A Novel Conformation of the Herpes Simplex Virus Origin of DNA Replication Recognized by the Origin Binding Protein*

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Alireza Aslani, Stina Simonsson, and Per Elias‡

From the Department of Medical Biochemistry, Göteborg University, Box 440, SE 405 30 Göteborg, Sweden

The Herpes simplex virus type I origin binding protein (OBP) is a sequence-specific DNA-binding protein and a dimeric DNA helicase encoded by the UL9 gene. It is required for the activation of the viral origin of DNA replication oriS*. Here we demonstrate that the linear double-stranded form of oriS* can be converted by heat treatment to a stable novel conformation referred to as oriS**. Studies using S1 nuclease suggest that oriS** consists of a central hairpin with an AT-rich sequence in the loop. Single-stranded oligonucleotides corresponding to the upper strand of oriS can adopt the same structure. OBP forms a stable complex with oriS**. We have identified structural features of oriS** recognized by OBP. The central oriS palindrome as well as sequences at the 5′ side of the oriS palindrome were required for complex formation. Importantly, we found that mutations have been shown to reduce oriS-dependent DNA replication also reduce the formation of the OBP-oriS** complex. We suggest that oriS** serves as an intermediate in the initiation of DNA replication providing the initiator protein with structural information for a selective and efficient assembly of the viral replication machinery.

Initiation of replication of viral and cellular chromosomes involves the assembly of multienzyme complexes, replisomes, at unique locations (1). Sequence-specific DNA-binding proteins, initiator proteins, identify the origins of DNA replication, and facilitate the conversion of duplex DNA to single-stranded templates for DNA synthesis. The origins of DNA replication display a structural diversity between species that might reflect variations in the mechanisms by which the origins are activated and controlled. In addition, it might also be important to create diversity to ascertain that chromosomes can be uniquely identified and preferentially replicated.

We have studied Herpes simplex virus type I to learn more about the molecular mechanisms that underlie initiation of DNA synthesis and subsequent events at the replication fork. HSV-1 DNA replication requires seven virally encoded proteins, initiator proteins, identify the origins of DNA replication and a dimeric DNA helicase encoded by the UL9 gene (4, 5). The origin binding protein, OBP, is encoded by the UL9 gene (4, 5). It is a sequence-specific DNA-binding protein with structural information for a selective and efficient assembly of the viral replication machinery.

DNA-binding protein ICP8 (8, 9). The putative viral replisome consists of a trimeric helicase-primase composed by the products of the UL5, UL8, and UL52 genes as well as a processive DNA polymerase containing the UL30 and UL42 gene products (2).

Initiation of HSV-1 DNA replication is dependent on the viral origins of replication oriS and oriL. The palindrome sequences characterized by two inverted copies of the recognition sequence for OBP, GTTCGCCAC (11). The two binding sites are referred to as box I and box II. A third site, box III, does not bind OBP with high affinity despite extensive sequence homology. Box I and box II are separated by a spacer sequence containing 18 alternating AT base pairs. The oriS palindrome is 46 nucleotides, and the oriL palindrome is 144 nucleotides. Mutations in boxes I, II, and III reduce DNA replication (12). Furthermore, the length of the AT-rich sequence affects DNA synthesis in a periodic fashion (13). It has also been clearly demonstrated that sequences immediately outside the minimal oriS have a profound effect on DNA replication (14, 15). These sequences may recruit additional trans-acting factors or affect the physical properties of DNA.

HSV-1 OBP appears to bind cooperatively as two dimers to the minimal oriS (16–18). The interaction results in a local unwinding of DNA that can be detected using electron microscopy (19). During this process unwound stem-loop structures are formed at oriS.

We describe here a series of investigations that demonstrate that HSV-1 oriS can be converted to a novel conformation referred to as oriS**. We also show that a complex with a high stability can be formed between OBP and oriS**. This complex can be readily detected in agarose gels. Using mutant forms of oriS and single-stranded oligonucleotides corresponding to the upper strand of oriS, we have been able to identify some of the structural features that are essential for the formation of a stable complex between HSV-1 oriS and OBP**. On the basis of these results we discuss a possible mechanism for the activation of oriS that involves an OBP-mediated extrusion of hairpin structures. Similar mechanisms for sequence-specific loading of DNA helicases may exist also for other organisms.

