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The Interaction of Herpes Simplex Type 1 Virus Origin-binding Protein (UL9 Protein) with Box I, the High Affinity Element of the Viral Origin of DNA Replication*

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The herpes simplex type 1 (HSV-1) origin binding protein, the UL9 protein, exists in solution as a homodimer of 94-kDa monomers. It binds to Box I, the high affinity element of the HSV-1 origin, Ori_s, as a dimer. The UL9 protein also binds the HSV-1 single strand DNA-binding protein, ICP8. Photocross-linking studies have shown that although the UL9 protein binds Box I as a dimer, only one of the two monomers contacts Box I. It is this form of the UL9 homodimer that upon interaction with ICP8, promotes the unwinding of Box I coupled to the hydrolysis of ATP to ADP and P_i. Photocross-linking studies have also shown that the amount of UL9 protein that interacts with Box I is reduced by its interaction with ICP8.

Antibody directed against the C-terminal ten amino acids of the UL9 protein inhibits its Box I unwinding activity, consistent with the requirement for interaction of the C terminus of the UL9 protein with ICP8. Inhibition by the antibody is enhanced when the UL9 protein is first bound to Box I, suggesting that the C terminus of the UL9 protein undergoes a conformational change upon binding Box I.

The herpes simplex virus type 1 (HSV-1)[1] genome contains three origins of DNA replication: Ori_L and the diploid Ori_s (1, 2). It also encodes three enzymes required for its replication: a heterodimeric DNA polymerase, a heterotrimeric helicase-primase or primosome, a single strand DNA-binding protein (ICP8), and an origin-binding protein (UL9 protein) (1, 2).

The 94-kDa UL9 protein specifically recognizes the Box I and II elements of Ori_s that are separated by an AT-rich sequence of 18 nucleotides (3, 4). The homogeneous UL9 protein has DNA-dependent ATPase and 3′-5′ helicase activities in addition to its origin binding activity (5–8). The N-terminal 400 amino acids of the UL9 protein contain the six motifs characteristic of the superfamily of DNA and RNA helicases (9, 10). The C-terminal 317 amino acids contain the DNA binding domain (11, 12). A comparison of the amino acid sequence of the HSV-1 UL9 protein with that from several other herpesviruses shows the helicase and DNA binding domains to be highly conserved (9, 13).

To unwind the high affinity Box I element of the Ori_s, we have found that the UL9 protein must interact with ICP8 bound to a 3′ single strand positioned downstream of the Box I sequence (Box I substrate, Fig. 1) (14). This orientation of the UL9 protein bound to the Box I permits the specific interaction of the C-terminal 27 amino acids of the UL9 protein with ICP8, thereby determining the 3′ → 5′ directionality of Box I unwinding.

The UL9 protein exists in solution as a homodimer (5, 6). Moreover, studies of its helicase and ATPase activities have shown the active form of the UL9 protein to be a dimer (6, 7). However, there have been conflicting findings regarding the interaction of the UL9 protein with Box I. In one study (15), only one of the two monomers of UL9 protein was found to bind to Box I. In two other studies (16, 17), both monomers of the UL9 protein were found to contact Box I. All of these studies were performed with a truncated 37-kDa form of the UL9 protein (t-UL9), which contains only the DNA binding domain and exists as a monomer in solution (18). Several lines of evidence support the suggestion that only one monomer of the UL9 protein contacts Box I sequence. (i) The affinity of the UL9 homodimer and the t-UL9 monomer for Box I is identical, suggesting that only one monomer is required for binding (19, 20). (ii) The DNase I protection pattern produced upon binding of the UL9 homodimer to the Box I sequence is indistinguishable from that found with the t-UL9 monomer (18, 20). (iii) A complex of Box I, the t-UL9 monomer, and ICP8 has been isolated whose molecular weight suggests that only one t-UL9 protein monomer binds to Box I (21). (iv) A 1:1 complex of the UL9 homodimer and ICP8 is required to unwind Box I and unwinding is unidirectional (14). In the experiments reported here we have found that only one of the two monomers of the UL9 homodimer contacts Box I and unwinds the Box I substrate when ICP8 is bound to the downstream 3′ single strand. We have also observed that the interaction between UL9 protein and ICP8 influences the UL9 protein-Box I interaction before unwinding. Finally, we have observed that the UL9 protein monomer very likely undergoes a conformational change upon binding of Box I.

