

Unwinding of a Herpes Simplex Virus Type 1 Origin of Replication (Ori_S) by a Complex of the Viral Origin Binding Protein and the Single-Stranded DNA Binding Protein

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A herpes simplex virus type 1 (HSV-1) Ori_S analogue in which the A+T sequence linking the box I and II elements was replaced by two single-stranded oligo(dT)₁₈s is unwound by the UL9 protein-ICP8 complex. Unwinding of wild-type Ori_S by the UL9 protein-ICP8 complex was also observed under conditions which destabilize the A+T sequence. These experiments support a model for the unwinding of Ori_S in which destabilization of the A+T sequence can generate a single-stranded DNA binding site for ICP8, which then associates with the UL9 protein bound to boxes I and II to promote the bidirectional unwinding of Ori_S.

Herpes simplex virus type 1 (HSV-1) encodes a 94-kDa origin binding protein, the product of the UL9 gene, which is essential for HSV-1 DNA replication (5, 10). The origin binding protein (UL9 protein) is a homodimer that binds the two inverted pentanucleotide repeats, boxes I and II of the HSV-1 origin of replication, Ori_S. Boxes I and II are linked by an A+T-rich sequence of 18 nucleotide residues (5). In addition to its origin binding activity, the UL9 protein possesses DNA-dependent ATPase and 3'-5' helicase activities (3, 6). Despite its helicase activity, we have been unable to detect the unwinding of duplex DNA containing Ori_S (9).

Previous studies have shown that a complex of the UL9 protein and the HSV-1-encoded single-stranded DNA binding protein, ICP8, can efficiently unwind a duplex box I if it possesses a 3' single-stranded tail at least 18 nucleotides in length, positioned downstream of box I (8). These findings suggested a model for the unwinding of Ori_S in which a complex of the UL9 protein bound to boxes I and II and ICP8 bound to single-stranded DNA generated at the A+T-rich linker, possibly as a consequence of transcription (4), unwinds the origin of DNA replication to provide access to the replication machinery, thereby permitting the initiation of DNA replication.

To test this model, we have investigated the ability of the UL9 protein-ICP8 complex to unwind an Ori_S analogue in which the A+T-rich linker has been replaced by two single strands each consisting of 18 deoxythymidylate residues. We have found this Ori_S analogue to be unwound by the complex. In addition, we have found that at temperatures that would be expected to destabilize the A+T-rich linker, some unwinding of wild-type Ori_S can be observed. Ori_S bearing mutations in boxes I and II which inhibit binding of UL9 protein could not be unwound under these conditions. These findings support the model in which destabilization of the A+T-rich linker to provide a binding site for ICP8 represents the initial event in the UL9 protein-promoted unwinding of Ori_S.

The structure of the duplex oligonucleotides used are shown in Fig. 1. The single-stranded oligonucleotides were purchased from Operon Technologies, Inc. They were purified prior to use by 16% polyacrylamide electrophoresis under denaturing

conditions. The concentration of single-stranded oligonucleotides was determined spectrophotometrically assuming that 1 absorbance unit at 260 nm is equivalent to 30 μg/ml. For double-stranded DNA 1 absorbance unit is equivalent to 50 μg/ml. Oligonucleotides were labeled with [γ -³²P]ATP, using T4 polynucleotide kinase according to a protocol supplied by the U.S. Biochemical Corp. Unincorporated nucleotide was removed by Sephadex G-50 spin column chromatography (Quickspin DNA; Boehringer Mannheim). Duplex oligonucleotides were formed by briefly mixing equimolar amounts of the two complementary single-stranded oligonucleotides in 50 mM Tris buffer, pH 8.0, containing 0.1 M NaCl at 90°C for 5 min and then allowing the mixture to cool slowly to room temperature overnight. The annealed DNA was passed

