 to 200 nucleotide 3'-terminated single strand tails in a buffer containing Mg²⁺ and then prepared for EM by adsorption to thin carbon supports, washing, air-drying, and rotary shadowcasting with tungsten (see Materials and Methods). Short single-stranded stubs are seen in a, suggesting that UL9 protein binds to the ss/ds junctions. c and d, Following incubation as described above but with limiting UL9 protein (see the text), ATP was added to 4 mM for five minutes and the samples prepared for EM as for a and b. Shown in reverse contrast. The bar represents 100 nm.
Helicase Action of HSV-1 UL9 Protein and ICP8

Table 1. Temporal appearance of structures involved in the unwinding of DNA by UL9 protein and ICP8

<table>
<thead>
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<th>Time (minutes)</th>
<th>1</th>
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<th>10</th>
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<td>Form I (%)</td>
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<tr>
<td>Form II (%)</td>
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<tr>
<td>Form IV (%)</td>
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<td>1</td>
<td>9</td>
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* DNA unwinding reactions employing UL9 protein and ICP8 were carried out as described for Figures 3 and 4 and in the text. In fields of molecules 100 to 150 complexes were scored for each incubation time.

duplex DNA. Complex II molecules were present after five minute incubations and persisted through 30 minutes, being most abundant at ten and 20 minutes (Table 1). The average diameter of the internally bound protein particles (which frequently appeared dimeric; arrows, Figure 4A) was 12 nm, indicative of their being UL9 protein dimers. In some cases unwound ssDNA chains could be visualized (arrows, Figure 4).

Complex III molecules consisted of compact DNA–protein coils or coiled-coils (Figure 5A to C). These structures were formed by the coiling of a regular DNA–protein filament and no protein-free regions of DNA were visible. The filaments appeared coiled in a right-handed manner as determined from shadowcasting (data not shown). Such structures were most abundant after 20 minute incubations (Table 1). Frequently (Figure 5B and C), they had extended filamentous tails that appeared identical to the presumptive ICP8-covered ssDNA tails in the complex II species. Some of the complex III molecules were very compact (Figure 5A) and may represent earlier stages along the unwinding pathway. These compact forms had an average diameter of 24 nm and a right-handed helical pitch of 14 to 15 nm. The average angle between the long axis and DNA–protein chain was %4(4 ° and there were 20 to 25 helical turns or about 120 to 150 bp of dsDNA or 120 to 150 nucleotides of ssDNA per turn. Other complex III species (Figure 5B and C) were more open and appeared as coiled-coils created by two DNA–protein filaments intertwined about each other. The individual filaments of the coiled-coils were similar in appearance to the ICP8-covered tails. Only rarely were intermediate structures between complexes II and III observed, suggesting that the formation of complex III coiled-coils is a fast process.

A fourth species, complex IV molecules, appeared only at the end of the incubation period (Figure 5D and E; Table 1). These molecules were, in general, indistinguishable from complexes formed by ICP8 binding to long ssDNA (see Ruyechan & Weir, 1984). Their increase, at the expense of the coiled and coiled-coil complex III species, argues that they represent ssDNA in which the two strands of the duplex had fully separated from each other.

A series of control experiments were carried out to more clearly define the parameters of the unwinding reaction. When the tailed DNA was incubated for 30 minutes with ICP8 or UL9 proteins alone and with or without 4 mM ATP, no unwound complexes (complexes III and IV) were observed (data not shown). Indeed in previous studies with ss tailed DNAs (Bortner et al., 1993) even high concentrations of ICP8 were not observed to unwind the DNA.