MATERIALS AND METHODS

Protein Purification—Recombinant UL9 protein and ICP8 were purified from baculovirus-infected Sf21 insect cells as described previously (14). Rabbit antiserum directed against the C-terminal 10 amino acids of the UL9 protein was a generous gift from Dr. Mark Challberg (NIH). Rabbit antibodies directed against the C-terminal 10 amino acids (anti-UL9 CT10) as well as full-length recombinant UL9 protein were purified with the use of a protein A column.

DNA Substrates—For the photocross-linking assays, two forms of Box I were prepared as described previously (14). A fully duplex Box I (24 bp) containing bromodeoxyuridine (B) in place of thymine was prepared by annealing 5′ ^{32}P-labeled 5′-CGCGAAGCGBBCGCACBBCGTCCC-3′ with 5′-GGGACGAAGTGCGAACGCTTCGCG-3′ (Fig. 1). Box I containing single strand DNA tails (Box I substrate, Fig. 1) was

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[1] The abbreviations used are: HSV-1, herpes simplex virus type 1; BrdUrd, bromodeoxyuridine.

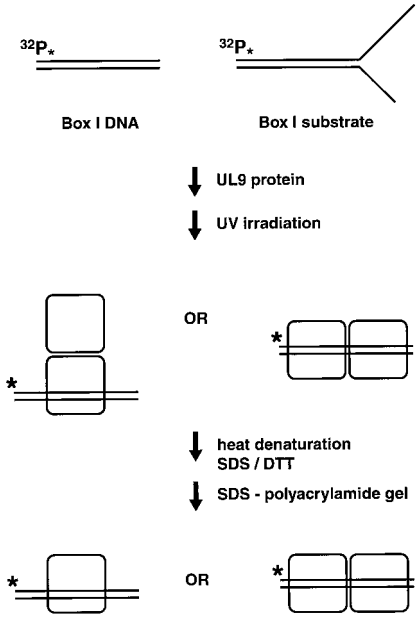

FIG. 1. **Scheme for the detection of UL9-Box I complexes by photocross-linking.** UL9 protein was incubated with either fully duplex Box I DNA containing BrdUrd or Box I substrate at 37 °C for 15 min. After irradiation at 300 or 260 nm, respectively, the complex was heated for 10 min at 90 °C in SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by 7% SDS-polyacrylamide gel electrophoresis. The molecular weights of the UL9 protein monomer or homodimer cross-linked to the Box I were determined by comparison with pre-stained marker proteins, dithiothreitol (*DTT*).

prepared by annealing the 5′ ^{32}P-labeled top strand (44 nucleotides) with the bottom strand (36 nucleotides) (14). Both substrates were gel purified.

Photocross-linking—Reaction mixtures (25 μl) containing 50 mM Hepes-KOH, pH 7.6, 7 mM $MgCl_2$, 100 mM NaCl, 5 mM dithiothreitol, 10 μg bovine serum albumin, 2 pmol of fully duplex BrdUrd-substituted Box I, and the indicated amounts of UL9 protein were incubated at 37 °C for 15 min. The samples were then transferred to an ice water bath and exposed under a hand-held UV probe (Fotodyne) at 300 nm for 5, 10, 20, 30, and 60 min, respectively. The reactions were stopped by adding SDS-polyacrylamide gel electrophoresis sample buffer and heated at 90 °C for 10 min. The UL9 protein-Box I complexes were separated by 7% SDS-polyacrylamide gel electrophoresis. The molecular weights of the complexes were estimated by comparison with several pre-stained molecular marker proteins. Quantitation of cross-linked UL9 protein-Box I complexes was performed with a PhosphorImager (Molecular Dynamics).