**EXPERIMENTAL PROCEDURES**

DNA and Plasmids—Restriction fragments containing oriS were derived from the pORI series (17) or the pCG series (12, 20). pORI—6AT has a 6-base pair deletion in the AT-rich spacer sequence of oriS of pORI(wt). pCG5 contains the wild type sequences of oriS. The plasmids pCG11, pCG16, and pCG20 have TT to GG transversions in boxes I, II, and III, respectively. pCG28, pCG35, and pCG42 have the same transversions in two of the binding sites. They are identified as pCG28—box I—box II (pORIBI), and pCG42—box I (pORIBI). pCG42—box I (pORIBI), pCG42—box I (pORIBI), and pCG42 (pORIBI) have point mutations in all three binding sites. SphiI-EcoRI fragments were labeled the 3′ end of the upper strand (21). EcoRI-HindIII fragments were labeled the 3′ end of the lower strand. The upper strand is defined by the nucleotide sequence from pORI(wt): 5′-CTGCAGGTCGACACTCTAGAGGATCCCCTGGTAAAAGAA- GTGAAAGCCGAAAGCGTGGGCATCCATATATATATATATAT-
ATTAGGCGGAAGCTGGCGCAGTGGCGCC-3

The restriction fragments were isolated by gel electrophoresis in agarose gels and transferred to DEAE membranes. The DNA fragments were eluted in a buffer containing 1 M NaCl, 0.1 mM EDTA, and 20 mM Tris-HCl, pH 8.0. They were labeled either at the EcoRI site using [α-32P]dATP or at the HindIII site using [α-32P]dGTP (3000 Ci/mmol).

The oligonucleotides used were as follows: PE17, 5′-GATCTGCGAAGCTGGCGCAATCTGGCCGAA-3′; PE18, 5′-GATCTGCGAAGCTGGCGCAATCTGGCCGAA-3′; PE45, 5′-GATCTAAGAAAGTGAGAACGCG-3′; PE46, 5′-GCTCCGGTGGCTACCTTCTTTAT-3′; PE146, 5′-GCTCCGGTGGCTACCTTCTTTAT-3′; and PE18, 5′-GATCTGCGAAGCTGGCGCAATCTGGCCGAA-3′.

Proteins—Escherichia coli SSB was from Amersham Pharmacia Biotech. OBP and ICP8 were purified to near homogeneity from Sf9 cells using recombinant baculovirus vectors obtained from Dr. N. D. Stow (Medical Research Council Virology Unit, Glasgow, UK) essentially as described (8). The protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad).

Preparation of oriS*—The reaction mixture (5 μl) contained 0.2 nm of radiolabeled restriction fragment containing oriS, 50 mM NaCl, and 20 mM Tris-HCl, pH 8.0. It was heated at 95 °C in a programmable thermo controller (MJ Research, Inc.) for 5 min and cooled directly on ice.

S1 Nuclease Mapping—The reaction mixture contained 0.5 nm oriS or oriS* and 1 μg of heat-denatured calf thymus DNA in 50 mM CH3COONa, pH 4.5, 280 mM NaCl, and 4.5 mM ZnSO4. S1 nuclease (Promega) was added to a final concentration of 0.003 units/μl in a total volume of 400 μl. The samples were incubated 3 min at 37 °C. The reaction was stopped by the addition of 5 μl of 0.5 mM EDTA and 10% sodium dodecyl sulfate. DNA was recovered by ethanol precipitation and dissolved in 4 μl of water. The samples were analyzed on a 8% polyacrylamide sequencing gels.

DNA Sequencing—DNA sequencing was performed using the Thermo Sequenase kit from Amersham Pharmacia Biotech. Standard G+A cleavage reactions were performed as described (22).

Agarose Electrophoresis of Protein-DNA Complexes—The reaction mixtures (10 μl) contained 0.1 nm end-labeled oriS or oriS*, 20 mM Tris-HCl, pH 7.6, 10% glycerol, 2.5 mM dithiothreitol, 3 mM MgCl2, 2.5 mM ATP·S, 200 μg/ml bovine serum albumin, 50 mM NaCl, and the indicated amounts of OBP and ICP8. The incubation was at 37 °C for 10 min. Protein-DNA complexes were analyzed on 1% agarose gels in a buffer containing 40 mM Tris acetate, pH 8.0, and 1 mM EDTA. The submarine gels were run at 7 V/cm at room temperature for 2.5 h. The gels were dried on DE81 paper (Whatman). They were the autoradiographed overnight at −80 °C or subjected to PhosphorImager analysis.

RESULTS

OBP Binds to an Alternative Conformation of Functional oriS—The origin binding protein of Herpes simplex virus binds in a sequence-specific manner to two sites in the viral origin of DNA replication referred to as box I and box II (Fig. 1). The two sites are separated by an AT-rich spacer sequence. A series of plasmids was made in which mutations were introduced into the AT-rich spacer sequence of oriS (17). They were either additions or deletions of AT dinucleotides. The mutations had opposite effects on DNA replication and the cooperative binding of OBP to oriS. For example, the deletion of 6 AT base pairs from the spacer sequence as in oriS*(−6 AT) completely inhibited DNA replication but strongly stimulated cooperative binding of OBP to oriS (17). We suggested that the ATP-dependent cooperative binding of OBP to oriS might induce a conformational change in DNA, thereby facilitating the initiation of DNA replication (17). The nature of this structural change has remained elusive.