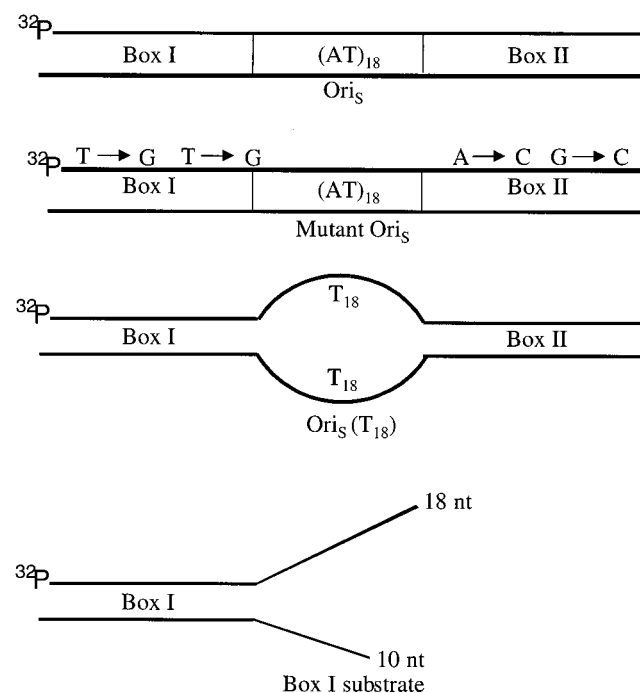


FIG. 1. Helicase substrates used. nt, nucleotides.

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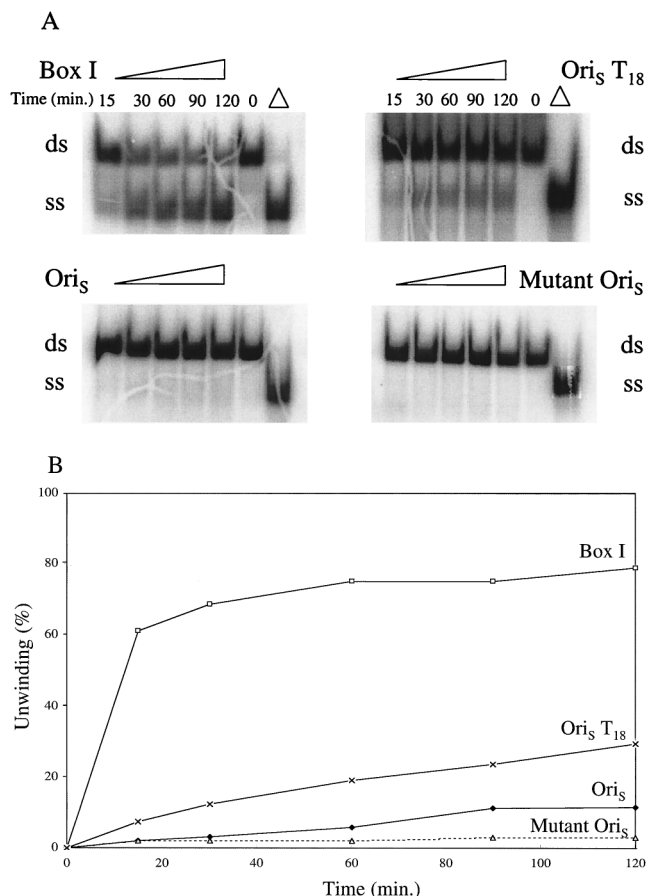


FIG. 2. Unwinding of Ori_S, mutant Ori_S, Ori_ST₁₈, and box I substrate by UL9 protein and ICP8. Individual reaction mixtures containing 1.0 pmol of Ori_S, 1.8 pmol of mutant Ori_S, 1.6 pmol of Ori_ST₁₈, or 2.0 pmol of box I substrate, 1.6 pmol of UL9 protein, and 1.6 pmol of ICP8 were prepared as described in the text. They were incubated at 37°C for the times indicated. The products were electrophoresed through a 15% polyacrylamide gel. (A) Autoradiogram; (B) quantitation of products with a PhosphorImager. Percent unwinding is the ratio of the intensities of the single-stranded DNA product and the double-stranded DNA substrate. Δ, double-stranded DNA substrate heated at 100°C for 5 min and then chilled on ice. ss, single stranded; ds, double stranded.

through a Sephadex G-50 spin column to remove single-stranded DNA.