To examine the effect of ICP8 on the UL9 protein-Box I substrate interaction, the indicated amounts of ICP8 were mixed with UL9 protein (2 pmol) and Box I substrate. Photocross-linking was performed under the same conditions as described above, except that the wavelength was 260 nm. The samples were analyzed by 7% SDS-polyacrylamide gel electrophoresis, and the amount of cross-linked UL9 protein-ICP8-Box I substrate complexes was quantitated with a PhosphorImager. Each of the cross-linking experiments was performed a minimum of four times.

Box I Unwinding Assay—Reaction mixtures (25 μl) containing 50 mM Hepes-KOH, pH 7.6, 7 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 5 mM dithiothreitol, 10 μg of bovine serum albumin, 2 pmol of Box I substrate, 2 pmol of UL9 protein, and the indicated amounts of ICP8 were assembled on ice (14). The reactions were initiated by adding ATP (4 mM) and continued for 30 min at 37 °C. The reactions were terminated by adding 6.5 μl of stop solution (100 mM EDTA, 1% SDS, 10 μg of proteinase K). The single-stranded DNA products were separated from the Box I substrate by nondenaturing 16% polyacrylamide gel electrophoresis at 10 V/cm, and quantitated with a PhosphorImager.

To examine the influence of anti-UL9 protein antibodies on unwinding of the Box I substrate, the indicated amounts of antibody were pre-incubated with UL9 protein (2 pmol) alone or UL9 protein (2 pmol) and the Box I substrate (2 pmol) at 37 °C for 30 min. The reactions were initiated by adding 4 pmol of ICP8 and 4 mM ATP, and incubated for an additional 30 min. The reaction mixtures were analyzed as described above. Each of the unwinding assays was performed a minimum of four times.

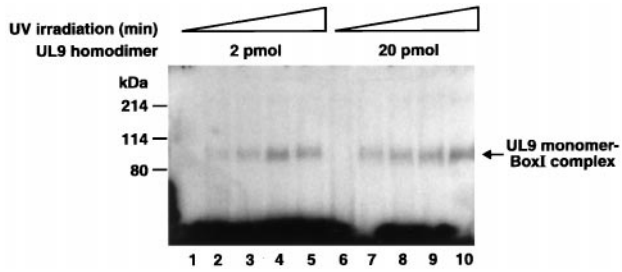

FIG. 2. **A UL9 monomer is cross-linked to fully duplex Box I.** The UL9 protein homodimer (2 pmol, *lanes 1–5*; or 20 pmol, *lanes 6–10*) was incubated with BrdUrd substituted Box I (2 pmol), followed by irradiation at 300 nm for 0 (*lanes 1* and *6*), 5 min (*lanes 2* and *7*), 10 min (*lanes 3* and *8*), 20 min (*lanes 4* and *9*), and 30 min (*lanes 5* and *10*). The samples were analyzed as described under "Materials and Methods" and in Fig. 1.

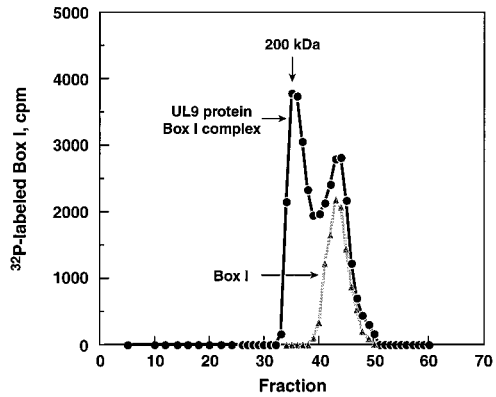

FIG. 3. **Sephacryl-200 gel filtration of UL9-Box I complex.** Photocross-linking and gel filtration chromatography were performed as described under "Materials and Methods." The radioactivity of 50-μl aliquots of each fraction was determined by scintillation counting.

