Herpes Simplex Virus 1 Single-strand DNA-binding Protein (ICP8) Will Promote Homologous Pairing and Strand Transfer

Carl Bortner¹, Thomas R. Hernandez², I. Robert Lehman² and Jack Griffith¹†

¹Linesberger Comprehensive Cancer Center
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7295, U.S.A.

²Department of Biochemistry, Beckman Center
Stanford University School of Medicine
Stanford, CA 94305-5307, U.S.A.

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The herpes simplex virus type 1 encoded ICP8 protein binds single-stranded (ss) DNA and is required for DNA replication in vitro. We have used electron microscopy to examine the ability of ICP8 to promote homologous pairing and strand transfer reactions. Visualization of M13 ssDNA–ICP8 complexes showed that they preferentially bound and enveloped homologous double-stranded (ds) DNA fragments; their deproteinization released ssDNA circles containing dsDNA segments, and an equal number of linear single strands. Optimal transfer required Mg²⁺ but not nucleoside triphosphates, and showed a fourfold preference for dsDNA fragments with a few bases recessed ends. Gel electrophoretic analysis confirmed the strand transfer activity of ICP8.

Keywords: ICP8 protein; electron microscopy; DNA strand transfer; herpes simplex virus

1. Introduction

The pathways of DNA replication, recombination and repair are often interrelated (Kornberg & Baker, 1991). Our understanding of the molecular mechanisms of homologous recombination began with detailed genetic analysis of bacteria and bacteriophage mutants that exhibited reduced frequencies of homologous recombination and increased sensitivity to DNA-damaging agents. These studies identified the RecBCD pathway in Escherichia coli of which the RecA protein is central (Clark, 1973), and the uvsX, Y, Z pathway in T4 phage (Cunningham & Berger, 1977) in which UvsX protein is a functional analog of RecA. Other pathways of homologous recombination may exist in E. coli, for example the recE and recF pathways (reviewed by Clark, 1991) that use a more restricted set of DNA templates than the broad range of DNAs utilized by RecA and UvsX proteins (for reviews, see Cox & Lehman, 1987; Griffith & Harris, 1988; Eggleston & Kowalczowski, 1991). Indeed, recent studies by Kolodner and colleagues (Luisi-DeLuca et al., 1988; Hall, Kane & Kolodner, personal communication) have shown that the recE gene encodes an exonuclease (exonuclease VIII) and a strand reannealing activity (RecT protein) suggesting that this pathway may catalyze transfers utilizing linear double-stranded (ds) DNA.

The identification of eukaryotic proteins with homologous pairing and strand transfer activity has followed a route beginning with their isolation and proceeding toward their genetic characterization. The activities of RecA protein suggested simple in vitro assays, in particular those that detect the transfer of a strand from a linear dsDNA fragment onto a homologous single-stranded (ss)DNA circle. Using such assays, transferase activities have been identified from a variety of eukaryotic sources (Hsieh et al., 1986; Kolodner et al., 1987; Fisher et al., 1988; McCarthy et al., 1988; Halbrook & McEntee, 1989). Like RecA and UvsX, these proteins are required in nearly stoichiometric amounts relative to the ssDNA. Unlike RecA and UvsX proteins, they do not require ATP, are larger (120,000 to 160,000 M₉ versus 38,000 to 40,000 M₉) and have not been shown to catalyze parametric

† Abbreviations used: ds, double-stranded; ss, single-stranded; HSV-1, herpes simplex virus type 1; kb, 10³ base-pairs; EM, electron microscopy; DMEM, Dulbecco's-modified Eagle's medium; bp, base-pair(s); SSB, single-stranded DNA-binding protein.
joining. Furthermore, the three best-characterized eukaryotic proteins, the human HPP-1 (Moore & Fishel, 1990), yeast SEP-1 (Johnson & Kolodner, 1991) and Droso phila Erp-1 (Sunder et al., 1991) proteins all contain an intrinsic or tightly associated exonucleolytic activity which may be required for the initial synapsis step.

