ATP-dependent Unwinding of a Minimal Origin of DNA Replication by the Origin Binding Protein and the Single-Strand DNA Binding Protein ICP8 from Herpes Simplex Virus Type I.

Running title: Activation of oriS

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Summary:

The Herpes simplex virus type I origin binding protein, OBP, is encoded by the UL9 gene. OBP binds the origin of DNA replication, oriS, in a cooperative and sequence specific manner. OBP is also an ATP dependent DNA helicase. We have recently shown that single-stranded oriS folds into a unique and evolutionarily conserved conformation, oriS*, which is stably bound by OBP. OriS* is contains a stable hairpin formed by complementary base-pairing between box I and box III in oriS. Here we show that OBP, in the presence of the single-strand DNA binding protein ICP8, can convert an 80 base pair double-stranded minimal oriS fragment to oriS* and form an OBP/oriS* complex. The formation of an OBP/oriS* complex requires hydrolysable ATP. We also demonstrate that OBP in the presence of ICP8 and ATP promotes slow but specific and complete unwinding of duplex minimal oriS. The possibility that the OBP/oriS* complex may serve as an assembly site for the herpes virus replisome is discussed.
Introduction

DNA replication starts at specific sites on chromosomes referred to as origins of DNA replication. Sequence-specific DNA binding proteins recognize the origins of replication and facilitate local unwinding of duplex DNA. Once single-stranded DNA has been exposed the remaining replication proteins may assemble into a multi-enzyme complex frequently referred to as the replisome (1). It is likely that the origins of DNA replication have structural features that contribute to the efficiency and regulation of initiation of DNA replication during one or both of these steps.

Initiation of Herpes simplex virus DNA replication depends on two largely homologous origins of replication in the viral genome: oriL and oriS (2-4). There are three copies of the recognition sequence, boxes I, II and III, for the origin binding protein, OBP, in oriS and they are arranged in two palindromes (5). Box I and box III are part of an evolutionarily conserved palindrome that forms a stable hairpin in single-stranded DNA. Box I and box II are separated by an AT-rich spacer sequence that varies in length and nucleotide composition between different alpha herpes viruses. In oriS from HSV-1 box I, box II and the AT-rich sequence form a 46 bp palindrome. Genetic analyses have demonstrated that the boxes I, II and III as well as their precise arrangement in oriS affect the efficiency of origin dependent DNA replication in vivo (5-11). We have previously demonstrated that heat-treated duplex oriS and single-stranded oligonucleotides co-linear with the upper strand of oriS adopt a novel conformation, oriS*, and OBP forms a very stable and specific complex with oriS* (5,12). OriS* is contains a stable hairpin formed by complementary base-pairing between box I and box III (5,12). Biochemical and genetic experiments suggest that this feature is important not only for the formation fo the OBP/oriS* complex but also for efficient initiation of DNA replication at oriS (12).
The initiator protein, OBP, is a sequence-specific DNA-binding protein and a DNA helicase (4). The DNA helicase activity resides in the N-terminal part of the protein which is composed of the ubiquitous helicase domains 1A and 2A (13,14). The C-terminal part of the protein, below referred to as ΔOBP, binds the sequence GTTCGCAC through interactions in the major groove of DNA (15-18). In addition ΔOBP forms a specific complex with the viral single-strand DNA- binding protein ICP8 (19). It has been shown that single-stranded DNA efficiently disrupts the complex between OBP and ICP8 (20).

We now demonstrate that an OBP/oriS* complex can be formed from an 80 base pair fragment of double-stranded minimal oriS. We also show that OBP and ICP8 together support specific unwinding of minimal oriS dependent on ATP. A model for the activation of HSV-1 oriS is presented. It suggests that the DNA sequence of the origin of replication directs the folding of DNA into a stable structure specifically bound by the initiator protein and that this complex may serve as an assembly site for the replisome.
Experimental Procedures

Nucleotides and DNA:

\[ \gamma^{32}P\]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia. ATP, ADP, ATP\(\gamma\)S and AMP-PNP were obtained from Roche.

Oligonucleotides were from DNA Technology A/S, Denmark. Unless otherwise stated they were 80 nucleotides long. The upper strands were labelled with \[\gamma^{32}P\]ATP by T4 polynucleotide kinase. They were used either as single-stranded oligonucleotides or to make double-stranded DNA. Complementary strands were annealed in a reaction mixture containing 20mM Tris-HCl pH 7.8 and 0.1 M NaCl. In the experiments described below oriS consists of double-stranded DNA, and oriS* is made up of a single-stranded 80-mer corresponding to the upper strand of oriS as shown in figure 1.