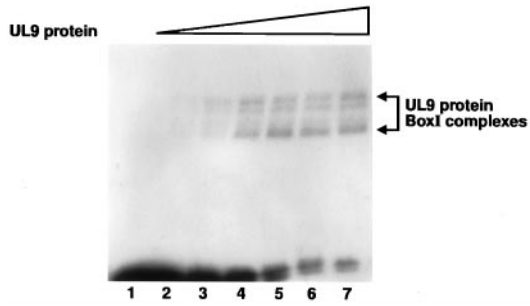

FIG. 4. **A UL9 monomer is cross-linked to Box I containing single strand tails (Box I substrate).** Increasing amounts of UL9 protein homodimer (*lanes 1–7*: 0, 0.1, 0.5, 1, 2, 4, and 8 pmol, respectively) were incubated with Box I substrate (2 pmol). The samples were irradiated 260 nm and treated as described under "Materials and Methods" and Fig. 1.

Sephacryl 200 Gel Filtration—UL9 protein (120 pmol) was incubated with the radiolabeled BrdUrd-containing fully duplex Box I (60 pmol) at 37 °C for 15 min, followed by photocross-linking at 300 nm for 20 min. The mixture (100 μl) was loaded onto a Sephacryl 200 gel filtration column (1.5 × 60-cm) equilibrated with a buffer consisting of 50 mM Hepes-KOH, pH 7.8, 10% glycerol, 1 mM dithiothreitol, and 200 mM NaCl. The column was monitored with Box I substrate and with molecular weight marker proteins, β-amylase (200 kDa), catalase (232 kDa), bovine serum albumin (66 kDa), and cytochrome *c* (12.4 kDa), before and after the sample was loaded. The radioactivity of 50 μl-aliquots of the fractions was determined by scintillation counting.

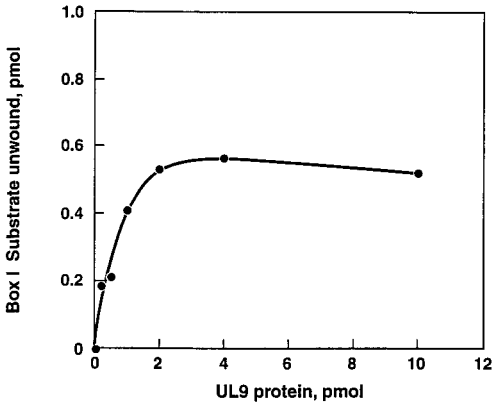

FIG. 5. **Unwinding of Box I substrate by a UL9 protein-ICP8 complex.** Increasing amounts of UL9 protein homodimer were incubated with 4 pmol of ICP8 and the Box I substrate (2 pmol). The reaction was initiated by addition of ATP (4 mM), and continued for 30 min at 37 °C. The reaction mixtures were electrophoresed through a 15% polyacrylamide gel as described under "Materials and Methods," and the single-stranded DNA products were quantitated with a PhosphorImager.

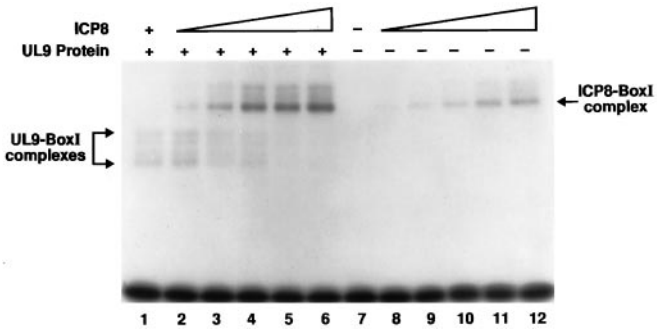

FIG. 6. **Effect of ICP8 on UL9 protein-Box I interaction.** UL9 protein homodimer (2 pmol) and increasing amounts of ICP8 were incubated with the Box I substrate (2 pmol), and irradiated at 260 nm for 5 min. The reaction mixtures were analyzed by 7% SDS-polyacrylamide gel electrophoresis as described under "Materials and Methods" and Fig. 1. *Lane 1*, UL9 protein homodimer alone; *lanes 2–6*, 0.5, 1, 2, 4, and 8 pmol of ICP8, in the presence of 2 pmol of UL9 protein homodimer; *lane 7*, Box I substrate alone; *lanes 8–12*, 0.5, 1, 2, 4, and 8 pmol of ICP8 in the absence of the UL9 protein.

