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Herpes Simplex Virus Type 1 ICP8: Helix-Destabilizing Properties

PAUL E. BOEHMER AND I. R. LEHMAN*

Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, California 94305-5307

Received 17 August 1992/Accepted 30 October 1992

The major single-stranded DNA-binding protein, ICP8, of herpes simplex virus type 1 (HSV-1) is one of seven virus-encoded polypeptides required for HSV-1 DNA replication. To investigate the role of ICP8 in viral DNA replication, we have examined the interaction of ICP8 with partial DNA duplexes and found that it can displace oligonucleotides annealed to single-stranded M13 DNA. In addition, ICP8 can melt small fragments of fully duplex DNA. Unlike a DNA helicase, ICP8-promoted strand displacement is ATP and Mg^{2+} independent and exhibits no directionality. It requires saturating amounts of ICP8 and is both efficient and highly cooperative. These properties make ICP8 suitable for a role in DNA replication in which ICP8 destabilizes duplex DNA during origin unwinding and replication fork movement.

ICP8, the major single-stranded DNA (ssDNA)-binding protein (SSB) of herpes simplex virus type 1 (HSV-1), is a 128-kDa zinc metalloprotein encoded by the UL29 gene (15, 23). UL29 mutants are defective in viral DNA replication (8, 18, 34), and ICP8 has been identified as one of seven virus-encoded polypeptides that are necessary and sufficient for HSV-1 DNA replication in vivo (21, 27, 37). ICP8 has also been implicated in the regulation of viral gene expression (12, 14).

In vitro, purified ICP8 interacts preferentially with ssDNA (17, 24, 25), to which it binds tightly and cooperatively (17, 24). Electron microscopic studies indicate that ICP8 binds to DNA in regular repeating units, holding the DNA in an extended conformation (24, 25). In the absence of DNA, ICP8 forms long helical filaments (20). The stoichiometry of binding of ICP8 to ssDNA as determined by electron microscopy and nuclease protection has been estimated to be 1 ICP8 per 40 and 12 nucleotides, respectively (20, 24). In addition, ICP8 shares the ability to lower the melting temperature of synthetic polynucleotides such as poly(dA)-poly(dT) with other SSBs, notably the *Escherichia coli* SSB and the bacteriophage T4 gene *32* protein (1, 22, 33, 36).

ICP8 stimulates the HSV-1 DNA polymerase (16, 25) as well as the helicase and DNA-dependent nucleoside triphosphatase activities of the HSV-1 origin-binding protein (UL9 protein) (2, 11) and is required for complete unwinding of duplex DNA by the HSV-1-encoded DNA helicase-primase (9). ICP8 also appears to interact directly with the HSV-1 DNA polymerase-UL42 protein complex and the alkaline nuclease (6, 18, 22, 28–31). Overall, these properties suggest that ICP8 is a classic SSB functioning in DNA replication in a manner similar to that of the *E. coli* SSB and the bacteriophage T4 gene *32* protein (reviewed in references 5, 7, and 19). ICP8 is also required for the organization of viral DNA replication enzymes into nuclear replication compartments (4, 10, 35).

To investigate further the role of ICP8 in HSV-1 DNA replication, we have examined the interaction of ICP8 with partially duplex DNA and found that the protein can efficiently melt such duplexes. The ability of ICP8 to destabilize a DNA duplex may help explain the manner in which ICP8 stimulates helicase activities of the HSV-1 origin-binding protein and the DNA helicase-primase.

MATERIALS AND METHODS

Materials. M13mp18 ssDNA (38) was obtained from U.S. Biochemical Corp. The 22- and 100-residue oligonucleotides used to generate M13 DNA hybrids were as described previously (2). Molar concentrations of nucleotides were determined by using extinction coefficients at 260 nm of 8,780 M^{-1} cm^{-1} for ssDNA and 10,000 M^{-1} cm^{-1} for oligonucleotides. These values were converted to molar concentrations of DNA molecules by dividing the molar concentration of nucleotides by the number of bases per molecule. ICP8 was purified from HSV-1-infected U35 cells essentially as described by Hernandez and Lehman (16). The protein was >95% pure as judged by densitometry of a Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel. Its concentration was determined by using an extinction coefficient at 280 nm of 82,720 M^{-1} cm^{-1}, which was calculated from the predicted amino acid sequence by using the formula of Gill and von Hippel (13). Buffer A contained 20 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 10% glycerol, and 0.1 mg of bovine serum albumin per ml. Buffers used in the pH titration were made up as 0.1 M stock solutions, and their pHs were adjusted with NaOH at room temperature.