We show below the sequences of the oligonucleotides representing the upper strand of oriS (Fig1). Mutations are underlined and highlighted in *italics*.

oriS(wt):

5´-AAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTA
TTAGGGCGAAGTGCGAGCACTGGCGCC-3´

oriS(-Box I):

5´-
AAAAGAAGTGAGAACGCGAAGCGCGCACTTCGCACTTCGTCCCAATATATATATATTA
TTAGGGCGAAGTGCGAGCACTGGCGCC-3´

oriS(-Box III):

5´-AAAAGAAGTGAGCGCGAAGCGTTCGCACTTCGTCCCAATATATATATATATTA
TTAGGGCGAAGTGCGAGCACTGGCGCC-3´

oriS(-Box I,III):

5´-AAAAGAAGTGAGCGCGAAGCGTTCGCACTTCGTCCCAATATATATATATATTA
TTAGGGCGAAGTGCGAGCACTGGCGCC-3´
5’-

AAAAGAAGTGAGCCGCAAGCGGGCGCAGTTACTGGCGCC
TTAGGGCGAAGTGCGAGCACTGGCGCC-3’

oriS(136-mer):
5’-

CTGCAGGTCGACTCTAGAGGATCCGGTAAGAAGTCGAGACGCAGCGTT
CGCACTTCTGCATTATATATATATTATATTAGGGCGAAGTGCGAGCACTGGCGCC
GTGCGCGATCCCGGCTCTCGAGCTCG -3’

oriS(Scrambled):
5’-AGTATGTCGTACAGCTGACAGTACGACTGTCAAGCAGACTGACAGTCATCA
ACTGCTACTCTGTAGTAGCTGAGACTC-3’

T65 is a 65-mer of oligodeoxythymidylicate.

**Proteins:**

OBP and ICP8 were purified from Sf9 cells using recombinant baculovirus vectors as described (19). The proteins were approximately 95 % pure as judged SDS polyacrylamide gel electrophoresis. Protein concentrations were determined using the Bio-Rad Protein Assay.

**Gel retardation assay:**

Ultra pure agarose was purchased from Gibco RBL(Life Technologies). Reaction mixtures, 10 µl, with 0.2 nM radiolabeled oriS or oriS* in a buffer containing 20 mM Tris-HCl, pH 8.0, 10% glycerol, 2.5 mM dithiothreitol, 3 mM MgCl₂, 200 µg/ml bovine serum albumin and 50 mM NaCl were supplemented with 40 nM OBP and 50 nM ICP8 as indicated. The reaction mixtures also contained 2.5 mM ATP or ATPγS, ADP and AMP-PNP as indicated. Samples were incubated at 37°C. The reactions were analyzed on 0.9% agarose gels in a buffer.
containing 40 mM Tris acetate, pH 8.0, and 1 mM EDTA. Submarine gels were run for 90 minutes at room temperature and a field strength of 7 V/cm. The gels were dried onto DE81 paper, Whatman, and they were either autoradiographed overnight at -80°C or subjected to PhosphorImager analysis.

**Exonuclease I footprinting:**

Exonuclease I at a concentration of 10 U/µl was from Amersham Biosciences. Single-stranded 80-mer oriS(wt) was radiolabelled at the 5’end. Double-stranded oriS(wt) was produced by reannealing complementary strands as described above. In the 40 µl reaction mixtures 0.8 nM of single-stranded oriS(wt), oriS*, or double-stranded oriS(wt), oriS, were incubated with 0.16 µM OBP and 0.2 µM ICP8 in a buffer containing 20mM Tris-HCl pH 8.0, 10% glycerol, 2,5 mM dithiothreitol, 3mM MgCl2, 200 µg/ml bovine serum albumin, 50 mM KCl and 10 mM ATP as indicated. Incubation was for 90 minutes at 37°C to allow formation of OBP/oriS* complexes from double-stranded DNA. To remove ICP8 from the protein-DNA complexes 2 µl T65 was added. After an incubation for one minute in 37°C 1 µl exonuclease I, 10U, was added as indicated and the incubation was allowed to proceed for another 15 minutes at 37°C. The samples were extracted by phenol and DNA was precipitated by ethanol. DNA was dissolved in 5 µl 10 mM Tris-HCl and 1 mM EDTA pH 8.0. 5µl formamide loading buffer was added and the samples were analysed on 8% polyacrylamide sequencing gels. Electrophoresis was for 2h at 600 V. The dried gels were analysed using PhosphorImager.

Single-stranded radiolabeled 44-mer, 54-mer and 63-mer oligonucleotides corresponding to the upper strand of oriS were used as markers.

**Unwinding of oriS:**
Metaphor® agarose was from BioWhittaker Molecular Applications. The reaction mixture was as described above. Double-stranded DNA radiolabelled at the upper strand was produced as described above. The incubation was carried out at 37°C. The reaction was stopped after 60 minutes by addition of sodium dodecyl sulphate to a final concentration of 0.1%. Samples were analysed on 3% Metaphor agarose gels in 40 mM Tris acetate, pH 8.0, and 1 mM EDTA. Electrophoresis was for 2 hours at room temperature at 100V. The gels were then dried onto DE81 paper, Whatman, and they were either autoradiographed overnight at -80°C or subjected to PhosphorImager analysis.
Results

An OBP-oriS* complex formed from double-stranded minimal oriS.