Herpes simplex virus type 1 (HSV-1), like T4 phage, has a large genome (152 kilobase-pairs: kb) that codes for many of its own DNA replication enzymes including a DNA polymerase, a single-strand DNA-binding protein, an origin binding protein, and a helicase-primase (reviewed by Crute et al., 1990; Olivo & Chalberg, 1990). It has a well-established genetics, and possibly several active recombination systems. An inversion occurs between the long and short (L and S) segments of HSV-1 DNA following infection (Sheldrick & Berthelet, 1975) and introduction of different HSV-1 strains into the same cell rapidly leads to the appearance of a mixture of the viral genomes in the progeny (reviewed in Roizman & Sears, 1990). The L-S inversion is typical of site-specific recombination while the phenotypic mixing is suggestive of homologous recombination. If HSV-1 codes for a protein that catalyzes homologous pairing and strand transfer, one candidate is ICP8 (Bayliss et al., 1975). Originally termed VP143 (Hones & Roizmann, 1973), ICP8 corresponds to the HSV-2 infected cell protein ICP 11/12 (Powell et al., 1981), and is an early protein of 138,000 M, that binds preferentially to ssDNA. ICP8 has been shown to stimulate DNA replication in vitro (Ruychan & Weir, 1984; Hernandez & Lehman, 1990) and to be involved in the nuclear localization of the HSV-1 replication machinery (Quinan et al., 1984). The location of the ICP8 gene on the HSV-1 genome has been mapped (Weller et al., 1983) and numerous mutants, both temperature sensitive and deletions affecting ICP8 function have been reported (Conley et al., 1981; reviewed by Gao & Knipe, 1989).

Here we report the use of electron microscopy (EM) to search for conditions under which ICP8 will catalyze homologous pairing and strand transfer. We find that the complexes formed between ICP8 and ssDNA rapidly bind and envelop homologous linear dsDNA. Deproteinization of these complexes releases products that are typical of a strand transfer reaction: ssDNA circles with dsDNA segments and an equal number of displaced ssDNA fragments. Strand transfer is dependent on Mg but not ATP or single-strand binding cofactors. The vigorous deproteinization required to observe the strand transfer products suggests that a complete cycle of strand transfer including the release of the products may require additional factors.

2. Materials and Methods

(a) DNA and enzymes

Phage M13 ss and ds DNAs were isolated as described (Regatte & Griffith, 1986). Growth of P-labeled M13 phage was carried out as described by Webster et al. (1981). Carrier-free 32P-orthophosphoric acid was purchased from New England Nuclear Inc. Restriction enzymes were purchased from Bethesda Research Laboratories (BRL) or New England Biolabs and used as indicated by the vendor.

(b) Purification of ICP8

ICP8 was purified from HSV-1 infected U3S cells, a cell line kindly provided by P. Schaeffer which overexpresses ICP8 when infected by HSV-1 (Orberg & Schaeffer, 1987). U3S cells from 40 confluent flasks (150 cm ) were seeded into 20 roller bottles (1750 cm ; Falcon) containing 500 ml Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Irvine Scientific Inc.). 0.1 ml non-essential amino acids, 2 mM L-glutamine, penicillin (100 units/ml) streptomycin (100 µg/ml) (Gibco), and 10 mM HEPES (pH 7.3) (culture media A). The media was removed from the roller bottles, and HSV-1 (R-Æ565) (Post et al., 1981) was added to a multiplicity of infection (moi) of 10 in 1 ml of DMEM. After adsorption of the virus for 1 h at 37°C, the inoculum was removed and 250 ml of culture media A (without non-essential amino acids or penicillin-streptomycin) was added. Following incubation at 37°C for 18 h, the cells were harvested, and nuclear and cytoplasmic extracts were prepared (Ellis et al., 1986) and frozen in liquid nitrogen.

ICP8 was purified from the nuclear extract as described (Hernandez & Lehmann, 1990; O'Donnell et al., 1987) through the phosphocellulose and heparin-Sepharose steps. ICP8 was identified as 10% (w/v) polyacrylamide-SDS gel electrophoresis followed by staining with Coomassie blue. The leading half of the ICP8 peak on heparin-Sepharose chromatography was essentially nuclease free and was used for all the experiments described. The remainder contained a contaminating exonuclease activity that could be removed by Superose 12 gel filtration. The preparation of ICP8 used was approximately 95% pure. The trace nuclease activity present in the heparin-Sepharose preparation used in the experiments could be removed by MonoQ column chromatography. However, the MonoQ preparation of ICP8 caused severe aggregation of the DNAs and did not promote strand exchange.

Routine nuclease assays were carried out as previously described (Crute et al., 1988). The final assays of exonuclease activity were measured using a J135I digest of pJClOOR that produces 6 fragments of 66 to 1419 base pairs (bp) with a 2-base recessed 3' end. The 5' exonuclease substrate was labeled with 32P at the 5' end, and the 3' substrate was labeled by filling in with a single 32P-labeled nucleotide. Exonuclease activity was expressed in terms of pmol of nucleotide excised per µg of ICP8 in a 10 min reaction using ratios of ICP8 to dsDNA and ionic conditions similar to that of the strand transfer reactions.