If complexes III and IV were to consist of mostly UL9 protein bound to the DNA, and the only role of ICP8 were to aid in loading UL9 protein onto the duplex DNA, then it is likely that much lower concentrations of ICP8 relative to the 1:1 ICP8/UL9 protein molar ratio used above would suffice to induce unwinding and the appearance of complexes III and IV. If on the other hand, complexes III and IV consist mostly of ICP8, then upon reduction of the levels of ICP8, the degree of unwinding and frequency of complexes III and IV should decline. To examine this, the ss tailed DNA (0.5 nM) was incubated with 0.3 μM UL9 protein and 4 mM ATP as described above but with 0.3, 0.15, 0.075, 0.032 or 0.016 μM ICP8. The experiments were then carried out as described above for 30 minutes and the samples examined by negative staining. Examination of the samples showed that upon reduction of ICP8 to even 0.15 μM complexes III and IV were no longer present and those complexes observed had an irregular and collapsed appearance (data not shown).

Thus, saturated concentrations of ICP8 and/or equimolar concentrations of UL9 protein to ICP8 are required for efficient formation of complex III and unwinding. These data argue that the major constituent of complexes III and IV is ICP8.

The DM27 mutant form of UL9 protein lacks the C-terminal 27 amino acid residues and has a greatly reduced affinity for ICP8 (Boehmer et al., 1994). Identical unwinding reactions were carried out employing this mutant protein and ICP8 over a 30 minute incubation period and the products examined by EM as described above. The same series of complexes (complexes I to IV) were observed (data not shown). Complex III appeared without any apparent intermediates between complex II and complex III, suggesting that here too the transition is rapid. One difference was that after 30 minutes there were a significant number of complex I species remaining, while few remained when wild-type UL9 protein was employed. Thus, direct interactions between UL9 protein and ICP8 are not absolutely required for the unwinding process and may be involved more in the loading of UL9 protein at the ssDNA tail than in the subsequent unwinding step, which may be driven by the build-up of local regions of unwound DNA, as discussed below.

Discussion

In this study, electron microscopy has been used to examine how HSV-1 UL9 protein and ICP8 act to unwind long segments of duplex DNA. Consistent with other results (Bruckner et al., 1991; Fierer & Challberg, 1992) negative staining revealed that in
solution, UL9 protein exists as a dimer and ICP8 as a monomer. Incubation of UL9 protein with duplex DNA containing 3’ extended tails showed that in the absence of ATP, UL9 protein localized to the ss tails (or ss/ds junctions), and that when supplied with ATP, it appeared internally in the duplex segments. The combination of ICP8 and UL9 protein unwound a 3 kb duplex DNA by a mechanism that progressed through distinct stages initiated by the binding of UL9 protein and ICP8 to the ss tails. Regular

Figure 4. Visualization of the initial stage of DNA unwinding by UL9 protein and ICP8. As described in the text, the 3’ ss-tailed dsDNA (100 ng) was incubated with 2.5 μg of UL9 protein, and 4 μg of ICP8 in a buffer containing Mg2+ for ten minutes at 37°C. ATP was then added to 4 mM and the incubation continued for one to 30 minutes. Aliquots were taken directly from the incubations and prepared by negative staining with 2% uranyl acetate as described for Figure 1. The complexes shown here were most abundant after ten to 20 minutes of incubation. The bar represents 100 nm.
Continued EM studies are warranted. First, it was not possible to determine if ICP8 and UL9 protein associate in solution to form a specific complex. Both proteins, in particular UL9 protein, had a tendency to aggregate, which obscured complexes of the size of an ICP8/UL9 protein dimer. In addition, attempts to localize UL9 protein and ICP8 on the nucleoprotein complexes (e.g. complexes II, III or IV) using polyclonal antisera were unsuccessful as the antiserum induced their aggregation and collapse. Nonetheless, comparison of the images obtained here with previously published micrographs of ICP8–ssDNA complexes (Ruyechan, 1983; Ruyechan & Weir, 1984) argued strongly that the ss tails in complex I molecules and the full length of the ssDNA in complex IV molecules were bound almost exclusively by ICP8. Further, the observation that ICP8 formed 26 nm diameter rings when added to 158 nucleotide ssDNAs, and that the diameter of the tightly twisted coils in complex III molecules was 24 nm and contained 120 to 150 nucleotides per coil, provides additional evidence for ICP8 being the major component of these coiled structures (complexes III and IV). This was further supported by the loss of these structures upon reduction of the amount of ICP8 in the incubations.