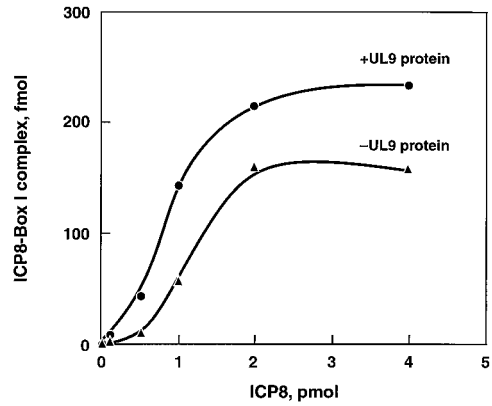

FIG. 7. **Effect of UL9 protein on ICP8-Box I substrate interaction.** Increasing amounts of ICP8 were incubated with Box I substrate (2 pmol) in the presence (●) or absence (▲) of UL9 protein homodimer (2 pmol) and UV-irradiated at 260 nm for 5 min as described under "Materials and Methods" and Figs. 1 and 6. ICP8-Box I substrate complexes were quantitated with a PhosphorImager.

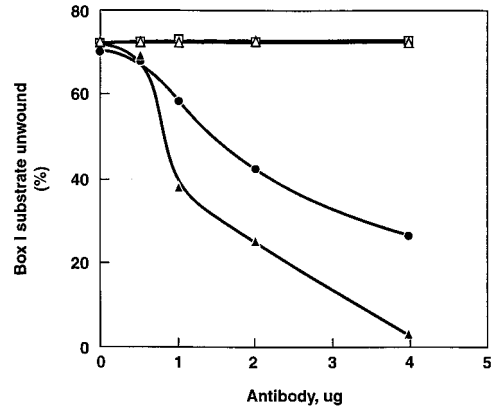

FIG. 8. **Inhibition of Box I unwinding by antibody directed against the C-terminal 10 amino acids of UL9 protein.** The UL9 protein homodimer (2 pmol) was incubated with ICP8 (4 pmol) and Box I substrate (2 pmol) in the presence of increasing amounts of antibody. The UL9 protein was preincubated with Box I substrate, followed by addition of antibody (▲), or the UL9 protein was preincubated with antibody before ICP8, ATP, and Box I substrate were added (●). Antibody against the full-length UL9 protein (△); pre-immune IgG (□). The reaction mixtures were electrophoresed through a 15% polyacrylamide gel as described undder "Materials and Methods," and the single-stranded DNA products were quantitated with a PhosphorImager.

RESULTS AND DISCUSSION

Only a Single Monomer of the UL9 Homodimer Contacts Box I—The UL9 protein exists as a stable homodimer in solution. In view of the uncertainty regarding the mode of interaction between Box I and the UL9 protein, we first wished to determine whether the UL9 protein contacts Box I as a monomer or dimer. The experimental protocol is illustrated in Fig. 1. UL9 protein was photocross-linked to fully duplex Box I DNA containing BrdUrd in place of thymine, and the products were analyzed by 7% SDS-polyacrylamide gel electrophoresis. The reaction conditions including the concentration of UL9 protein and Box I were similar to those used for the Box I unwinding assay. If both UL9 monomers (apparent molecular mass, 83 kDa) contact Box I (molecular mass of single-stranded 44-mer, 13.3 kDa) an approximately 180-kDa complex should be formed. If only one monomer of the UL9 homodimer interacts with Box I DNA, the molecular mass of the complex should be approximately 93 kDa (Fig. 1).

As shown in Fig. 2, when equivalent amounts of UL9 protein and Box I were irradiated at 300 nm, a 93-kDa complex of UL9 protein cross-linked to Box I was formed. The presence of the UL9 protein in the complex was verified by immunoprecipitation with antibody directed against the UL9 protein (data not shown). Formation of this complex was UL9 protein-specific and dependent upon UV-irradiation. The molecular mass of the complex indicated that only one monomer of UL9 homodimer contacted Box I. To test whether an excess of UL9 protein can induce formation of a UL9 protein-Box I complex in which the two monomers are cross-linked to Box I, a 10-fold excess of UL9 protein was incubated with Box I and irradiated as before. These conditions are comparable to those used by Fierer and Challberg (17). As shown in Fig. 2, only the 93-kDa complex was formed, similar to that observed with equimolar amounts of UL9 protein and Box I. We can therefore conclude that in agreement with Gustafsson *et al.* (21) only one of the two monomers of the UL9 dimer contacts the Box I sequence.