DNA substrates. Oligonucleotides (10 pmol) were labeled at their 5′ termini with [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham) using T4 polynucleotide kinase according to a protocol supplied by U.S. Biochemical Corp. Unincorporated nucleotide was removed by spin column chromatography (Quickspin DNA, G-25; Boehringer). M13 DNA hybrids were formed by annealing the 5′-^{32}P-labeled oligonucleotides with M13mp18 ssDNA at a ratio of 2:1 in TE buffer (26) containing 0.1 M NaCl. Following a 10-min incubation at 65°C (98°C for the M13–100-mer), the mixture was slowly cooled to <35°C. The M13 DNA hybrids were separated from unannealed oligonucleotide by gel filtration on Bio-Gel A-15m (Bio-Rad).

The DNA substrate used in the directionality experiment was prepared by labeling 50 fmol of M13–100-mer DNA with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear) by using the Sequenase version 2.0 protocol from U.S. Biochemical Corp. Unincorporated nucleotide was removed as

* Corresponding author.

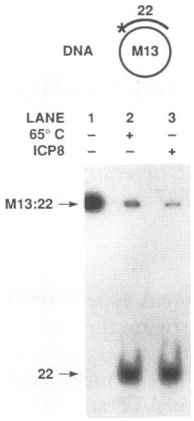

FIG. 1. ICP8-promoted strand displacement. The assay was performed in the presence of M13–22-mer substrate and 0.42 μM ICP8 as described in Materials and Methods. Positions of the DNA substrate and the displaced oligonucleotide are indicated.

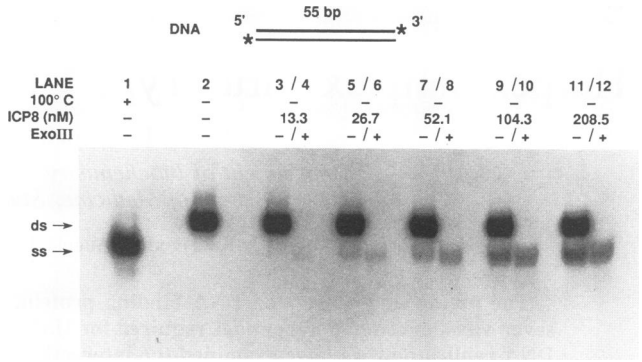

FIG. 2. ICP8-promoted melting of short DNA duplexes. Reactions were performed in duplicate in the presence of 10 nM (molecules) DNA substrate and the indicated concentrations of ICP8 as described in Materials and Methods. Following incubation with ICP8, one set of reactions was treated with 2 U of exonuclease III (ExoIII; New England BioLabs) for 10 min at 37°C to remove residual duplex DNA. Duplex and ssDNA were separated by electrophoresis through a 15% polyacrylamide TBE gel (26). Positions of the native (ds) and heat-denatured (ss) DNAs are indicated.

described above. The 22-residue 3′-^{32}P-labeled and 29-residue 5′-^{32}P-labeled fragments were generated by digestion of the M13–100-mer DNA with restriction endonucleases *Eco*RI and *Hin*dIII (New England BioLabs), respectively. The directionality of strand displacement was also determined with the synthetic 60-mer–20-mer DNA hybrids described by Boehmer et al. (2).

Linear duplex DNA fragments of 55 and 350 bp were prepared by digesting plasmids pUC19 (38) and pBR322 (3) with restriction endonucleases *Eco*RI and *Hin*dIII and with *Bam*HI and *Hin*dIII (New England BioLabs), respectively. The DNA fragments were filled in at their 3′ termini in the presence of [α-^{32}P]dATP (3,000 Ci/mmol; New England Nuclear) by using the Sequenase version 2.0 protocol from U.S. Biochemical Corp. Unincorporated nucleotide was removed as described above.

Strand displacement assay. Unless otherwise indicated, reactions (10 μl) were performed at 37°C for 15 min in buffer A containing 0.5 nM (molecules) DNA substrate and the given concentrations of ICP8. Reactions were terminated by the addition of 2 μl of 90 mM EDTA (pH 8.0)–6% sodium dodecyl sulfate–30% glycerol–0.25% bromophenol blue–0.25% xylene cyanol, and the products were electrophoresed through 12% polyacrylamide TBE gels (26). Following electrophoresis, the gels were dried onto DE81 paper (Whatman) and exposed to X-ray film (X-OMAT AR 5; Kodak) at −80°C. The products were quantitated by counting the radioactive gel slices in Ready-Safe scintillant (Beckman).