To form the box I substrate, the 44-mer 5'-CGCGAAGCG TTCGCACTTCGTCCTCCCGCCTTCCTGCGCCTTCCTGT-3' was annealed to the 36-mer 5'-CATGCTCGCAGCGGGACG AAGTGCGAACGCTTCGCG-3' to generate a 26-bp box I duplex with an 18-nucleotide 3' and a 10-nucleotide 5' single-stranded tail.

To form the duplex Ori_S, 5'-CGCGAAGCGTTCGCACTT CGTCCAATATATATATATTATTAGGGCGAAGTGCG AGCACTGGC-3' was annealed to 5'-GCCAGTGCTCGCA CTTCGCCCTAATAATATATATATATTGGGACGAAGT GCGAACGCTTCGCG-3'. The mutant Ori_S was generated by annealing 5'-CGCGAAGCGGGCGCACTTCGTCCTCCAATA TATATATATTATTAGGGCGAAGTGCGCCCACTGGC-3' to 5'-GCCAGTGCGCGCACTTCGCCCTAATAATA TATATATATTGGGACGAAGTGCGCGCTCCTCGCG-3'. Ori_ST₁₈ was generated by annealing 5'-CGCGAAGCGTTCG CACTTCGTCCTTTTTTTTTTTTTTTTTTTTGGGCGAAGT GCGAGCACTGGC-3' to 5'-GCCAGTGCTCGCACTTCGC CCTTTTTTTTTTTTTTTTTTTTGGGACGAAGTGCGAACG CTTCGCG-3'.

To measure the unwinding of the duplex oligonucleotides, reaction mixtures (25 μl) containing 50 mM HEPES-KOH (pH 8.15), 10 mM NaCl, 10 mM MgCl₂, 2.0 mM dithiothreitol, 10% (vol/vol) glycerol, 10 μg of bovine serum albumin, 2 pmol of box I substrate, 1.0 pmol of Ori_S, 1.8 pmol of Ori_ST₁₈, 1.8 pmol of mutant Ori_S, 1.6 pmol of ICP8 (8), and 1.6 pmol of UL9 protein (8) were incubated on ice for 5 min. ATP (50 μM) was added, and the reaction mixtures were incubated at 37°C for 60 min. The reactions were terminated by the addition of 6.5 μl of stop solution (100 mM EDTA, 1% sodium dodecyl sulfate, 20 μg of proteinase K) for an additional 10 min. at 37°C, followed by electrophoresis through a 15% polyacrylamide gel at 10 V/cm. The gel was then dried on Whatman DE81 paper at 80°C under vacuum and quantitated with a PhosphorImager (Molecular Dynamics) or exposed to Kodak XAR X-ray film.

Unwinding of Ori_ST₁₈. Earlier studies with the box I substrate had led to a model in which destabilization of the A+T sequence linking boxes I and II to provide a binding site for ICP8 was the first step in the unwinding of Ori_S by the UL9 protein (8). Unwinding of Ori_S is required to provide an entry site for the replication machinery needed to initiate DNA replication. In vivo, destabilization could result from transcription from promoter sites flanking Ori_S (4). To test this notion, we generated an Ori_S duplex, Ori_ST₁₈, in which the A+T region was replaced by two unpaired single strands, each containing 18 thymidylate residues, a structure analogous to Ori_S in which the A+T sequence had been unwound (Fig. 1). As shown in Fig. 2, in the presence of UL9 protein and ICP8, substantial (20%) unwinding of this structure occurred in 60 min; at 120 min, 30% if the Ori_ST₁₈ was unwound. Under the same conditions, the extent of unwinding of the wild-type Ori_S duplex was 5 and 8%, respectively. The extent of unwinding of the wild-type Ori_S, though small, is nevertheless significant, since less than 2% of the mutant Ori_S was unwound under these conditions. This mutant contained base changes in both boxes I and II and did not bind the UL9 protein (7).