To verify that the UL9 protein bound to Box I as a dimer, a mixture containing UL9 protein (120 pmol) and ^{32}P-labeled Box I (60 pmol) was irradiated at 300 nm then subjected to Sephacryl 200 gel filtration. Two radiolabeled peaks appeared. The first eluted at a molecular mass of 200 kDa, which is consistent with the UL9 protein dimer and radiolabeled Box I. The second peak consisted of free Box I (Fig. 3). These results

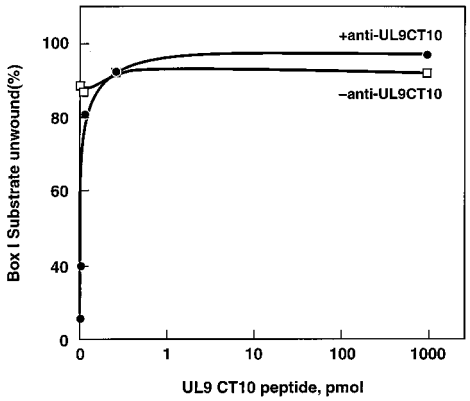

FIG. 9. **Effect of a synthetic peptide (UL9 CT10) on the inhibition of Box I unwinding by antibody directed against the C-terminal 10 amino acids of the UL9 protein.** Increasing amounts of peptide were added to the reaction mixtures in the presence (●) or absence (□) of antibody. The reaction mixtures were prepared and analyzed as described in Fig. 8.

demonstrate that although the UL9 protein binds Box I as a dimer, only one of the two monomers actually contacts Box I. This conclusion is supported by the finding that the electrophoretic mobility of the ^{32}P-labeled Box I substrate-UL9 protein complex was significantly increased upon treatment with SDS (data not shown).

To examine the interaction between the UL9 protein and the Box I substrate during the unwinding reaction, increasing amounts of the UL9 protein were mixed with the Box I substrate and irradiated at 260 nm for 5 min, followed by heating at 90 °C for 10 min (Fig. 1). As shown in Fig. 4, two UL9 protein-Box I complexes were formed: The predominant one migrated with a molecular mass of 99 kDa and the second at 115 kDa. These forms correspond to a UL9 monomer cross-linked to the single-stranded 44-mer (13.3 kDa) and the UL9 monomer cross-linked to the duplex Box I (24.3 kDa). The latter likely resulted from some renaturation of the complementary single strands of the Box I substrate. These results demonstrate that as in the case of the fully duplex Box I, the Box I substrate contacted only one of the two UL9 monomers. Quantitation of the UL9 monomer-Box I complex formed showed that 2 pmol of UL9 homodimer was sufficient to saturate 2 pmol of the Box I substrate. As shown in Fig. 5, maximal unwinding of the Box I substrate (2 pmol) also occurred in the presence of an equivalent amount of UL9 dimer (2 pmol). In these experiments, multiple unwinding events were prevented by the addition of limiting amounts (4 pmol) of ICP8, which is required in stoichiometric amounts (8).

UL9 Protein-Box I Substrate Interaction Is Weakened by ICP8—Earlier studies (14, 22) had shown, using a C-terminal deletion mutant of UL9, that a specific interaction between the C-terminal portion of the UL9 protein and ICP8 is required to unwind the Box I substrate. To better define the role of ICP8 in the unwinding reaction, we examined the effect of ICP8 on the UL9 monomer-Box I interaction using the photocross-linking assay. Equivalent amounts of UL9 homodimer and Box I substrate were incubated together with increasing amounts of ICP8 in the absence of ATP. As shown in Fig. 6, the amount of UL9 protein-Box I complex formed following UV-cross-linking decreased significantly with increasing amounts of ICP8.