(c) ICP8-DNA complex formation

M13 ssDNA was diluted into 20 mM-Tris-Cl (pH 7.5), 80 mM-NaCl, 10 mM-MgCl2, 1 mM-dithiothreitol at a concentration of 1 µg/ml and unless otherwise indicated, all 4 deoxynucleoside triphosphates were added to 300 µM. The mixture was warmed to 37°C for 10 min. ICP8 was then added to a final concentration of 40 µg/ml (from a stock at 500 µg/ml) and the mixture incubated at 37°C for 10 min followed by addition of the dsDNA to 2 µg/ml and continued incubation.
(d) Purification of the DNA products and staining with single-strand binding protein

Following incubation as described above, SDS was added to 1%, and the samples were warmed to 37 to 55°C for 5 min. Proteinase K (100 μg/ml) was then added for 15 min, followed by an equal amount of Proteinase K for an additional 15 min at 55°C. The samples were chroma-tographed over 2 ml Bio-Gel A5M columns equilibrated with 20 mM-Hepes (pH 7.5), 0.1 mM-EDTA. For EM analysis, single-strand DNA-binding protein (SSB) was added to 3 μg/ml at 20°C for 10 min, followed by glutaral-dehyde to 0.6% for 10 min more.

(e) Electron microscopy

Glutaraldehyde fixed complexes of DNA with SSB or ICP8 were adsorbed to freshly glow-charged carbon films in a buffer containing 2 mM-uridine, washed, air-dried and rotary shadow casts with chymot as described (Gri-fth & Christiansen, 1978). Preparation of samples by fast-freezing was carried out as described (Bortner & Griffith, 1980). Briefly, ICP8 complexes were prepared and without fixation, quickly passed through Bio-Gel A5M columns equilibrated with the reaction buffer and immediately absorbed for 5 s onto a thin carbon film supported by a copper mesh grid. The sample was washed in water for 1 min before being plunged into liquid ethane chilled by liquid nitrogen. The frozen sample was transferred to a Wilek-modified Balzers 300 freeze-etch system and the water sublimed for 2 h at -85°C and 1 h at -20°C. The sample was then rotary shadow cast with tungsten at -170°C and 13 x 10^-6 Pa. Micrographs were taken on a Philips EM400 TEG.

3. Results

(a) ICP8-ssDNA complexes formed under recombination conditions are compact filamentous loops

Complexes of ICP8 and ssDNA have been examined by EM before (Rueyetchan, 1983; O'Donnell et al., 1987). However, it was important to examine complexes formed under ionic strengths and cofactor conditions optimal for strand transfer catalyzed by known strand transfer proteins, and using newer EM techniques that do not employ fixation. Nucleic acid protection experiments by O'Donnell et al. (1987) showed that at or above a mass ratio of 37 μg of ICP8 to 1 μg of ssDNA the ssDNA was fully protected. Here unless otherwise noted, ICP8 and M13 ssDNA were incubated together at a mass ratio of 40:1 in a buffer that included 10 mM-MgCl2 and 80 mM-NaCl at 37°C for 30 minutes and then fixed and prepared for EM (see Materials and Methods). Very compact structures (Fig. 1) were observed that depended on both ICP8 and ssDNA. A more gentle means of preparing samples for EM, termed fast-freezing (Heuser, 1983; Bortner & Griffith, 1990) substitutes very rapid freezing in the reaction buffer for chemical fixation followed by removal of the ice by sublimation. ICP8-M13 ssDNA complexes observed following fast-freezing were very similar to those which had been fixed, although finer structural resolution was afforded and occasional complexes appeared as relatively open loops of an ICP8-ssDNA fiber following fast-freezing (Fig. 1A). Using higher or lower ratios of ICP8-ssDNA did not produce any greater extension of the filaments and at 80:1 aggregation of the filaments became more pronounced.

Examination of ICP8-ssDNA complexes formed in the presence of 0 to 100 mM-sodium acetate, with or without 300 μM of each of the four deoxyribonucleoside triphosphates, and with or without 3 mM-ATP revealed similar structures. No binding of ICP8 to dsDNA alone was observed and when ICP8 was incubated in 2 to 10 μM-MgCl2 at 4°C for ten minutes in the absence of DNA, filaments were observed as described by O'Donnell et al. (1987); these filaments also did not bind dsDNA (not shown).