OBP forms a specific and stable complex with either heat treated double-stranded oriS or single-stranded oligonucleotides corresponding to the upper strand of oriS (Fig.1a) (5). The novel conformation of oriS recognized by OBP is referred to as oriS*. OriS* consists of stable hairpin formed by complementary base-pairing between box I and box III (5). The 3´ part of oriS* is made up of the AT-rich spacer sequence and it may also adopt a hairpin conformation (Fig.1 b). A secondary structure prediction using the mfold program, however, suggests that it is an unstable hairpin that easily may become single-stranded as temperature is increased from 37°C to 45 °C (Fig.1c) (21-23). We have previously shown that a mutant version of oriS referred to as oriS(-6AT) lead to reduced ability to form an OBP/oriS* complex (10,12). A secondary structure prediction using mfold indicates that the putative AT-rich hairpin becomes more stable in this mutant (results not shown). We have also seen that OBP forms a complex with single-stranded DNA containing a boxI/boxIII hairpin and a 3´ single-stranded tail (5). This DNA ligand not only supports the formation of a stable complex with OBP, but it also greatly stimulates ATP hydrolysis by OBP (5). It is therefore likely that oriS* exists as a hairpin with a single-stranded tail when bound by OBP.

We have now searched for conditions that would allow an 80 bp double-stranded oligonucleotide containing minimal oriS to be converted to single-stranded oriS* by OBP. The short version of oriS was formed by annealing of two 80-mer complementary DNA strands. The single-strand DNA binding protein ICP8 and ATP were together with OBP included in the reaction mixture. The production of oriS* was monitored by the appearance of a complex between OBP and oriS* detected by agarose gel electrophoresis under native conditions. The results were initially difficult to interpret because ICP8 will form a stable and
specific complex with the C-terminus of OBP (19). This complex can, however, be efficiently disrupted by single-stranded DNA (20). In the gel retardation experiments described below we have therefore added a 65-mer of oligodeoxythymidy late, T65, to the reaction mixture immediately prior to analysis of protein-DNA complexes by agarose gel electrophoresis. In this way we can distinguish between the OBP/oriS* complex and the ternary complex formed between OBP, ICP8 and oriS*.

We first examined the requirements for the formation of an OBP/oriS* complex from double-stranded minimal oriS (Fig.2). Incubation of OBP, ICP8 and ATP with double-stranded minimal oriS was carried out for 60 minutes at 37°C. T65 was then added as indicated to the reaction mixtures before gel electrophoresis. We found that incubation of double-stranded minimal oriS with OBP and ATP produced two complexes referred to as complex I and complex II (Fig.2 5th lane). These complexes have been previously characterized (10). Complex I represents OBP bound to box I, whereas complex II represents OBP cooperatively bound to boxes I and II. The addition of T65 did not significantly affect the formation of complexes I and II (Fig.2, 6th lane). ICP8 did not bind double-stranded oriS (Fig.2, 7th and 8th lanes). Simultaneous incubation of OBP, ICP8 and ATP resulted in the formation of complexes which migrated slower than complex II in agarose gels (Fig.2, 9th lane). Strikingly, addition of T65 reduced the amount of slowly migrating complexes and produced a unique complex with an electrophoretic mobility similar to the OBP/oriS* complex (Fig.2, 2nd and 10th lanes).

A separate experiment was performed in order to further examine the electrophoretic properties of the putative OBP/oriS* complex (Fig.3). In the sample analyzed in the first lane OBP was mixed with a single-stranded oligonucleotide corresponding to the upper strand of minimal oriS prior to agarose gel electrophoresis. In the sample analysed in the second lane OBP, ICP8 and ATP was first incubated with double-stranded minimal
oriS(wt). Competing T65 was then added prior to agarose gel electrophoresis. The gel was subjected to PhosphorImager analysis. The results show that the two complexes have identical electrophoretic mobilities.

To further authenticate the OBP/oriS* complex produced from double-stranded oriS an exonuclease I footprinting assay was developed. Exonuclease I is a 3´-5´ processive single-strand specific exonuclease. The single-stranded oriS(wt) 80-mer oligonucleotide oriS* will be completely degraded by exonuclease I without significant pause sites. In contrast double-stranded oriS(wt) is completely resistant to exonuclease I (results not shown). In the presence of OBP and ICP8, however, two prominent pause sites are detected during the digestion of oriS* (Fig.4). The first pause site corresponds approximately to a 54-mer, whereas the second pause site corresponds to 38 and 39-mers. The addition of T65, which disrupts the interaction between OBP and ICP8, does not affect the location of the pause sites (Fig.4). When double-stranded oriS is examined we find that incubation with OBP, ICP8 and ATP prior to exonuclease allows detection of the very same pause sites. Addition of T65 does not affect the location of the pause sites. Importantly, in the absence of ATP double-stranded oriS is completely resistant to exonuclease I, and no pause sites are detected (Fig.4). The results argue that OBP, in the presence of ICP8 and ATP, is able to form an OBP/oriS* complex from double-stranded oriS. It also indicates that the helicase domains of OBP may cover between 4 and 20 nucleotides of single-stranded DNA extending from the box I/box III hairpin towards the 3´end.