We have now found that heat-induced denaturation of a 140-base pair restriction fragment containing oriS gives rise to a novel conformation of that DNA referred to as oriS**(Fig. 2). oriS** was only produced quantitatively at low DNA concentrations, arguing that intramolecular base pairing prevents reannealing of the complementary single strand (results not shown). The new DNA species migrated slower than the corresponding linear duplex DNA in agarose gels (Fig. 2). The binding of OBP to oriS**(wt) and oriS**(−6 AT) as well as oriS**(wt) and oriS**(−6 AT) were then examined (Fig. 2). We found that OBP bound more efficiently to oriS**(−6 AT) than to oriS**(wt) in agreement with previous results (17). In contrast, OBP bound oriS**(wt) more efficiently than oriS**(−6 AT). Apparently mutations in the AT-rich spacer sequence that reduce DNA repli-
A New Conformation of oriS Bound by OBP

Stability of the OBP-oriS* Complex—To characterize the OBP-oriS* complex we performed an experiment to determine the half-life of the complex (Fig. 3). Previous experiments have shown that a complex between OBP and a duplex oligonucleotide containing box I has a half-life of 34 s at 22 °C (23). A preformed OBP-oriS* complex was now challenged with a 1000-fold excess of the unlabeled duplex oligonucleotide, PE17/18, containing box I. The time of challenge varied between 0 and 30 min at 37 °C. The samples were then immediately examined by agarose gel electrophoresis. We found that the OBP-oriS* complex was remarkably stable (Fig. 3, A and C). The half-life of the OBP-oriS* complex was determined from several independent experiments to be approximately 20 min at 37 °C (results not shown). In contrast, complexes between OBP and oriS dissociated immediately after the addition of the competing duplex oligonucleotide (Fig. 3, B and C). This finding implies that additional sequences and novel structures present in oriS* stabilize the complex.

Structure of oriS*—The structure of oriS* was first examined by S1 nuclease digestion. This experiment demonstrated that the AT-rich spacer sequence was readily accessible to the nuclease (Fig. 4A). The sequences at either side of the spacer were resistant to cleavage by S1. This finding indicated that a hairpin was formed at the site of the oriS palindrome. The sequences surrounding the putative hairpin were moderately accessible to S1, indicating that they adopt a partially single-stranded conformation. We could also show that oriS* but not oriS was bound by the HSV-1 encoded single-stranded DNA-binding protein ICP8 as well as E. coli SSB (Fig. 4, B and C).

A single-stranded oligonucleotide corresponding to the upper strand of oriS, oriS136, was synthesized. It behaved as oriS* in agarose gels and in the binding experiments further discussed below. We have used this oligonucleotide to perform DNA melting experiments. These experiments showed that oriS136 underwent a structural transition characterized by a sharp increase in hyperchromicity with a Tm of 65 °C in 0.1 M NaCl (results not shown).

In summary, oriS* appears to consist of a stable hairpin with the AT-rich spacer sequence in the loop. The stem contains complementary sequences from box I and box II. It may constitute a double-stranded binding site for the HSV-1 origin binding protein. The hairpin is surrounded by 5‘ and 3‘ tails. They appear to be partially single-stranded DNA inasmuch as they provide binding sites for single-stranded DNA-binding proteins.

Recognition of oriS* by OBP—Further insight into the structural requirements for the formation of an OBP-oriS* complex was obtained when we investigated the ability of OBP to bind to oriS* formed by the upper and lower strands of oriS. We noted that OBP bound preferentially to oriS* formed by the upper strand (Fig. 5). This finding argues that the OBP-oriS* complex consists of one OBP dimer bound to only one of the two available strands formed by heat denaturation of oriS. It also implies that there are important structural differences between oriS* formed by the upper strand and oriS* formed by the lower strand.

The importance of structures outside the oriS* palindrome was further investigated using synthetic single-stranded oligonucleotides. They were as follows: oriS136 corresponds to oriS* formed by the upper strand of the Sph/EcoRI fragment from pORI(wt); oriS93 is devoid of the 5‘ part of oriS*; oriS108 is missing the 3‘ part of oriS*; oriS80 has a short 5‘ sequence and no 3‘ part; oriS80tdT has a 5‘ part made up of poly(dT). The radiolabeled oligonucleotides were incubated with OBP, and the reaction mixtures were analyzed on agarose gels (Fig. 6). The results show that oriS* formed by heat denaturation of duplex DNA, oriS136, and oriS108 all supported the formation of OBP-DNA complexes of similar mobilities. oriS93 did not support the formation of a complex with OBP. The results suggest that sequences on the 5‘ side of the oriS palindrome but not on the 3‘ side of the oriS palindrome are essential for the formation of a stable complex with OBP.