(b) ICP8-ssDNA complexes promote pairing with homologous duplex DNA

Although interactions between ICP8 and dsDNA were not observed it was possible that, by analogy with RecA protein, ICP8-ssDNA complexes might bind homologous dsDNA. Thus ICP8-M13 ssDNA complexes were formed (as in Fig. 1A), incubated with a blunt-ended 1325 bp Ball-Ilpav M13 dsDNA fragment (5 dsDNA molecules per ssDNA circle) and then prepared for EM by fixation or fast-freezing. As shown (Fig. 1B and C) protein-free dsDNA was observed attached to the ICP8-M13 ssDNA complexes by one or both dsDNA ends, with a variable amount of the dsDNA buried within the ICP8-ssDNA complex. In this experiment, 17% of the ICP8-ssDNA complexes had a 1325 bp M13 dsDNA fragment attached after five minutes of incubation. By 15 and 30 minutes, 20% and 38%, respectively of the ICP8-ssDNA complexes had the dsDNA fragment attached.

To provide a population of M13 dsDNA fragments of various sizes that could interact with the ICP8-M13 ssDNA complexes, M13 dsDNA was cleaved with various restriction endonucleases. Cleavage of M13 dsDNA with TaqI produces ten fragments 240 to 2000 bp in length with two-base 3'-recessed ends. Upon incubation of the DNA fragments at 37°C with the ICP8-M13 ssDNA complexes (10 dsDNA fragments per M13 ssDNA circle) for 30 seconds, 33% of the ICP8-ssDNA complexes had an M13 dsDNA fragment attached. Continued incubation for 5, 25, 5, 10, 20, 30 and 60 minutes yielded the following values: 55%, 50%, 47%, 42%, 43%, 36%, and 36%, respectively. The decline may be due to the dsDNA being fully enveloped within the ICP8 complexes and thus invisible since less free dsDNA was observed on the supports at the later times. Incubation of supercoiled M13 dsDNA with the ICP8-M13 ssDNA complexes produced no significant binding, unlike the parallel reaction with RecA or UvX proteins in which rapid, efficient (paranemic) joining is observed.

For comparison, pBR322 DNA was digested with NcoI which produces ten 35 to 725 bp fragments containing single-base 3'-recessed ends. Upon incu-
Figure 1. Visualization of ICP8–DNA complexes. A, ICP8 was assembled onto M13 ssDNA for 30 min in a buffer containing 20 mM-Hepes (pH 7.5), 80 mM-NaCl, 10 mM-MgCl₂. The complexes were quickly chromatographed in this buffer to remove unbound protein and then rapidly frozen. The ice was removed by sublimation at high vacuum and the detail revealed by rotary shadowcasting with tungsten (Materials and Methods). B and C, ICP8–M13 ssDNA complexes were formed (as in A) and then incubated with a fully homologous 1325 bp M13 ssDNA fragment. Here the samples were fixed and processed for EM by a method employing fixation, washing, air-drying and tungsten shadowcasting. Shown in reverse contrast, bar represents 0.1 μm (A), 0.7 μm (B, C).

Determination of the pBR322 DNA with the ICP8–M13 ssDNA complexes for 30 minutes, no interaction between the ICP8 complexes and the pBR322 dsDNA was observed. However, upon prolonged storage of the ICP8 at −70°C, increased aggregation and binding of non-homologous dsDNA was observed. The observation that the ICP8–ssDNA complexes bind and envelop homologous dsDNA suggested that strand transfers may have occurred within these structures.

(c) ICP8 mediates strand transfer

To search for products to strand transfer, ICP8 was assembled onto M13 ssDNA, TaqI digested M13 dsDNA fragments were added (10 dsDNA fragments per ssDNA circle) and the mixture incubated for 30 minutes at 37°C. The samples were deproteinized with SDS and Proteinase K. E. coli SSB protein was then added to thicken and extend the ssDNA making it easy to distinguish from dsDNA by EM. Examination showed that 35% of the
ssDNA circles contained a duplex segment (heteroduplex circles) whose length was within the size range of the TaqI dsDNA fragments (Fig. 2A to D). In addition, short linear ssDNAs bound by SSB protein representing the displaced strand were observed. Heteroduplex circles in the same abundance were observed when the M13 dsDNA was cleaved with other restriction endonucleases (e.g., NlaIII) and (see below) when other deproteinization schemes were employed. When the reactions were carried out using the same weight-to-weight ratio of ICP8 to DNA as above and with equal molar ratios of M13 ssDNA and full length linear dsDNA, no transfers were observed whether the dsDNA ends were blunt (HpaI cleaved) or had a one-base 3' overhang (MboII overhang).

When the strand transfer reactions were carried out in the absence of ICP8, or ICP8 was replaced by E. coli SSB protein, the number of heteroduplex circles counted by EM ranged from 0 to 3% of 150 M13 ssDNA circles scored. The 3% value was observed only when the ratio of dsDNA fragments to ssDNA circles was increased to 40:1 and required scoring any questionable molecule as a heteroduplex. If the non-homologous XhoI digest of pBR322 DNA was substituted for the TaqI digest of M13 dsDNA, no heteroduplex circles were observed in over 300 ssDNA circles examined.