We have also analysed the effect of mutations in oriS on the formation of the OBP/oriS* complex (Fig.5). Box III is of special interest. It does not interact with OBP in double-stranded oriS in a sequence-specific manner, but it is essential for the formation of oriS* through complementary base-pairing with box I (5,15). The results again show that double-stranded minimal oriS(wt) can be converted to an OBP/oriS* complex in the presence
of ICP8 and ATP (Fig 5a,b lane 4). In contrast, a mutant version of minimal oriS, oriS (−box III), fails to support the formation of an OBP/oriS* complex. In this case, the addition of competing T65 to a reaction mixture containing OBP, ICP8 and ATP results in reappearance of complexes I and II (Fig. 5a,b lane 8). It should also be noted that a complex between OBP and a single-stranded oligonucleotide corresponding to the upper strand of oriS (− box III) is formed with a reduced efficiency when compared to the OBP/oriS* complex, and it has a different electrophoretic mobility (Fig. 5a,b lanes 1 and 5). These properties probably reflect a DNA conformation characterized by complementary base-pairing between boxes I and II as previously discussed (12).

The role of ATP binding and ATP hydrolysis was investigated in a similar set of experiments. We found that ADP, ATPγS and AMP-PNP did not support the formation of a complex migrating as an OBP/oriS* complex in agarose gels (Fig.6). In contrast, when ATP was present in the reaction mixture we found that slowly migrating complexes were produced, and that these complexes were disrupted by T65 and replaced by an OBP/oriS* complex (Fig.6, 5th and 6th lanes). We also noted that in the presence of ADP complexes accumulated in the wells (3rd and 4th lanes). The DNA retained in the wells is not single-stranded since the addition of sodium dodecyl sulphate will only release duplex DNA (results not shown, see also fig.9b below). The aggregation of OBP/DNA complexes presumably reflects a conformational difference between the OBP/ATP complex and the OBP/ADP complex. Previous studies have also shown the specific OBP/DNA complexes formed in the absence of the nucleotide co-factor may aggregate but complexes formed in the presence of nucleoside triphosphates readily enters the gel (10,24).

The time course for the formation of the OBP/oriS* complex was also examined. We noted that incubation for 30 minutes or more at 37°C was required in order to produce significant amounts of the OBP/oriS* complex from duplex DNA (Fig. 7).
These results argue that OBP together with ICP8 can convert double-stranded DNA containing minimal oriS to an activated form in a reaction dependent on hydrolysis of ATP. Activated minimal oriS can be isolated in complex with OBP and ICP8. When these complexes are exposed to single-stranded DNA ICP8 dissociates, but OBP remains bound to activated minimal oriS. The complex between OBP and activated minimal oriS has an electrophoretic mobility identical to the previously characterized OBP/oriS* complex. These complexes also present the same exonuclease I digestion patterns. Both complexes also depend on an intact version of box III. Since box III is involved in complementary base-pairing with box I in oriS* we suggest that this base-pairing scheme also occurs in the activated form of minimal oriS produced by OBP, ICP8 and ATP. Together these observations suggest that the OBP/oriS* complexes formed from single-stranded oriS* and double-stranded DNA oriS have the same composition and structure. It remains to be determined whether the OBP/oriS* complex is a product of an unwinding reaction or an intermediate formed during the process leading to complete strand separation.

Unwinding of minimal oriS.

Several attempts have been made to demonstrate that OBP can specifically unwind oriS containing double-stranded DNA. A study using electron microscopy has successfully demonstrated this phenomenon (25). In contrast, biochemical studies of OBP dependent unwinding of oriS have been less successful (26). It was recently demonstrated that OBP can catalyse complete unwinding of a short version of oriS provided that the AT-rich spacer sequence existed in a preformed single-stranded conformation (26). The unwinding of fully double-stranded oriS was, however, much less efficient. Encouraged by our observation that an OBP/oriS* complex could be formed from double-stranded minimal oriS we looked for
experimental conditions that would facilitate analysis of unwinding of minimal oriS. It was essential to identify experimental conditions that allowed complete separation of single-stranded oriS* and double-stranded oriS. We found that this could be achieved in gels containing 3% Metaphor agarose (Fig. 8). Using experimental conditions that favour the formation of an OBP/oriS* complex we could now demonstrate complete unwinding of an 80 bp minimal oriS duplex (Fig. 8a). Approximately 30% of double-stranded minimal oriS was converted to single strands (Fig. 8b). The reaction required OBP and ICP8, and it only occurred in the presence of hydrolysable ATP (Fig. 9a and b).

**Sequence requirements for OBP-dependent unwinding of oriS.**

Sequence requirements for initiation of DNA replication are complex. Studies of HSV-1 oriS have indicated that box I and box II are required for cooperative binding of OBP to duplex DNA (15-17, 27). In addition the distance and sequence between box I and box II must be correct (10). Finally, box III is needed for complementary intra-strand base-pairing with box I (5). We have now surveyed the effects of a selected set of oriS mutations on OBP dependent unwinding.