Unwinding of Ori_S. In another approach, unwinding of wild-type Ori_S was measured under conditions which would be expected to destabilize preferentially the A+T region, i.e., elevated temperature. As shown in Fig. 3, increasing the temperature of the reaction from 40 to 45°C resulted in an increase in the extent of unwinding from 5 to 18%. Previous experiments had demonstrated that the nonspecific helicase

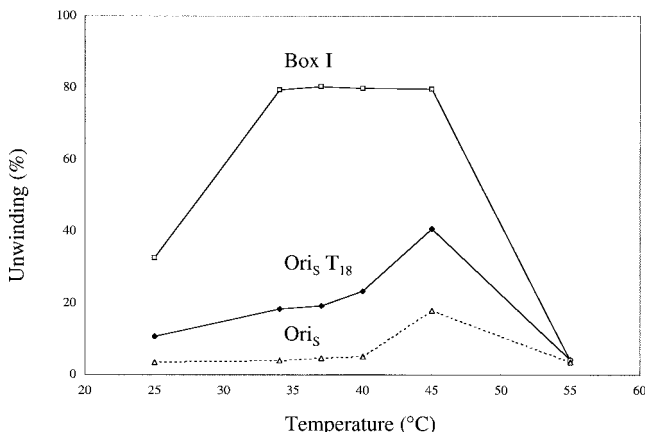


FIG. 3. Effect of temperature on unwinding of Ori_S, Ori_ST₁₈, and box I substrate by UL9 protein and ICP8. Reaction mixtures were prepared as described in the text and incubated for 60 min at the temperatures indicated. The products were electrophoresed through a 15% polyacrylamide gel and quantitated with a PhosphorImager.

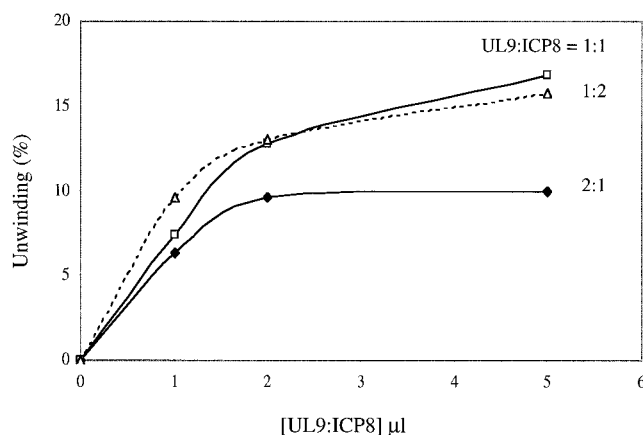


FIG. 4. Effect of the UL9 protein/ICP8 ratio on unwinding of $\text{Ori}_S\text{T}_{18}$. Reaction mixtures were prepared as described in the text. Increasing amounts of enzyme solution containing the indicated UL9 protein/ICP8 ratios were added, and the reaction mixtures were incubated for 60 min. The products were electrophoresed through a 15% polyacrylamide gel and quantitated with a PhosphorImager.

activity of the UL9 protein increased linearly from 33 to 45°C, then decreased at 55°C (2), where denaturation of the enzyme occurs (data not shown). The greater-than-threefold increase in the extent of unwinding of Ori_S with a 5°C increase in the temperature of incubation is consistent with the destabilization of the A+T sequence linking boxes I and II to provide a single-stranded DNA binding site for ICP8. In contrast to Ori_S , the difference in the extent of unwinding of the $\text{Ori}_S\text{T}_{18}$ analogue between 40°C and 45°C was only 1.6-fold, an increase to be expected from the Q_{10} of the enzyme (2). The box I substrate, which is efficiently unwound over a broad range of temperatures, was maximally unwound (>80%) at 35°C.

Effect of the UL9 protein/ICP8 ratio. ICP8 presumably serves two functions in the unwinding of Ori_S : (i) as a component of the UL9 protein-ICP8 complex which constitutes the active helicase (8) and (ii) as a single-stranded DNA binding protein that binds the single strands generated as a conse-

quence of helicase action, thereby preventing their reannealing (1, 11). We therefore investigated the effect of varying the concentration of ICP8 relative to the UL9 protein on the extent of unwinding of the Ori_S analogue $\text{Ori}_S\text{T}_{18}$. As shown in Fig. 4, in accord with a dual function for ICP8, the maximal extent of unwinding was significantly reduced at a UL9 protein to ICP8 ratio of 2:1.

It is also possible that an excess of UL9 protein may have a dominant negative effect on unwinding.

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