RESULTS

Helix destabilization activity of ICP8. ICP8 was tested for its ability to displace a 22-residue oligonucleotide annealed to M13 ssDNA. As shown in Fig. 1, saturating concentrations of ICP8 were capable of destabilizing the M13–22-mer hybrid, resulting in the appearance of free oligonucleotide. Similar results were obtained when the M13–100-mer was used (see Fig. 5). In addition, ICP8 could promote the melting of a fully duplex 55-bp DNA fragment (Fig. 2). However, melting of the 55-bp fragment was not as efficient and did not appear to be cooperative. In contrast, ICP8 could not melt a 350-bp duplex DNA fragment even at ICP8 concentrations exceeding saturating levels (>100 nM) and irrespective of the presence of a 4-nucleotide overhang (data not shown). Similarly, ICP8 could not denature linearized pUC19 DNA (2,686 bp) (data not shown).

Lack of directionality of strand displacement. The DNA substrate diagramed in Fig. 3 was used to determine whether ICP8-promoted strand displacement proceeded with a defined polarity. As shown in Fig. 3, ICP8 displaced the 3′- and 5′-end-labeled oligonucleotides with equal efficiency. Similarly, ICP8 could displace 20-residue oligonucleotides annealed to both the 5′ and 3′ ends of a 60-residue oligonucleotide (data not shown). Thus, ICP8-promoted strand displacement does not proceed with a specific directionality.

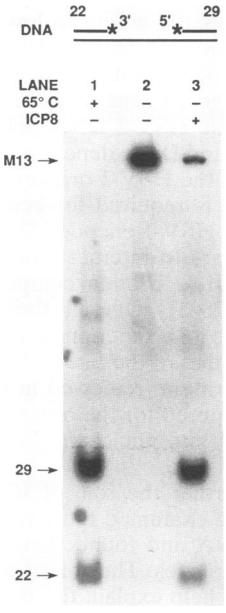

FIG. 3. ICP8-promoted strand displacement lacks directionality. The assay was performed in the presence of 0.27 μM ICP8 as described in Materials and Methods. Positions of the DNA substrate and the displaced oligonucleotides are indicated.

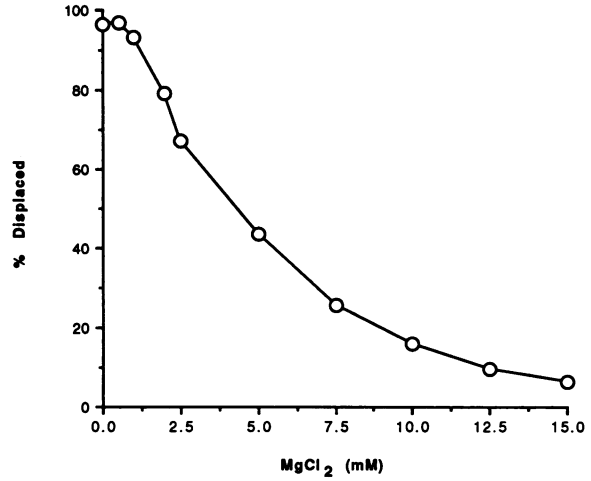

FIG. 4. Strand displacement as a function of $MgCl_2$ concentration. The assay was performed in the presence of M13–22-mer substrate, 0.27 μM ICP8, and the indicated concentrations of $MgCl_2$ as described in Materials and Methods.

Strand displacement is inhibited by Mg^{2+}. As shown in Fig. 4, the ICP8-promoted strand displacement reaction does not require Mg^{2+}. It is in fact inhibited at moderate Mg^{2+} concentrations, with approximately 90% inhibition at 15 mM Mg^{2+}. ATP had no effect on strand displacement (data not shown).

Strand displacement is highly cooperative. The experiments described thus far were performed at saturating concentrations of ICP8, assuming a stoichiometry of 1 ICP8 per 22 nucleotides of ssDNA (16). As shown in Fig. 5, the strand displacement reaction was highly cooperative for both the 22-residue and the 100-residue oligonucleotides. The concentration of ICP8 required for displacement of the 22-mer corresponded to a ratio of approximately 1 ICP8 molecule per 18 nucleotides of ssDNA. Displacement of the 100-mer required somewhat higher levels of ICP8, corresponding to a ratio of 1 ICP8 per 12 nucleotides of ssDNA.