We first compared unwinding of an 80 bp duplex oligonucleotide corresponding to wild type minimal oriS, oriS(wt), with either a 80 bp duplex oligonucleotide with the same nucleotide composition as oriS but with a scrambled nucleotid sequence, oriS(Scrambled), or an 80 bp minimal oriS duplex with mutations in box I, oriS(- BoxI). The results show that the scrambled duplex was a poor substrate for OBP dependent unwinding. We estimate that less than 4% of total DNA became single-stranded during the incubation period (Fig. 10). The unwinding of the box I mutant was reduced but not abolished. Approximately 12% of total DNA was retrieved as single strands (Fig. 10). Cooperative binding of OBP to the box I
mutant presumably accounts for the fact that some unwinding albeit reduced was observed in this instance (15,17,27).

To explore the connection between unwinding and the formation of the OBP/oriS* complex we examined oriS with mutations either in box I, oriS(- Box I), or box III, oriS(- Box III). These mutations severely reduce formation of the OBP/oriS* complex (12). In addition, a double mutant that restores complementary base-pairing between boxes I and III, oriS(- boxI,III), was used. We found that unwinding of the mutant versions of oriS was reduced compared to the wild type sequence, oriS(wt) (Fig. 11). It is interesting to note, however, that double mutations in box I and box III did not have an additive effect (Fig.11). This observation correlates with observations on the behaviour of the double mutant in transient replication experiments. In this instance an oriS containing plasmid with mutations in box I replicated as efficiently as a plasmid with a box I/box III double mutant (9). Our results argue that there is a good correlation between the formation of the OBP/oriS* complex and unwinding of double-stranded minimal oriS.

We also wanted to examine if the length of double-stranded DNA would affect the efficiency of OBP dependent unwinding of oriS. A longer oriS containing duplex, oriS(136-mer), was studied (Fig.11). We found that unwinding of oriS(136-mer) was much less efficient than unwinding of oriS(wt). This observation might indicate either that unwinding by OBP and ICP8 alone is restricted in vivo to minimal oriS or that long DNA substrates favour reannealing of complementary strands in vitro.
Discussion.

The results presented here demonstrate that the Herpes simplex virus type I initiator protein OBP collaborates with the single-strand DNA-binding protein ICP8 to promote ATP-dependent activation of HSV-1 oriS. Both OBP and ICP8 are needed to form the OBP/oriS* complex as well as to carry out complete unwinding of an 80 bp duplex minimal oriS. Below we will address the conformational changes that double-stranded oriS will undergo during this process. We will also discuss the role of ICP8 during initiation of DNA replication. Finally, a comparison will be made between OBP and structurally related DNA helicases that have different biological roles but may share important mechanistic properties with OBP.

Binding of initiator proteins to cognate origins of DNA replication lead to the induction of substantial conformational changes in DNA. In most instances enzymatic or chemical probes have been used to detect new conformations. The conformations of the activated origins of DNA replication remain poorly characterized from a structural point of view. One example could be mentioned. Binding of SV-40 T-antigen to its origin of replication starts with sequence-specific recognition of inverted repeats containing the pentanucleotide 5´GAGGC 3´, and it is accompanied by structural changes in the early palindrome and the AT-rich sequence surrounding the central palindrome (28). Ultimately double hexamers of T-antigen assemble at the origin and encircle the DNA strands. The precise nature of conformational changes the DNA molecule undergoes during this process is not known. The activation of the HSV-1 origin of replication oriS seems to follow a related pathway. Initially the origin binding protein binds cooperatively to two binding sites in duplex DNA (10,15). In the presence of the single-strand DNA binding protein ICP8 duplex DNA is destabilized as determined by an increased hyperchromicity and sensitivity to the single-strand specific endonuclease P1 (29). The reaction appears to be ATP independent. The DNA substrate used in these studies was, however, lacking the box III region of oriS,
and the two strands do not appear to become fully separated. We now show that OBP and ICP8 in the presence of a DNA substrate containing box III is able to completely unwind double-stranded minimal oriS in an ATP dependent manner. Unwinding of double-stranded oriS leads to the formation of a stable DNA structure, oriS*, consisting of a boxI/boxIII hairpin followed by a single-stranded tail comprising the AT-rich spacer sequence. OBP and oriS* forms a stable complex, and oriS* is a very efficient activator of hydrolysis of ATP by OBP (5,12). Unwinding of oriS and formation of the OBP/oriS* complex are dependent on intact box I as well as box III sequences. Our observations suggest that origins of replication contain not only DNA sequences that are easily unwound, but they may also contain sequences that allow formation of stable DNA structures that can be recognized by the initiator protein or other components of the replication machinery.