Previous studies (Bruckner et al., 1991; Fierer & Challberg, 1992; Boehmer et al., 1993) showed that UL9 protein by itself will unwind only short dsDNA fragments. We noted that incubation of large amounts of UL9 protein alone with ss-tailed DNA in the presence of ATP induced the formation of collapsed structures, apparently the result of multiple protein–protein interactions. It is likely that the formation of large disorganized structures would inhibit the separation of the DNA strands even at high concentrations of U19 protein. In further studies, Boehmer et al. (1993) showed that the addition of ICP8 would allow UL9 protein to unwind long DNAs, a finding confirmed here. Boehmer et al. (1993) also noted a lag period in the helicase reactions which could be eliminated by incubating the DNA and UL9 protein together prior to adding ATP. The authors suggested that either an inactive UL9 protein monomer must assemble into an active multimer, or that an inactive multimer must disaggregate to form an active monomer. Our data suggest that the lag period reflects the time required to form a UL9 protein helicase complex at the ss/ds junction.

A plausible model for the unwinding of dsDNA by UL9 protein and ICP8 can be drawn. In the first stage of unwinding, UL9 protein is loaded onto the ssDNA tails and localizes to the ss/ds DNA junction. Loading appeared to require Mg2+ but not ATP. Because ICP8 binds ssDNA very rapidly, UL9 protein loading likely occurs on ICP8-complexed ssDNA and ICP8 may facilitate loading via interactions between ICP8 and the C terminus of UL9 protein. Of note, other SSBS such as the E. coli protein appear to inhibit binding of UL9 protein to ss tails (Boehmer et al., 1993). Further, when the DM27 mutant of UL9 protein was used in the

Figure 5. Visualization of later stages in DNA unwinding by UL9 protein and ICP8. Aliquots taken from incubations as described for Figure 4 showed condensed coils (A) and coiled-coils (B and C) most frequently after 20 to 30 minutes of incubation. D and E, Long filaments typical of ssDNA covered by ICP8 were most abundant at the end of the incubations. The bar represents 100 nm.
experiments, a greater number of complex I molecules (protein bound to the ss tails but no protein internally on the dsDNA) remained throughout the reaction (data not shown) as contrasted to reactions with wild-type UL9 protein. This may reflect diminished loading capacity due to the reduced ICP8-UL9 protein interactions. Once UL9 protein had loaded onto the ss tails, the hydrolysis of ATP would allow it to move internally into the dsDNA. It has been shown that the direction of movement is 3' to 5' relative to the strand containing the 3'-extended ssDNA tail and that movement depends on the integrity of this strand but not the other one (Villani et al., 1994). Complex II molecules thus represent ones in which multiple UL9 protein dimers have moved inwards from both DNA ends.

The most abrupt structural transition observed was from complex II to complex III molecules. Because no structural intermediates between the two states were observed, this transition is likely rapid and cooperative. When an individual UL9 protein dimer moves along DNA, the amount of local ssDNA generated may be below the site size required for a large protein such as ICP8 to bind, and further, the DNA may rapidly close back on itself behind the moving dimer. If, however, the density of UL9 protein dimers becomes high enough, then segments of DNA between dimers may remain single stranded. Such segments were observed in the micrographs (arrows, Figure 4) and these regions would be targets for ICP8 binding. This may be a reversible process if moving UL9 protein dimers can displace short tracts of ICP8. Once the density of single stranded segments had reached some critical level across the length of the DNA, a highly cooperative polymerization of ICP8 would ensue displacing UL9 protein. It is known that the binding of ICP8 to ssDNA is rapid and highly cooperative (Lee & Knipe, 1985; Ruyechan, 1983). This inrush of ICP8 would generate a complex in which both strands were coated with ICP8 and were highly coiled about themselves. Such condensed coils were seen (Figure 5A). Their slow relaxation would then generate the looser coiled-coils and eventually the linear ICP8-ssDNA filaments.