Complete removal of ICP8 from the DNA at the end of the reaction required vigorous deproteinization. Unless otherwise noted, the samples were treated with 1% (w/v) SDS and two sequential additions of proteinase K at 55°C over a 35 minute period. SDS alone did not fully remove ICP8, resulting in aggregation and an artificially low yield of heteroduplex circles. To eliminate any concern over the use of 55°C for deproteinization, strand transfer reactions were carried out as described above for 30 minutes using both the TaqI and NlaIII digests of M13 dsDNA, then trypsin was
added (a 200-fold weight excess over ICP8) for a one hour incubation at 37°C in the presence of 200 mM-Tris (pH 8.0) to digest the ICP8. Strand transfer products were observed at levels comparable to the previous experiments for both dsDNA sets and no heteroduplex circles were observed when ICP8 was omitted.

The heteroduplex circles shown in Figure 2 could result from a strand transfer reaction, or by the passive annealing of a complementary single strand produced by either a helicase that could separate the two strands of the dsDNA, or an exonuclease that could reduce the duplex fragments to single-stranded molecules. Helicase contamination of the ICP8 was unlikely since incubation of dsDNA fragments with ICP8 and SSB protein (to complex any ssDNA produced) yielded no ssDNA molecules as seen by EM, and it was unlikely that SSB would have inhibited any putative helicase action inside the compact ICP8-DNA complexes. Also, as shown below, strand transfer did not require nucleotide cofactors, which are required by all known helicases.

Significant exonuclease contamination of the ICP8 also appeared unlikely. One concern was that a minor fraction (e.g. 10 to 20%) of the dsDNA molecules were rendered fully or extensively single-stranded due to a nuclease action and that these molecules were the ones that participated in the subsequent pairing and strand transfers. To examine this possibility, several different dsDNA fragments were incubated with ICP8 (40:1 mass ratio) under strand transfer conditions for 60 minutes at 37°C, deproteinized and stained with SSB protein. Using SSB staining and EM it is possible to detect single-stranded tails of 20 to 30 nucleotides or greater. In several experiments no SSB-stained tails were detected on over 100 dsDNAs scored, nor were any fully single-stranded fragments observed. Further if ICP8 was loaded onto the ends of dsDNA by providing single-stranded 3' or 5' tails of 20 to 30 nucleotides in length, incubation of these complexes at 37°C under strand transfer conditions did not lead to the elongation of the ssDNA tails as measured by SSB staining and EM. To quantitate the residual exonuclease in the ICP8 following the heparin-Sepharose purification, dsDNA fragments labeled only at their 3' or 5' ends were prepared and the exonuclease activity was measured using the ratios of ICP8 to dsDNA and ionic conditions employed in the strand transfer reactions (see Materials and Methods). The results revealed that in a ten minute reaction 0.14 picomoles of nucleotide was excised per microgram of ICP8 from the 5'-labeled substrate (0.4 pmol ends) and 0.018 picomoles of nucleotide was excised from the 3' labeled substrate (0.4 pmol ends).

A signature of a strand transfer reaction between the DNA molecules used here is the generation of an equal number of heteroduplex circles and displaced single strands. To determine if the ICP8-catalyzed reactions meet this requirement, an 870 bp TaqI fragment of M13 dsDNA was incubated with M13 ssDNA circles and ICP8 (7 dsDNA molecules per ssDNA circle) for 30 minutes at 37°C followed by deproteinization and staining with SSB. In two separate experiments the ratio of heteroduplex circles to the number of displaced ssDNAs as counted by EM was 46:43, and 59:55 or, approximately 1:1. Further, measurement of the length of the duplex segment on the M13 ssDNA circles yielded mean values of 830 bp (n = 35) and 873 bp (n = 29) and the displaced strands had a mean length of 900 and 917 bases. These results provide additional strong evidence against the production of strand transfer products via a direct nucleic acid.

