ICP8, the herpes simplex virus type-1 single-strand DNA-binding protein, was recently shown to promote strand exchange in conjunction with the viral replicative helicase (Nimonkar, A. V., and Boehmer, P. E. (2002) \textit{J. Biol. Chem.} 277, 15182–15189). Here we show that ICP8 also catalyzes strand invasion in an ATP-independent manner. Thus, ICP8 promotes the assimilation of a single-stranded donor molecule into a homologous plasmid, resulting in the formation of a displacement loop. Invasion of a homologous duplex by single-stranded DNA requires homology at either 3′ or 5′ end of the invading strand. The reaction is dependent on the free energy of supercoiling and alters the topology of the acceptor plasmid. Hence, strand invasion products formed by ICP8 are resistant to the action of restriction endonucleases that cleave outside of the area of pairing. The ability to catalyze strand invasion is a novel activity of ICP8 and the first demonstration of a eukaryotic viral single-strand DNA-binding protein to promote this reaction. In this regard ICP8 is functionally similar to the prototypical prokaryotic recombinase RecA and its eukaryotic homologs. This strand invasion activity of ICP8 coupled with DNA synthesis may explain the high prevalence of branched DNA structures during viral replication.

The Herpes Simplex Virus Type-1 Single-strand DNA-binding Protein (ICP8) Promotes Strand Invasion

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ICP8, the herpes simplex virus type-1 single-strand DNA-binding protein, was recently shown to promote strand exchange in conjunction with the viral replicative helicase (Nimonkar, A. V., and Boehmer, P. E. (2002) \textit{J. Biol. Chem.} 277, 15182–15189). Here we show that ICP8 also catalyzes strand invasion in an ATP-independent manner. Thus, ICP8 promotes the assimilation of a single-stranded donor molecule into a homologous plasmid, resulting in the formation of a displacement loop. Invasion of a homologous duplex by single-stranded DNA requires homology at either 3′ or 5′ end of the invading strand. The reaction is dependent on the free energy of supercoiling and alters the topology of the acceptor plasmid. Hence, strand invasion products formed by ICP8 are resistant to the action of restriction endonucleases that cleave outside of the area of pairing. The ability to catalyze strand invasion is a novel activity of ICP8 and the first demonstration of a eukaryotic viral single-strand DNA-binding protein to promote this reaction. In this regard ICP8 is functionally similar to the prototypical prokaryotic recombinase RecA and its eukaryotic homologs. This strand invasion activity of ICP8 coupled with DNA synthesis may explain the high prevalence of branched DNA structures during viral replication.

1 The herpes simplex virus type-1 (HSV-1) is a double-stranded DNA virus with a genome of ~152 kbp (1). The HSV-1 genome undergoes a high frequency of homologous recombination in a process that is temporally linked to viral DNA replication (2). Recently, we proposed a model for HSV-1 recombination in which strand exchange is mediated by two essential DNA replication proteins and follows a single-strand annealing and helicase-mediated heteroduplex extension mechanism (3). In particular, ICP8, the viral single-strand DNA-binding protein (SSB) utilizes its helix destabilizing and reannealing activities to promote intermolecular pairing of homologous DNA. Heteroduplex DNA intermediates formed in this fashion are further processed by helicase-mediated branch migration catalyzed by the replicative DNA helicase-primase.

Viral DNA replication intermediates include a high prevalence of branched structures that presumably arise due to strand invasion coupled to DNA synthesis (4). To account for this phenomenon, we examined the ability of ICP8 to promote strand invasion. Here we describe the novel finding that ICP8 promotes assimilation of single-stranded (ss) DNA into homologous supercoiled acceptor DNA, resulting in the formation of a displacement loop (D-loop).

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—\textit{Escherichia coli} SSB (E-SSB), RecA, and exonuclease I were purchased from U. S. Biochemical Corp. RecA concentrations are expressed in moles of monomeric protein, while those of E-SSB are expressed in moles of tetramer protein. DNA topoisomerase I (calf thymus) and proteinase K were purchased from Amersham Biosciences and Roche Molecular Biochemicals, respectively. Bacteriophage T4 polynucleotide kinase, E. coli, RecA, and all restriction endonucleases were obtained from New England Biolabs. ICP8 was purified as described previously (5). Its concentration, expressed in moles of monomeric protein, was determined using an extinction coefficient of 82,720 M cm⁻¹ at 280 nm calculated from its predicted amino acid sequence (6). ATP (disodium salt) and chloroquine (diphosphate salt) were purchased from Sigma. γ-[^32]P]ATP (4,500 Ci/mmol) and H_3[^32]PO_4 were purchased from ICN Biomedicals.