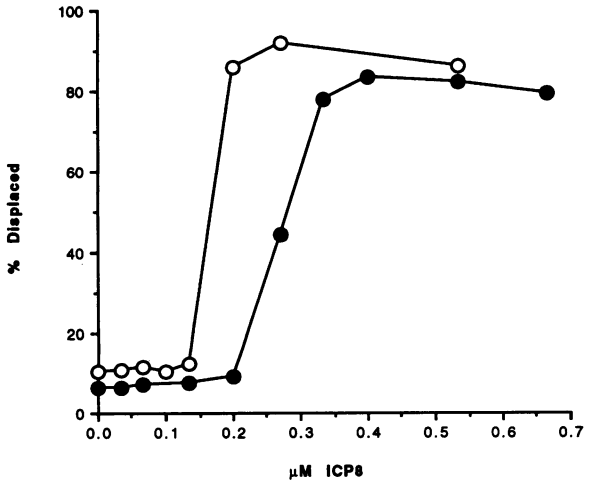

FIG. 5. Cooperative strand displacement promoted by ICP8. The assay was performed in the presence of M13–22-mer (○) and M13–100-mer (●) substrates and the indicated concentrations of ICP8 as described in Materials and Methods.

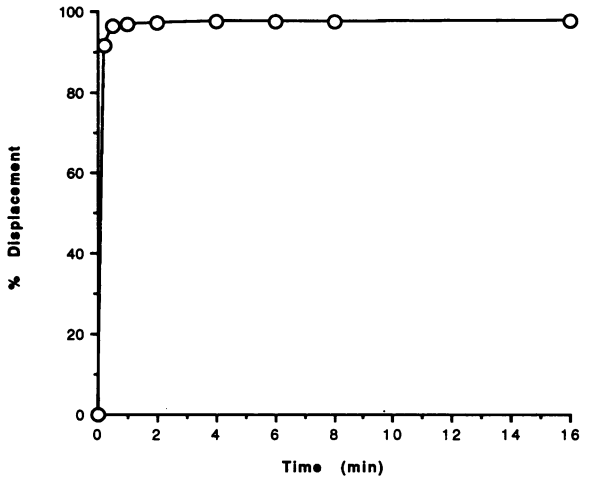

FIG. 6. Kinetics of strand displacement. The assay was performed in reaction mixtures (85 μl) containing M13–22-mer substrate and 0.27 μM ICP8 as described in Materials and Methods. At the times indicated, 10-μl aliquots were removed to determine the extent of the reaction.

Kinetics of strand displacement. The strand displacement reaction is extremely rapid, reaching completion within 30 s. The reaction was also very efficient, proceeding to nearly 100% displacement (Fig. 6).

Effects of ionic strength and pH on strand displacement. Figure 7 shows the effects of increasing ionic strength and pH on the strand displacement reaction. The addition of salt had an inhibitory effect (Fig. 7A). $(NH_4)_2SO_4$ was more inhibitory than NaCl, which in turn was more inhibitory than potassium glutamate. Depending on the type of salt, there was less than 20% strand displacement at physiological ionic strength (150 mM). Strand displacement occurred over a wide range of pHs (pH 6.0 to 8.5) (Fig. 7B). However, the activity decreased to approximately 10% at pHs greater than 10.

DISCUSSION

We have investigated the interaction of the HSV-1-encoded SSB ICP8 with partial DNA duplexes, and we have shown that ICP8 is capable of rapid and efficient displacement of oligonucleotides annealed to M13 ssDNA. ICP8 can also promote the melting of short (55-bp) but not long (350-bp) fully duplex DNA fragments.

The observations that ICP8-promoted strand displacement is ATP independent and that it proceeds without directionality suggest that the reaction does not proceed by a conventional helicase mechanism. These characteristics are consistent with the helix-destabilizing properties of other SSBs. Collectively, these proteins have a higher affinity for ssDNA than for double-stranded DNA and thus promote the melting of duplex DNA by trapping single-stranded regions of DNA that occur spontaneously as a result of "breathing." The finding that increasing Mg^{2+} and ionic strengths inhibit ICP8-promoted strand displacement is probably due to their stabilization of duplex DNA (32). However, higher salt concentrations and alkaline pH may reduce the binding of ICP8 to DNA, thereby causing inhibition (7). Given that ICP8 is known to interact cooperatively with ssDNA (17, 24), it is not surprising that the strand displacement reaction is highly cooperative, requiring saturating levels of protein.