(d) ICP8-mediated strand transfer requires Mg2+ but not nucleoside triphosphates, and products form rapidly

EM was used to follow the appearance of the heteroduplex circles dependent on the presence of Mg2+, nucleoside triphosphates, time of incubation, and the ratio of ICP8 to ssDNA. In titrations of ICP8 to ssDNA (with all 4 deoxyribonucleotides present) and scoring the percent product by EM, at ratios of 10:1 or 20:1 (levels below which O'Donnell et al. (1987) had found to saturate the ssDNA (37:1)) less than 1% strand transfer product molecules were observed. When a ratio of 40:1 was used, roughly 50% more product molecules were observed than at ratios from 37:1 to 4:1. However, given the substantial numbers of product molecules observed at a ratio of 40:1, the greater degree of aggregation seen at the higher levels of ICP8 and the effort to maintain the residual nucleic acid to the minimum, a value of 40:1 was used throughout these studies. Strand transfer required Mg2+, with the extent of the reaction being similar between 2 and 10 mM-MgCl2. Inclusion of 300 μM-ATP, 300 μM of each of the four deoxyribonucleotides, or 300 μM-beta-ribomethylene-TPP, a non-hydrolyzable dTTP mimic, did not stimulate or inhibit the strand transfer reaction significantly (Table 1).

The time course of strand transfer by ICP8 was compared with the E. coli RecA protein in the presence of E. coli SSB (Fig. 3). In both reactions, the protein was assembled onto the ssDNA and the reaction initiated by the addition of dsDNA. Heteroduplex circles were observed as early as 30 seconds after addition of dsDNA for the ICP8-catalyzed reaction, but not until 90 seconds for the RecA protein-catalyzed reaction. The reaction reached a plateau at ten minutes (30 to 35% product) for ICP8, and at 30 minutes for RecA, where over 90% of the ssDNA was in the form of heteroduplex circles.

(e) ICP8 promotes strand transfer most readily with dsDNA that has ends resected by several bases

The nature of the ends of the dsDNAs engaged in strand transfer have been found to be important for
Table 1
Dependence of ICP8-catalyzed strand transfer on Mg2+ and lack of dependence on nucleoside triphosphates

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Mg2+ (mM)</th>
<th>Heteroduplex circles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>ATP</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>β-β-MTTP</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>AI 4-dNTPs</td>
<td>2</td>
<td>47</td>
</tr>
<tr>
<td>AI 4-dNTPs</td>
<td>10</td>
<td>43</td>
</tr>
</tbody>
</table>

ICP8 M13 ssDNA complexes were formed as in Fig. 1 by incubation of ssDNA and ICP8 for 30 min in a buffer containing 20 mM Tris-HCl (pH 7.5), 80 mM-NaCl, 1 mM-dithiothreitol, and supplemented with Mg(OAc)2 and nucleoside triphosphates as indicated. M13 dsDNA cleaved with Taql was added for 20 min more (10 dsDNA fragments per ssDNA circle). The samples were then deproteinized, stained with SSB, and the fraction of heteroduplex circles in at least 150 M13 ssDNAs counted by EM. All nucleotides were added to 300 μM each, β-β-MTTP is the non-hydrolyzable analog. β,β-Methylene TTP. Molecules showing 2 or more transfers were scored as a single heteroduplex circle.

RecA as well as for the eukaryotic strand transferases. RecA protein shows a several-fold higher preference for dsDNA that has a four-base 5'-recessed end over DNA that is fully blunt ended (Soltis & Lehman, 1984). To compare the effects of different ends on dsDNA molecules, M13 dsDNA was cleaved with three different restriction enzymes.

The Taql restriction enzyme cleaves M13 dsDNA into ten fragments having 3'-recessed ends with

Table 2
Dependence of ICP8-catalyzed strand transfer on DNA ends

<table>
<thead>
<tr>
<th>dsDNA end</th>
<th>Overhang (bases)</th>
<th>Heteroduplex circles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' recessed</td>
<td>43</td>
<td>98</td>
</tr>
<tr>
<td>Blunt</td>
<td>10</td>
<td>41</td>
</tr>
<tr>
<td>5' recessed</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

ICP8-catalyzed strand transfer was carried out and the results scored as described in Table 1 (with all 4 dNTPs and 10 mM-Mg2+), using M13 ssDNA that had been digested with Taql (3'-recessed ends), StuI (5'-recessed ends), or HaeIII (blunt ends) at an equal mass ratio of ssDNA to ssDNA. Reactions employing E. coli RecA and SSB proteins with these ssDNAs including the source of the proteins are described by Regarat et al. (1987). Incubation was for 30 min at 37°C.