ICP8 appears to be required for initiation of DNA replication as well as elongation since a mutant version of OBP that lacks the sequences at the C-terminus required for binding to ICP8 is deficient in DNA synthesis as measured in transient replications experiments using expression plasmids encoding the seven HSV-1 replication proteins (30). ICP8 may act solely by affecting properties of the initiator protein through a specific interaction with the C-terminus of OBP (19,30). It is also possible that ICP8 will bind to single-stranded regions of oriS exposed during the process of initiation and prevent reannealing of complementary strands. There is some genetic evidence that ICP8 may participate in two separate reactions. The HSV-1 mutants TL4 and TL5 which map in the N-terminus of ICP8 appears to fully complement the HSV-1 mutant n11SV in which 28 amino acids at the very C-terminus have been replaced by the SV40 nuclear localization signal (31). A distinct role for the C-terminus of ICP8 is also indicated by the fact that the mutant dl105 with a deletion mapping at the C-terminus acts in a dominant-negative way and inhibits viral DNA replication (32). Together these observations argue that ICP8 has at least two distinct roles. Perhaps the binding of ICP8
to single-stranded DNA at the replication fork and binding of ICP8 to OBP during initiation of DNA replication are differentially affected in the aforementioned mutants.

The crystal structure analyses of the DNA helicases Rep and PcrA have highlighted universal structural features characterizing DNA and RNA helicases (14,33,34). The RecA-like domains 1A and 2A provide the scaffold for the conserved helicase motifs involved in binding and hydrolysis of ATP. It is more difficult to pinpoint specific roles for the remaining domains. It appears, however, that these enzymes have composite DNA binding sites that target the enzymes to appropriate DNA structures. In the case of OBP, sequence-specific binding to duplex DNA by the C-terminal domain is accompanied by recognition of a 3′ single-stranded tail by the N-terminal helicase domains (5). Both of these interactions have to be establish in order to efficiently trigger hydrolysis of ATP. In a similar way PriA appears to recognize a specific DNA structure at the same time as it binds a 3′ single-stranded tail (35). Interestingly, binding of DNA is highly dependent on the length of the tail. The $K_D$ for binding duplex DNA is decreased approximately 200-fold when the tail length is increased from 8 to 12 nucleotides (35). A complementary observation was made for OBP. The hydrolysis of ATP is dramatically stimulated when the tail length is altered from 7 to 10 nucleotides (12). The exonuclease I digestion experiments presented here indicate that the helicase domains of OBP cover between 4 and 20 nucleotides of single-stranded DNA at the 3′ side of the boxI/box III hairpin. These observations are compatible with the crystal structure analysis of the SF2 RNA helicase NS3 from hepatitis virus C. In this instance eight nucleotides of single-stranded deoxyuridylate bind in a groove between the helicase domains and a C-terminal domain (36).

We suggest that the OBP/oriS* complex is formed during the activation of HSV-1 oriS and that it might serve as an intermediate during initiation of DNA replication. It could act as an assembly site for the viral replisome. This model could be tested in several ways.
For example, does the OBP/oriS* complex specifically interact with helicase-primase and DNA polymerase? It should also be possible to extend the unwinding experiments described above. What DNA sequences affect the efficiency of unwinding of oriS? Can OBP unwind extensive stretches of DNA or is it restricted to act on minimal oriS only? Such studies should facilitate experiments aiming at establishing a reconstituted system for HSV-1 DNA replication.
References

1. Kornberg, A., and Baker, T. (1992) *DNA replication Second Edition*, W.H. Freeman and Company, New York

2. Stow, N. D., and McMonagle, E. C. (1983) *Virology* 130(2), 427-38.

3. Weller, S. K., Spadaro, A., Schaffer, J. E., Murray, A. W., Maxam, A. M., and Schaffer, P. A. (1985) *Mol Cell Biol* 5(5), 930-42.

4. Boehmer, P. E., and Lehman, I. R. (1997) *Annu Rev Biochem* 66, 347-84

5. Aslani, A., Macao, B., Simonsson, S., and Elias, P. (2001) *Proc Natl Acad Sci U S A* 98(13), 7194-9.

6. Lockshon, D., and Galloway, D. A. (1986) *J Virol* 58(2), 513-21.

7. Lockshon, D., and Galloway, D. A. (1988) *Mol Cell Biol* 8(10), 4018-27.

8. Weir, H. M., and Stow, N. D. (1990) *J Gen Virol* 71(Pt 6), 1379-85.

9. Hernandez, T. R., Dutch, R. E., Lehman, I. R., Gustafsson, C., and Elias, P. (1991) *J Virol* 65(3), 1649-52.

10. Gustafsson, C. M., Hammarsten, O., Falkenberg, M., and Elias, P. (1994) *Proc Natl Acad Sci U S A* 91(11), 4629-33.

11. Hammarsten, O., and Elias, P. (1997) *Nucleic Acids Res* 25(9), 1753-60.

12. Aslani, A., Simonsson, S., and Elias, P. (2000) *J Biol Chem* 275(8), 5880-7.

13. Abbotts, A. P., and Stow, N. D. (1995) *J Gen Virol* 76(Pt 12), 3125-30.

14. Subramanya, H. S., Bird, L. E., Brannigan, J. A., and Wigley, D. B. (1996) *Nature* 384(6607), 379-83.

15. Elias, P., Gustafsson, C. M., and Hammarsten, O. (1990) *J Biol Chem* 265(28), 17167-73.
16. Hazuda, D. J., Perry, H. C., Naylor, A. M., and McClements, W. L. (1991) *J Biol Chem* **266**(36), 24621-6.