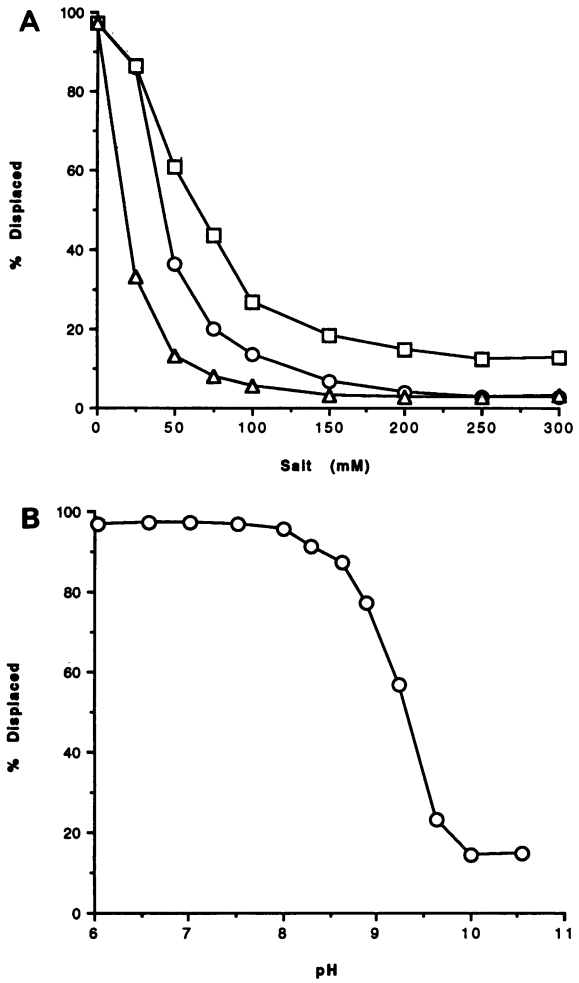

FIG. 7. Effect of ionic strength and pH on strand displacement. The assays were performed in the presence of M13–22-mer substrate and 0.27 μM ICP8 as described in Materials and Methods. (A) Effect of increasing concentrations of NaCl (○), potassium glutamate (□), and $(NH_4)_2SO_4$ (△). (B) pH titration. The following buffers were used at 50 mM: MES [2-(*N*-morpholino)ethanesulfonic acid], pHs 6.03 and 6.58; MOPS [3-(*N*-morpholino)propanesulfonic acid], pH 7.01; HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.51; EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid)], pHs 8.01, 8.3, and 8.63; TAPS [*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid], pHs 8.9 and 9.24; CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid], pHs 9.64 and 10.0; and CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], pH 10.54.

The concentration of ICP8 required for strand displacement corresponds to a ratio of 1 ICP8 per 18 and 12 nucleotides for the M13–22-mer and the M13–100-mer, respectively. These values are in the same range as the site size reported in nuclease protection experiments (20) and for the stimulation of the HSV-1 DNA polymerase (16). They are, however, considerably smaller than the site size of 40 nucleotides estimated by electron microscopy (24). As expected for cooperative binding, the strand displacement reaction is very fast and efficient.

E. coli SSB and bacteriophage T4 gene *32* protein can promote the denaturation of synthetic polynucleotides but are incapable of melting natural DNAs (1, 36). In contrast, ICP8 has the ability to denature natural DNAs as well as synthetic polynucleotides. ICP8 may have a stronger affinity for ssDNA than its prokaryotic counterparts and as a consequence acts as a more efficient helix destabilizer. However, ICP8 is limited in the length of DNA that it will denature. It is possible that once ICP8 has gained access to a frayed end of a DNA duplex, it will melt the duplex only if the ratio of ends to internal base pairs is high. Alternatively, the ability of ICP8 to denature duplex DNA may be dictated by the melting properties of the DNA itself (32). Consequently, the longer substrates may not be denatured because they contain regions of high internal stability.

The strand displacement activity of ICP8 may help explain its stimulation of the helicase activities of the HSV-1-encoded DNA helicase-primase and origin-binding protein (2, 9). Thus, ICP8 may participate directly and specifically in the DNA unwinding mediated by these two enzymes by shifting the equilibrium towards denatured DNA and maintaining this equilibrium. Consequently, these results are consistent with a model in which ICP8 participates in viral DNA replication by aiding in the unwinding of the origin and during propagation of the replication fork.

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