† In this population of circles, 10% had undergone multiple exchanges producing circles that were nearly 100% duplex.

overhanges of two bases. StuI cleaves M13 ssDNA into 13 fragments (2 of which are very small) having 5'-recessed ends with a base overhang of one base, and HaeIII produces ten blunt-ended M13 dsDNA fragments. Mixing these dsDNA digests with M13 ssDNA at an equal weight ratio of ss:ds resulted in similar ratios of dsDNA fragments to ssDNA circles. Upon incubation of these ssDNAs for 30 minutes with ICP8-M13 ssDNA complexes, the dsDNA fragments with either 3'-internal or 5'-internal ends yielded between 30% and 45% heteroduplex circles, while the blunt-ended dsDNA produced less than 10% heteroduplex circles (Table 2). By comparison, the RecA and SSB proteins catalyzed 98%, 84%, and 41% heteroduplex circle formation for dsDNAs with 3'-internal ends, 5'-overhangs, or blunt ends, respectively. For the dsDNA with 3'-overhangs, RecA and SSB proteins drove 16% of the ssDNA circles into nearly fully duplex structures resulting from multiple exchanges. Efforts to examine the effects of longer (20 to 30 base) tails on the dsDNA failed due to the aggregation of the DNA at their ICP8-complexed ends resulting in the dsDNA being sequestered from the reaction.

(F) Nuclease digestion and gel electrophoresis confirms that ICP8 promotes net strand transfer

The heteroduplex circles visualized by EM contain a duplex segment whose length corresponds to the length of the duplex fragment(s) in the incubation. To demonstrate this by a method other than EM, M13 ssDNA was uniformly labeled with 32P and the conversion of a segment of the ssDNA into a duplex form was measured with S1 nuclease. ICP8 was assembled onto 32P-labeled M13 ssDNA and unlabeled M13 dsDNA (Taql digest) was added. After a 30 minute incubation the sample was deproteinized and treated with S1 nuclease to remove any ssDNA. The S1 nuclease-resistant material was electrophoresed in a 9% (w/v) polyacrylamide gel. As shown in Figure 4, a portion of the labeled ssDNA appeared as a series of bands

Figure 3. Time course of strand transfers. Strand transfer reactions were carried out with ICP8 (●) or RecA and SSB proteins (○). For ICP8, the conditions as described in Table 1 using 10 mM-Mg(OAc)2 were employed, and for RecA and SSB, the conditions were as described in Table 2. Samples were taken at the times indicated, deproteinized, complexed with SSB, and prepared for EM as described in the legend to Fig. 1B and C. Percent product refers to the fraction of heteroduplex circles scored in at least 160 M13 ssDNA circles visualized by EM.
that co-migrated with a $^{32}$P-labeled TaqI-digested M13 dsDNA. In general, the patterns were very similar. The differences in intensity of several of the bands may suggest that transfers are inhibited at some sites as contrasted to others, possibly due to local secondary structure in the ssDNA. The appearance of ssDNA at these mobilities in the gel could only have occurred if the ssDNA had been protected from digestion by its annealing with an unlabeled dsDNA strand. Thus, we conclude that a net strand transfer had occurred in a manner consistent with the EM assay.

4. Discussion

We have described the use of EM to examine the ability of HSV-1-encoded ICP8 protein to promote the pairing and strand transfer reactions of homologous recombination. EM revealed that complexes formed between ICP8 and ssDNA readily bind and envelop homologous linear dsDNA fragments. When the dsDNA was an 870 bp M13 dsDNA fragment, an equal number of M13 ssDNA circles containing an 870 bp duplex segment and 870-base displaced single strands were observed, diagnostic of a strand transfer reaction. Strand transfer required $\text{Mg}^{2+}$, but no nucleoside triphosphate or SSB cofactors. The extent of the reactions approached 50% product (heteroduplex circles), and there was a several-fold preference for dsDNAs having single-strand ends of a few bases. There was no evidence for pairing at sites internal to the ends of the dsDNA. Gel electrophoretic analysis confirmed the basic conclusion.

(a) Use of EM to detect and characterize pairing and strand transfer

Our finding that ICP8 has homologous pairing and strand transfer activity results from the use of EM to identify conditions under which synaptic complexes form, and then to search for strand transfer products, and optimize their production. Over 7000 molecules were scored in this study. Strand transfer by ICP8 required stringent deproteinization, use of less than full length M13 dsDNA fragments, and was favored by dsDNAs having resected ends of a few bases in length. These specific requirements explained why we and others (O'Donnell et al., 1987) previously failed to detect these activities.

Because the dsDNA was partially or fully enveloped within the compact ssDNA-ICPS filaments, we were unable to elucidate the ultrastructure of the joints formed between the ssDNA and dsDNA. Pairing occurred rapidly with blunt-ended DNA suggesting that the nature of the dsDNA ends may be relatively unimportant in the pairing step, but were important in the subsequent strand displacement step as the use of blunt-ended DNA resulted in fewer product molecules. Joints were not observed when super-twisted DNA was employed.