17. Hazuda, D. J., Perry, H. C., and McClements, W. L. (1992) *J Biol Chem* **267**(20), 14309-15.

18. Simonsson, S., Samuelsson, T., and Elias, P. (1998) *J Biol Chem* **273**(38), 24633-9.

19. Boehmer, P. E., and Lehman, I. R. (1993) *Proc Natl Acad Sci U S A* **90**(18), 8444-8.

20. Gustafsson, C. M., Falkenberg, M., Simonsson, S., Valadi, H., and Elias, P. (1995) *J Biol Chem* **270**(32), 19028-34.

21. SantaLucia, J., Jr. (1998) *Proc Natl Acad Sci U S A* **95**(4), 1460-5.

22. GCG (2000) *Wisconsin Package Version 10.1*, Genetics Computer Group, Madison, WI

23. Zuker, M. (1989) *Science* **244**(4900), 48-52.

24. Elias, P., and Lehman, I. R. (1988) *Proc Natl Acad Sci U S A* **85**(9), 2959-63.

25. Makhov, A. M., Boehmer, P. E., Lehman, I. R., and Griffith, J. D. (1996) *Embo J* **15**(7), 1742-50.

26. He, X., and Lehman, I. R. (2000) *J Virol* **74**(12), 5726-8.

27. Elias, P., Gustafsson, C. M., Hammarsten, O., and Stow, N. D. (1992) *J Biol Chem* **267**(24), 17424-9.

28. Borowiec, J. A., Dean, F. B., Bullock, P. A., and Hurwitz, J. (1990) *Cell* **60**(2), 181-4.

29. He, X., and Lehman, I. R. (2001) *Proc Natl Acad Sci U S A* **98**(6), 3024-8.

30. Boehmer, P. E., Craigie, M. C., Stow, N. D., and Lehman, I. R. (1994) *J Biol Chem* **269**(46), 29329-34.

31. Gao, M., Bouchez, J., Curtin, K., and Knipe, D. M. (1988) *Virology* **163**(2), 319-29.

32. Chen, Y. M., and Knipe, D. M. (1996) *Virology* **221**(2), 281-90.
33. Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) *Cell* **90**(4), 635-47.

34. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) *Cell* **97**(1), 75-84.

35. Nurse, P., Liu, J., and Marians, K. J. (1999) *J Biol Chem* **274**(35), 25026-32.

36. Kim, J.L., Morgenstern, K.A., Griffith, J.P., Dwyer, M.D., Thomson, J.A., Murcko, M.A., Lin, C., and Caron, P.R. (1998) *Structure* **6**, 89-100.
Footnotes

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Abbreviations

OBP, origin binding protein; ICP8, infected cell protein 8; SDS, sodium dodecyl sulphate
Figure legends

**Fig.1 Conformations of HSV-1 oriS.**

a. Double-stranded HSV-1 minimal oriS. The binding sites for OBP, boxes I, II and III, are indicated. Two prominent palindromes in oriS are also shown.
b. Structure of oriS* as predicted by mfold at 37°C.
c. Structure of oriS* as predicted by mfold at 45°C.

**Fig 2. Formation of an OBP/oriS* complex from double-stranded minimal oriS.**

Autoradiograph of a gel retardation experiment. Radiolabelled single-stranded or double-stranded minimal oriS(wt) were added to incubation mixtures containing OBP, ICP8 and the OBP/ICP8 complex as indicated. The samples were incubated at 37°C for 60 minutes in the presence of ATP. 2µM T65 was added immediately prior to agarose gel electrophoresis. Complexes between OBP and double-stranded oriS are referred to as Complex I and Complex II. An asterisk indicates the position of the OBP/oriS* complex.

**Fig 3. Electrophoretic mobility of the OBP/oriS* complex.**

A gel retardation experiment analysed by autoradiography and densitometry using the Phosphoimager. Lane 1 (solid line): OBP was mixed with a single-stranded oligonucleotide corresponding to the upper stand of oriS(wt). Lane 2 (dotted line): OBP, ICP8, ATP and double-stranded oriS(wt) was incubated at 37°C for 60 minutes. 2µM T65 was added immediately prior to agarose gel electrophoresis. The arrowhead shows the position of free DNA.
Fig 4. Exonuclease I footprinting of OBP/oriS* complexes formed from single-stranded and double-stranded substrates.

Autoradiograph of a sequencing gel. Single-stranded and double-stranded 80-mer oriS* and oriS was incubated with OBP, ICP and ATP to allow formation of OBP/oriS* complexes. Oligodeoxythymidylate, T65, was added to disrupt the interaction between ICP8 and OBP. Two characteristic pause sites appearing during exonuclease I digestion of OBP/oriS* complexes correspond approximately to 38 and 39-mers and a 54-mer.