The novel aspect of this process is that unwinding occurs by a nearly explosive mechanism across the entire length of the DNA as contrasted to local unwinding at a fork that begins at the end of a DNA followed by its inwards movement. Numerous examples of the latter mechanism have been described (Wessel et al., 1990a,b, 1992) and the paradigm of this mechanism is the unwinding of DNA at a replication fork by replication-associated helicases. Relevance of the observations made here to HSV-1 replication may be found in our recent study (Makhov et al., 1996) in which it was shown that UL9 protein binds to the HSV-1 OriS origin of replication as a pair of dimers that appear to be oriented in opposite directions. In the presence of ATP, they initiate an unwinding of the origin that extrudes a loop of partially unwound DNA that can be up to 1 kb in size. Possibly in vivo, once such a partially unwound segment had grown to a certain size, then the inrush of ICP8 would generate a long fully unwound region about the origin of replication.

Materials and Methods

Proteins and DNAs

HSV-1 UL9 protein, mutant UL9 protein (UL9 DM27) lacking the C-terminal 27 amino acid residues and ICP8 were purified from nuclear extracts prepared from S9 cells infected with Autographa california nuclear polyhedrosis virus recombinant for the UL9, UL9DM27 and UL29 genes as described (Boehmer & Lehman, 1993b; Boehmer et al., 1994). E. coli exonuclease VIII was the gift of Dr R. Kolodner (Harvard). Biogel A-5m was purchased from BioRad Inc., and uranyl acetate was from Ladd Research, Inc. All other chemicals were purchased from Sigma Chemical Co.

Plasmid pYW11 (Wang et al., 1991) (~3 kb) was cleaved at a single site with SalI restriction endonuclease (New England Biolabs) and 2 μg of DNA in a 100 μl volume was treated with 3 μg of exonuclease VIII for one minute at 37°C in a buffer containing 20 mM Hepes (pH 7.5), 0.3 mM MgCl₂, 10 mM β-mercaptoethanol. The reaction was stopped by the addition of EDTA to 5 mM and SDS to 1% (v/v). The DNA was extracted with a 1:1 (v/v) phenol/chloroform mixture, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris (pH 7.5), 1 mM EDTA). EM examination revealed that >90% of the ends of the molecules were single-stranded, with a mean length of 150 to 200 nt.

UL9 protein and ICP8-DNA binding reactions

Helicase reactions were as described by Boehmer et al. (1993). In a typical 100 μl reaction, ss-tailed DNA (0.5 nM) was mixed with UL9 or UL9 DM27 protein, and if included, ICP8, at 0.3 μM for each protein in binding buffer which contained 20 mm Tris-HCl (pH 7.5), 30 mm NaCl, 3.5 mm MgCl₂, 1.0 mM dithiothreitol for ten minutes at 37°C. When included, ATP was then added to 4 mM and the incubation continued for times described in the text. In reactions not involving negative staining, glycerol was included to 10% (v/v).

Electron microscopy

For examination of DNA–protein complexes by rotary shadowcasting, aliquots from the incubations were fixed with 0.6% (v/v) glutaraldehyde for ten minutes at 20°C and the complexes chromatographed through Biogel A-5m equilibrated in TE buffer to remove fixatives and unbound proteins. The samples were mixed with spermidine hydrochloride to a concentration of 2 mM and applied to thin carbon foils, washed, dehydrated, and rotary shadowcast with tungsten (Griffith & Christiansen, 1978). Micrographs were taken on a Philips CM12 electron microscope.

Images recorded on sheet or 35 mm film were digitized using a Cohu CCD camera and a Data Translation Quick Capture board on a Macintosh computer. The NIH IMAGE software was used to adjust contrast in the images, which were transferred to 35 mm film using a GCC film recorder. Dimensions in the negatively stained
images were determined using the criteria of Steven et al. (1988) and NIH IMAGE software. Contour lengths were measured using a Sugagraphics digitizing tablet coupled to a Macintosh computer programmed with software developed by J.D.G.

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References


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