Nucleic Acids—The following oligodeoxyribonucleotides, each with the indicated region of complementarity to the minus strand of pUC18, were synthesized by Sigma: PB9, 22-mer (422–444) (7); PB11, 100-mer (379–478) (7); PB136 (5′-GTA AAA CGA CGG CCA GTG CCA AGC TTG CAT GCC TGG TAC AGC ACT GTA TGG CTC GAT CTC CAG TCT CAC AGT GCT C GAG GAT CCC CGG GTA CCG AGC TCG AAT ATG TGA CTG GTA ACT GTC CGT CAG TCG CAG CTC CAT ACA CTC CAA AGT GCT C), complementarity (379–428); PB137 (5′-ATG TGA CTG GTA ACT GTC CTC GGT CAG CTC CAG TCT CAC ACA CTC CAA AGT GCT C GAG GAT CCC CGG GTA CCG AGC TCG AAT ATG TAA TCA TCA GTG CTT CT), 104-mer with 50 nucleotides 5′ complementarity (379–428); and PB142 (5′-GTT ATT GCA TGA TGA AAG CCC GGC TG), 68-mer with 22 nucleotides internal complementarity (422–444). Their concentrations were determined using extinction coefficients at 260 nm of 208918.6, 939208.1, 951107.4, 3.5 nM, and incubation was continued for 30 min at 30 °C. RecA-acceptor plasmid. Strand invasion was initiated by adding pUC18 form I DNA in a buffer containing 25 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, and 100 μg/ml bovine serum albumin. Strand invasion was initiated by adding pUC18 form I DNA (3.5 nM), and incubation was continued for 30 min at 30 °C. RecA-mediated strand invasion was performed under the same conditions using 3.5 μM protein. The ability of E-SSB to promote strand invasion was also examined under the same conditions using 0.25 μM protein.

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Reactions were quenched by the addition of termination buffer (final concentration: 2% SDS, 50 mM EDTA, and 3 μg/ml protease K) followed by incubation for 10 min at 30 °C. The reaction mixtures were resolved by electrophoresis through 1% agarose-Tace acetate EDTA, pH 7.6, gels at 8 V/cm for 1.5 h. The gels were dried onto DE81 chromatography paper (Whatman), analyzed, and quantitated by storage phosphor analysis with an Amersham Biosciences Storm 820. When necessary, prior to drying, gels were stained with 0.1 μg/ml ethidium bromide (EtBr) for 45 min and analyzed by ultraviolet transillumination using a Bio-Rad VersaDoc 1000 imaging system.

Chloroquine-Agarose Gel Electrophoresis—Strand invasion reaction products were extracted using the Promega Wizard DNA cleanup system and electrophoresed through 1% agarose-Tace acetate EDTA, pH 7.6, gels containing 0, 2, 4, 10, 25, 100, and 200 μg/ml chloroquine at 4.5 V/cm for 3.5 h (8). Chloroquine was also included in the electrophoresis and loading buffers at the respective concentrations.

RESULTS

ICP8 Catalyzes Strand Invasion—We have examined the ability of ICP8 to catalyze strand invasion in vitro. The substrates for this reaction were acceptor DNA, consisting of form I pUC18 DNA, and donor DNA, consisting of a homologous 5′-32P-labeled 100-mer (PB11). Pairing between these substrates was measured with an electrophoretic mobility shift assay of deproteinized reaction products as described under “Experimental Procedures.”

As shown in Fig. 1A, ICP8 results in the assimilation of the 100-mer into homologous form I acceptor DNA, presumably leading to the formation of a D-loop (lane 1). The concentration of ICP8 used was 2.5-fold in excess to that required to coat the 100-mer (9). Under these conditions, up to 15% of the form I acceptor DNA participated in D-loop formation. Strand invasion does not require a high energy co-factor, since D-loops were formed in the absence of ATP (lane 2). ICP8 and homologous acceptor DNA are indispensable for D-loop formation, since products failed to form in the absence of ICP8 (lane 3) or pUC18 (lane 4). Substitution with a heterologous acceptor DNA (pUC19) also prevented D-loop formation (lane 5). The reaction is greatly stimulated by Mg2+, since its omission resulted in drastic reduction in D-loop formation (lane 6). At a concentration of E-SSB equivalent to that of ICP8 and 4-fold in excess to that required to coat the 100-mer (assuming a site size of 40 nucleotides/tetramer), only negligible amounts of D-loops were formed (lane 7). Likewise, no significant D-loops were formed with coating concentrations of human replication protein A (RP-A) or T4 gp32 protein (data not shown). The products of ICP8-mediated strand invasion exhibited a electrophoretic mobility identical to those formed by the action of RecA (lane 8).