Fig 5. Box III is required for the formation of an OBP/oriS* complex from double-stranded minimal oriS.

a. Autoradiograph of a gel retardation experiment. Radiolabelled single-stranded or double-stranded oriS(wt) and oriS(- box III) were added to incubation mixtures containing OBP, ICP8 and the OBP/ICP8 complex as indicated. The samples were incubated at 37°C for 60 minutes in the presence of ATP. 2µM T65 was added immediately prior to agarose gel electrophoresis. Complexes between OBP and double-stranded oriS are referred to as Complex I and Complex II. An asterisk indicates the position of the OBP/oriS* complex.

b. Analysis by densitometry using the Phosphoimager. The solid lines correspond to lanes 1-4 and the dotted lines represent lanes 5-8. The arrowhead shows the position of free DNA.

Fig 6. ATP but not ADP or ATP analogues support formation of an OBP/oriS* complex from double-stranded minimal oriS.

Double-stranded oriS was incubated with OBP and ICP8 in presence of either 2.5 mM ATP, ADP, ATPγS or AMP-PNP. The reaction was incubated for 60 minutes at 37°C. 2µM T65 was added prior to agarose gel electrophoresis. Complexes between OBP and double-
stranded oriS(wt) are referred to as Complex I and Complex II. An asterisk indicates the position of the OBP/oriS* complex.

**Fig 7. OBP/oriS complexes precede the OBP/oriS* complex.**

The time course for the formation of an OBP/oriS* complex from duplex minimal oriS is shown by an autoradiograph of a gel retardation experiment. Complexes between OBP and double-stranded oriS are referred to as Complex I and Complex II. An asterisk indicates the position of the OBP/oriS* complex.

**Fig 8. Unwinding of HSV-1 minimal oriS.**

a. An autoradiograph of a Metaphore agarose gel showing a time course for the formation of single-stranded DNA, ssDNA, from double-stranded minimal oriS, dsDNA. Reaction conditions are described under Experimental Procedures. The samples were treated with sodium dodecyl sulphate prior to electrophoresis.

b. Quantitative analysis of the experiment described above using the Phosphorimager.

**Fig 9. Unwinding of minimal oriS requires OBP, ICP8 and ATP.**

a. An autoradiograph of a Metaphore agarose gel showing unwinding of double-stranded minimal oriS, dsDNA. Reaction mixtures are described under Experimental Procedures. They all contain 2.5 mM ATP. OBP and ICP8 were added as indicated.

b. ATP dependent unwinding of oriS. Reaction mixtures as described in Experimental Procedures were supplemented with nucleotides as indicated. Samples were analysed on Metaphor agarose gels described above.

**Fig 10. Sequence specific unwinding of minimal oriS.**
a. An autoradiograph of a Metaphore agarose gel showing unwinding of double-stranded minimal oriS, dsDNA. Reaction conditions are described in Experimental Procedures. The double-stranded DNA substrates were oriS(wt); an 80-mer oriS(Scrambled); a mutant containing two T-G transversions in box I referred to as oriS(–Box I).

b. Quantitative analysis of the experiment described above using the Phosphorimager.

**Fig 11. Mutations in the recognition sequence for OBP and the length of the oriS fragment affect the efficiency of unwinding.**

a. An autoradiograph of a Metaphore agarose gel showing unwinding of double-stranded minimal oriS, dsDNA. Reaction conditions are described in Experimental Procedures. The double-stranded DNA substrates were oriS(wt); a mutant containing two T-G transversions in box I referred to as oriS(–Box I); a mutant containing two G-T transversions in box III referred to as oriS(-Box III); a mutant combining the previously described transversions referred to as oriS(–Box I,III); a 136-mer containing the wild type sequence of HSV-1 oriS referred to as oriS(136-mer).

b. Quantitative analysis of the experiment described above using the Phosphorimager.
Fig. 1a

Fig. 1b

Fig. 1c
Fig. 2
Fig. 3
|        | 54mer | oriS^- | oriS^-+OBP-ICP8 | 44mer | 54mer | 63mer | oriS^- | oriS^-+OBP-ICP8 | 63mer |
|--------|-------|--------|------------------|-------|-------|-------|--------|------------------|-------|
| ATP    | -     | -      | +                | -     | -     | -     | +      | +                | -     |
| T65    | -     | -      | +                | -     | -     | -     | +      | +                | -     |
| exo I  | -     | -      | +                | -     | -     | -     | +      | +                | -     |

Fig. 4
Fig5a
Fig. 6
| Time (min) | 60 | 0 | 10 | 20 | 30 | 40 | 50 | 60 |
|-----------|----|---|----|----|----|----|----|----|
| OBP       | +  | + | +  | +  | +  | +  | +  | +  |
| ICP8      | -  | + | -  | +  | +  | +  | +  | +  |
| T65       | -  | - | +  | -  | +  | -  | +  | -  |

Fig. 7
Fig. 8a

Fig. 8b
Fig. 9a

| Nucleotide | – | – | ADP | ATP | ATP-γS |
|------------|---|---|-----|-----|--------|
| OBP + ICP8 | – | – | +   | +   | +      |

Fig. 9b

ds DNA

ss DNA
Fig. 10a

Fig. 10b
ATP-dependent unwinding of a minimal origin of DNA replication by the origin binding protein and the single-strand DNA binding protein ICP8 from herpes simplex virus type I

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