The interaction between the UL9 protein and ICP8 also influenced the binding of ICP8 to the single-stranded DNA tails of the Box I substrate. As shown in Fig. 7, ICP8 interacted with the single-stranded DNA tails of the Box I substrate in the absence of UL9 protein. However, the amount of ICP8 cross-linked to the Box I substrate was increased 2–3-fold by the UL9

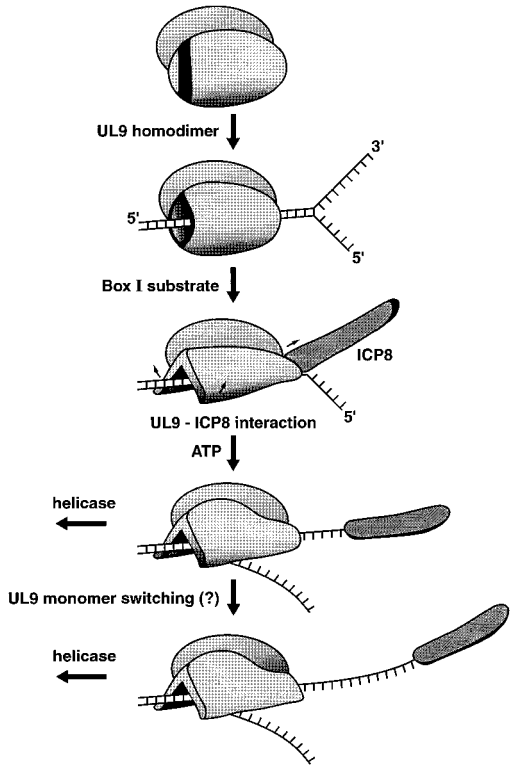

FIG. 10. **A model for the unwinding of the Box I element of Ori$_S$ by the UL9 protein-ICP8 complex.** See text for details.

protein. In contrast, there was no effect of UL9 protein on the interaction of ICP8 with a single strand the same length (18 nucleotides) as the 3′ single-stranded DNA tail of the Box I substrate. There was also no effect of ICP8 on the binding of UL9 protein to the fully duplex Box I substrate (data not shown).

Antibody Directed Against the C-terminal 10 Amino Acids of UL9 Protein Inhibits Box I Unwinding Activity—The C-terminal 27 amino acids of the UL9 protein are required for maximal Ori$_S$-dependent DNA replication *in vivo* (22). They are also required for the interaction with ICP8, as well as the unwinding of Box I (14). To explore further this interaction on the unwinding of Box I, the effect of the antibody directed against the C-terminal 10 amino acids of the UL9 protein was examined. As shown in Fig. 8, preincubation of the UL9 protein with the antibody significantly inhibited unwinding. In contrast, antibody directed against full-length UL9 protein, which recognizes several native epitopes of the UL9 protein but not the C-terminal portion of the molecule, had no effect on unwinding (Fig. 8). Thus binding of the antibody to the C-terminal 10 amino acids of the UL9 protein prevents interaction between the UL9 protein and ICP8 and thereby inhibits unwinding. Addition of a peptide consisting of the C-terminal 10 amino acids of UL9 protein (UL9 CT10) prevented inhibition of Box I unwinding by the antibody, confirming that the inhibition results from an interaction between the UL9 protein bound to the Box I and the antibody directed against the peptide (Fig. 9). Finally, when the antibody was added to a UL9 protein-Box I complex, followed by ICP8 and ATP, unwinding was completely inhibited (Fig. 8), suggesting that when the CT10 epitope of the UL9 protein is bound to the Box I substrate, it becomes more accessible to the antibody. These observations taken together confirm that the C-terminal portion of the UL9 protein, when bound to Box I, is required for its interaction with ICP8. They also suggest that the UL9 protein undergoes a significant conformational change upon binding Box I. Such a conformational

change may be required to facilitate helicase activity. A model for the unwinding of the Box I element of Ori_s by the dimeric UL9 protein and ICP8, which encompasses these phenomena, is presented in Fig. 10.

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