Fig. 1, B and C, are EtBr-stained and storage phosphor images of the same gel that compare the ability of three topological forms of acceptor DNA to participate in D-loop formation. D-loops were formed with form I (lane 1) but not with forms IV or III (lanes 2 and 3) DNA, indicating that strand invasion is dependent on the free energy of supercoiling.

ICP8 Promotes Complete Assimilation of a 100-mer into a Homologous Plasmid—To determine whether ICP8 can facilitate complete invasion of the donor 100-mer into the acceptor DNA, we examined the susceptibility of the resulting D-loops to digestion with ssDNA exonucleases. D-loops were resistant to digestion with exonuclease I (3′-5′ exonuclease) as well as RecJ (5′-3′ exonuclease) when analyzed by electrophoresis through a 1% agarose gel (Fig. 2A). Quantitative analysis indicates that the amount of 100-mer protected from both exonuclease I and RecJ digestion is equal to the amount of 100-mer participating in D-loop formation (~5%). It should be noted that exonuclease digestion products (5′-32P-labeled dGMP and d[pGpT]) for RecJ and exonuclease I, respectively) migrate more slowly through 1% agarose gels than the 100-mer. Fig. 2B confirms that the concentrations of exonucleases used were sufficient to completely degrade the donor 100-mer. Since the 32P label of the invading 100-mer is at the 5′ end, resistance to RecJ demonstrates that the 5′ end of the 100-mer was stably assimilated into the acceptor DNA (Fig. 2A, lane 3). To ascertain that the 3′ end of the 100-mer was fully incorporated into the donor DNA, exonuclease I digestion products were also resolved by denaturing polyacrylamide gel electrophoresis. Fig. 2C shows that the 100-mer involved in D-loops was not shortened by treatment with exonuclease I, thereby indicating that the 3′ end of the 100-mer was also stably integrated into the acceptor DNA (compare lanes 3 and 4). The amount of 100-mer that was resistant to degradation in Fig. 2C, lane 4 (~5%), corresponds to the amount of 100-mer participating in D-loop formation (~5%) (Fig. 2A, lane 1).

ICP8-mediated D-loop Formation Requires a Homologous End—D-loop formation was examined with a variety of oligonucleotide donors that were either completely homologous to the acceptor plasmid (PB11, 100-mer and PB9, 22-mer) or possessed heterology at their 3′ (PB136, 100-mer), 5′ (PB137, 104-mer), or both (PB142, 68-mer) ends. Fig. 3 shows that long
oligonucleotides possessing heterology at either 3' or 5' ends (data not shown). However, oligonucleotide PB142 (68-mer) possessing a 23-nucleotide heterology at both ends failed to participate in D-loop formation (lane 8). A control oligonucleotide (PB9) with an equivalent length of homology as PB142 (22 nucleotides) was efficiently assimilated into the acceptor plasmid, indicating that the failure of PB142 to form D-loops was not due to insufficient homology but rather due to the lack of homology at the ends (lane 10).

ICP8-promoted Strand Invasion Perturbs the Topology of the Superciled Acceptor Plasmid—Upon electrophoresis through a 1% agarose gel, D-loops and acceptor DNA have a similar mobility (Fig. 4A, lanes 2 and 3). To resolve differences in topology between these two species, we examined their migration through 1% agarose gels containing varying concentrations of chloroquine. Intercalation of chloroquine into negatively supercoiled DNA leads to DNA unwinding (10). This at first reduces the superhelicality (relaxation) of the plasmid, concomitantly reducing its electrophoretic mobility (compare lanes 2 of Fig. 4, A–D). However, as more chloroquine intercalates into the DNA, the relaxed plasmid is converted into positively supercoiled DNA, increasing its electrophoretic mobility (compare lanes 2 of Fig. 4, D–G). Chloroquine does not reduce the electrophoretic mobility of D-loops, indicating that they are not unwound by the intercalating agent. On the contrary, D-loops migrate faster with increasing chloroquine concentrations (compare lanes 3 of Fig. 4, A–G). This suggests that assimilation of the 100-mer alters the superhelicity density by overwhelming the acceptor plasmid. Electrophoresis in the presence of chloroquine also resolves an additional species. We believe that this species is form X DNA, which is presumably generated due to ICP8-mediated unwinding of the acceptor DNA involved in D-loops (Fig. 4G, lane 3). This observation is similar to that hypothesized for RecA where extensive RecA filament formation extending from the D-loop into the duplex portion of the plasmid causes unwinding and accumulation of positive supercoils in the plasmid (11, 12).