(b) Evidence against artifactual involvement of nuclease or helicases

Numerous lines of evidence indicated that the strand transfer products observed here by EM and gel electrophoresis were not the result of contaminating helicases or nucleases providing full length single-stranded fragments to pair with the complementary ssDNA circles, but rather arose from a legitimate strand transfer reaction. The most compelling was the quantitative measurement of low nuclease activity in the ICP8 protein. Similarly, helicase activity could not be detected as judged by the failure to observe displacement of ssDNA upon incubation of dsDNA with ICP8. ICP8 can displace oligonucleotides 20 to 100 nucleotides in length from M13 ssDNA. However, this reaction is almost entirely inhibited at the concentrations of $\text{Mg}^{2+}$ and NaCl employed in these experiments (P. Boehmer & I. R. Lehman, unpublished results). A more subtle concern was that a minor fraction of the dsDNA fragments in the incubation were rendered highly
single-stranded due to a contaminating exonuclease and that these molecules led to strand transfer products. However, incubation of ICP8 with linear dsDNA for 60 minutes followed by SSB staining and EM showed that no molecules with tails of 20 to 30 nucleotides or greater could be detected in over 100 molecules scored; nor were any fully single-stranded fragments observed. Additional observations argue against a primary involvement of a contaminating nuclease. The very rapid appearance of heteroduplex products (within 30 s) followed by the reaction reaching a plateau is not consistent with a nuclease-limited reaction. Equal numbers of heteroduplex circles and displaced strands (which remained full length) were observed, and finally, synopsis and pairing with homologous but not nonhomologous dsDNA was extremely fast and efficient. None the less, we do not know the exact requirement in these reactions for the dsDNA ends. It is possible that some nuclease action is activated upon the binding of ICP8 to ssDNA and formation of a homologous synopsis. Detailed future studies will be required to address these questions adequately.

(c) Parallels with other strand transferases

The conclusion that ICP8 has homologous pairing and strand transfer activity is bolstered by the parallels with RecA, UvsX, SEP-1, HPP-1 and Rrp-1. Under conditions optimal for strand exchange, RecA protein binds ss but not ds DNA, and the protein filaments formed by RecA protein alone in the presence of Mg2+ do not bind dsDNA. However, the RecA-ssDNA filaments bind homologous dsDNA avidly, and with the appropriate templates, the dsDNA is pulled into the filaments and a net strand transfer ensues (see Griffith & Harris, 1988). ICP8 showed the same behavior. Further, the time course of strand transfer and preference for ends resected by a few bases, as contrasted to blunt ended DNA (Solits & Lehman, 1984), was similar for RecA protein and ICP8. Unlike RecA or UvsX proteins, ICP8 was unable to catalyze strand transfer with full-length M13 dsDNA. Since only a rare ICP8-ssDNA complex was well extended, strand transfer with full-length dsDNA, which may have been too large to be fully contained within the partially extended ICP8-ssDNA complexes, may have been inhibited. The inclusion of factors which might produce more extended filaments might in turn result in strand transfer with longer DNAs. Additional differences between ICP8 and RecA revealed strong parallels between ICP8 and SEP-1, HPP-1, Rrp-1. ICP8 is similar in size to SEP-1, HPP-1 and Rrp-1, and to date, these proteins, like ICP8, have been observed to promote pairings involving only homologous linear dsDNA. ICP8, like HPP-1, SEP-1 and Rrp-1, did not require ATP for in vitro pairing and strand transfer (Moore & Fishel, 1990; Johnson & Kolodner, 1991; Sander et al., 1991). This apparent independence of ATP may reflect the current view that a major role of ATP is to release RecA protein from the strand exchange products at the end of each cycle (Menetski et al., 1990). Strand transfer products catalyzed by ICP8 (or SEP-1) are not observed unless vigorous deproteination is used, suggesting that it and other ATP-requiring proteins may complete the cycle.

RecA and UvsX proteins mediate a broad array of molecular reactions. ICP8, like SEP-1, HPP-1 and Rrp-1, may possibly mediate only a limited set of these reactions, e.g. pairing and exchange involving a linear dsDNA. The recent observations of Kolodner and colleagues showing that the recE pathway of E. coli involves a 140,000 M, protein with exonuclease activity (recE) and a strand reannealing activity (recT) (Hall, Kane & Kolodner, personal communication) suggests that the analogs to ICP8, SEP-1 and HPP-1 and Rrp-1 may reside in these proteins rather than RecA.

Herpes simplex viruses undergo a high level of homologous recombination during their replication (reviewed by Roizman & Sears, 1990). This high level may be a consequence of the production of a herpes-encoded recombinase or the generation of recombinogenic replication intermediates, or both. By virtue of its strand transfer capacity, ICP8 could very well serve as a homologous pairing and strand transfer protein in the HSV-1 life cycle.

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