Since D-loops exhibit a modified topology (Fig. 4), we predicted that they would show altered sensitivity to restriction endonucleases. D-loops were digested with the following enzymes: HindIII and EcoRI that cleave in the region of pairing at co-ordinates 399 and 450, respectively, AluNI and AflIII that cleave outside the region of pairing at co-ordinates 1217 and 806, respectively. The EtBr-stained image serves as an internal control to show that the acceptor DNA (pUC18) was linearized by all four enzymes (Fig. 5A, lanes 1, 2, 4, and 5). Storage phosphor analysis of the same gel shows that acceptor DNA involved in D-loops was cleaved by HindIII and EcoRI but not by AluNI or AflIII (Fig. 5B, lanes 1, 2, 4, and 5). In addition, D-loops were resistant to cleavage by BarBI and DraI both of which have three recognition sites outside of the region of pairing at coordinates 498, 739, and 2540, respectively, with specific mobilities extending from the D-loop into the duplex portion of the plasmid (Fig. 4F, lanes 7–9). These results suggest that the D-loops were resistant to cleavage by the restriction endonucleases that are located outside of the region of pairing.
reaction. The concentration of chloroquine is indicated below each panel. The solid lines /H9261 (lower G lanes 1 respectively.

**Experimental Procedures**

Wizard DNA purification system. Samples were resolved on a 1% agarose gel (H9262) and 1% agarose gels containing 2, 4, 10, 25, 100, and 200 μg/ml chloroquine (B–G). Lane 1 of each panel, 32P-labeled λ-HindIII marker; lane 2 of each panel, 32P-labeled pUC18 form I; lane 3 of each panel, D-loop reaction. The concentration of chloroquine is indicated below each panel. The solid lines indicate the migration of the ~23 kbp (upper) and 2 kbp (lower) λ-HindIII fragments. The dashed line indicates the positions of form I and D-loops in A, in the absence of chloroquine. The positions of D-loops and of form X DNA are indicated in G.

**FIG. 5.** ICP8-promoted D-loops are resistant to the action of restriction endonucleases. Susceptibility of D-loops to restriction endonuclease digestion. D-loops were formed as described under “Experimental Procedures” and digested with 20 units of enzyme for 1 h. A and B are EtBr-stained and storage phosphor images of the same gel, respectively. Lanes 1, 2, 4, and 5, HindIII, EcoRI, AluNI, and A/III, respectively; lane 3, untreated D-loops. C and D are EtBr-stained and storage phosphor images of the same gel, respectively. Lanes 1–3, untreated D-loops, DraI, and BsrBI, respectively. The positions of 100-mer, D-loops, and of DNA forms I and III are as indicated. ✂ indicate the relative cleavage sites of HindIII (❶), EcoRI (❶), AluNI (❶), and A/III (❶). The block in cleavage for AluNI and A/III is as indicated. The schematics shown above A and B depict the topologies of form I DNA and D-loops. The arrowheads at the right of C indicate the positions of the DraI and BsrBI digestion products. It should be noted that the 1.9-kbp BsrBI digestion product co-migrates with pUC18 form I.

with deproteinized D-loops as well as with ongoing D-loop reactions (i.e. in the presence of ICP8), indicating that triple helical structures, which would presumably interfere with cleavage, are not the stable end products.

**DISCUSSION**

Using a classical *in vitro* assay, we have demonstrated that ICP8 promotes strand invasion. This reaction is distinct to its helix destabilizing and reannealing activities and to its ability to promote strand exchange in conjunction with the viral replicative helicase (3). To our knowledge, ICP8 is the first eukaryotic viral SSB shown to mediate this reaction. It has previously been pointed out that several thermodynamic parameters of ICP8 are closer to those of known recombinases (e.g. *E. coli* RecA and T4 UvsX protein) than they are to other SSBS (e.g. E-SSB, RP-A, and T4 gp32) (9). Thus, like *bona fide* recombinases, ICP8 stretches ssDNA, possibly to facilitate the search for homology, forms complexes with ssDNA that are stable at high salt concentrations and exhibits both weaker and lower cooperativity ssDNA binding than other SSBS. Although ICP8 promotes D-loop formation, its ability to do so is relatively weak (up to 15% product formation) when compared with the prototypical *E. coli* RecA recombinase (~40% product formation under our reaction conditions that lack an ATP regenerating system). However, the efficiency of the reaction promoted by ICP8 is comparable with that achieved by eukaryotic RecA counterparts such as yeast Rad51, which only promotes ~2% product formation (13).

An important feature of ICP8-mediated strand invasion is that it does not require ATP. This distinguishes ICP8 from the RecA-type recombinases (e.g. *E. coli* RecA and eukaryotic Rad51) that require ATP binding to catalyze strand invasion (14, 15). ICP8 presumably utilizes the energy stored in the negatively supercoiled form I DNA to drive the reaction. D-loop formation was minimal in the absence of Mg2+, whereas concentrations ranging from 5 to 50 mM Mg2+ greatly stimulated the reaction. The low efficiency of D-loop formation in the absence of Mg2+ can be rationalized by the fact that ICP8 is more proficient at helix destabilization in the absence of Mg2+ and may therefore lead to dissociation of D-loops under these conditions (3). In addition, stimulation by Mg2+ may be related to the fact that ICP8-mediated pairing is Mg2+-dependent (3).

Our data indicate that ICP8 mediates complete assimilation of a donor 100-mer into the acceptor plasmid, which, to our knowledge, has not been demonstrated with other strand invasion proteins such as RecA or Rad51. The stability of D-loops formed by ICP8 following deproteinization indicates that these structures entail pleiotropic DNA interactions. The results obtained with oligonucleotides possessing heterology at 5’ or 3’ ends provides evidence that ICP8-mediated D-loop formation
can initiate at either end. Moreover, donor DNA with heterology at both ends did not form D-loops, thereby indicating the need for a homologous end. It may be possible for a substrate with double heterology to form parameric D-loops. However, such intermediates are unstable and would dissociate upon deproteinization. The lack of bias toward an end is in contrast to either RecA or Rad51, which preferentially lead to assimilation of 3' and 5' ends, respectively (13).

Strand invasion by ICP8 alters the topology of the acceptor plasmid as evidenced by anomalous migration in chloroquine-containing gels and resistance to restriction endonucleases that cleave outside the area of pairing. ICP8 also generates form X DNA, which has previously been described for RecA-mediated D-loop formation (11, 12). Generation of form X DNA requires protein-induced unwinding of the acceptor DNA, starting at the point of assimilation of the donor DNA. This generates compensatory positive supercoils allowing such structures to be resolved from normal D-loops by agents (e.g. chloroquine) that alter the writhing number.

The exact mechanism for strand invasion remains unclear. Two competing mechanisms have been proposed (reviewed in Ref. 16). According to the first mechanism (R-form hypothesis), the donor nucleoprotein filament forms a canonical DNA triple helix involving non-Watson-Crick base pairing. The second mechanism (base-flipping model) states that the donor nucleoprotein filament induces base-flipping in one strand of the acceptor DNA, thereby permitting a homology search between the protein bound oligonucleotide and the flipped bases based on Watson-Crick interactions. Our data suggest that ICP8-mediated D-loop formation occurs by the later model. This conclusion is based on the susceptibility of D-loops to restriction endonucleases that cleave in the region of pairing, whereas they would otherwise be resistant if the D-loops were to possess triple helical character (17).

Another protein that has been shown to promote strand invasion is E. coli RecT (18). Although RecT- and ICP8-mediated D-loop formation occur under similar conditions and at similar protein to ssDNA ratios, the ICP8-mediated reaction is greatly stimulated by Mg2+2, while the RecT-mediated reaction is Mg2+-independent and inhibited with increasing Mg2+. The major difference between these two proteins, however, is that ICP8 functions as an SSB, while the RecT-mediated reaction is dependent on its interaction with both ss and duplex DNA (1, 18).

During its replicative cycle, multiple concatemeric HSV-1 genomes are generated by rolling circle replication. Highly branched networks of DNA are prevalent at later times during replication (4). These may arise from intra- and interconcatemeric recombination. The strand invasion activity of ICP8 may be pivotal in the formation of such structures. Thus, we envisage that ICP8 mediates the invasion of ssDNA into homologous regions. Invading 3’-terminal strands would presumably prime DNA synthesis and lead to the formation of replication forks that would ultimately result in the formation of branched DNA structures. This process may be initiated by double-strand DNA breaks that arise due to a variety of reasons (e.g. collapsed replication forks, endonuclease G cleavage at viral a sequences (19), and DNA damaging agents). ssDNA required for strand invasion may be generated by the helix destabilizing activity of ICP8, possibly in conjunction with the viral replicative helicase or by exonucleolytic processing of broken DNA ends (3).

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