To further investigate the role of the 5‘ end we have carried out an experiment in which increasing amounts of OBP were added to the radiolabeled oligonucleotides oriS80 and oriS80poly(dT). The amount of complex formed was measured after agarose gel electrophoresis using a PhosphorImager. oriS80 supported the formation of a complex with OBP as efficiently as oriS136. oriS80poly(dT) did not form a stable complex with OBP (results not shown). This result indicates that there exist specific recognition elements in oriS80 that are absent in oriS80poly(dT). It strongly suggests that nucleotide sequence and secondary structure of the 5‘ part of oriS* contribute significantly to the stability of the OBP-oriS* complex.

The results obtained using S1 nuclease digestion of oriS* indicated that the AT-rich spacer constitutes a single-stranded loop of a hairpin in which the stem contain complementary sequences from box I and box II. The sequences upstream and downstream the central oriS palindrome hairpin may adopt a single-stranded conformation. To characterize the role of these regions we performed experiments in which oligonucleotides either co-linear or complementary to the 5‘ and 3‘ tails of oriS* were incubated with radiolabeled oriS* before or after the addition of OBP (Fig. 7). We found that an oligonucleotide complementary to the 5‘ tail, PE 46, completely prevented the formation of a complex when added prior to OBP (Fig. 7A). A co-linear oligonucleotide, PE 45, did not affect complex formation. Similar experiments were also performed to study the role of the 3‘ tail (Fig. 7B). We found that an oligonucleotide complementary to the 3‘ tail was unable to inhibit the formation of the OBP-oriS* complex. Our observations infer that the OBP interacts directly with structural elements present at the 5‘ side of the oriS* hairpin. We argue that the formation of the OBP-oriS* complex requires sequence- and structure-specific interactions with the 5‘ part of oriS*.

Mutations in oriS That Affect the OBP-oriS* Complex—Biochemical experiments have revealed the existence of two sites in oriS, box I and box II, that bind OBP in a sequence-specific way. These sites are also required for efficient DNA replication (12). A third site, box III, is homologous to boxes I and II. However, when box III is present in a double-stranded oligonucleotide it fails to bind OBP in a sequence-specific way (20). Extensive mutational analyses have demonstrated that sequences outside boxes I and II contribute significantly to the efficiency of DNA replication (13, 14, 15, 24). A biochemical understanding of the role these sequence elements play during the initiation of DNA replication is lacking. We have now examined how mutations in boxes I, II, and III affect the formation of the OBP-oriS* complex (Fig. 8).

The mutations were all TT to GG transversions in the recognition sequence, GTTCCGAC, for OBP. Restriction fragments containing wild type and mutant forms of oriS were heat-treated, and the products were analyzed on agarose gels (Fig. 8). They were all shown to give rise to a DNA species that migrated as oriS* in agarose gels. Their ability to bind OBP was also examined. We found that oriS* (−box I) had a reduced ability to bind OBP. oriS* (−box II) showed normal levels of complex formation. The double mutant oriS* (−box I, −box II) did not support the formation of an OBP-oriS* complex.
FIG. 3. Stability of the OBP-oriS* complex. The half-life of the OBP-oriS* complex was determined by challenging a preformed complex with a 1000-fold excess of an unlabeled competitor. A, autoradiograph of a gel retardation experiment. The reaction mixture contained 2 fmol radiolabeled oriS* and 0.4 pmol of OBP. 1 μM of the duplex oligonucleotide PE17/18 corresponding to box I of oriS was added to the preformed complex. The incubation continued for 0, 5, 10, 15, 20, 25, or 30 min. The reaction mixtures were loaded on agarose gels immediately before electrophoresis. B, autoradiograph of a gel retardation experiment. The reaction mixture contained 2 fmol of radiolabeled oriS and 0.4 pmol of OBP. 1 μM of the duplex oligonucleotide PE17/18 corresponding to box I of oriS was added to the preformed complex. Incubation and analysis of the samples were as above. C, a quantitative analysis of the autoradiographs using the PhosphorImager. The filled circles are results from the experiment with oriS*. The open circles are results from the experiment with oriS. The squares represent the amount of free DNA seen in the absence of the competitor. The open square is from the experiment with oriS, and the filled square is from the experiment with oriS*.

FIG. 4. oriS* contains a hairpin with partially single-stranded tails. A, S1 nuclease digestion of oriS*. An autoradiograph of a sequencing gel is shown. S1 digestions of oriS and oriS* were performed as described under “Experimental Procedures.” A standard G+A cleavage reaction of oriS was included as a marker. Equal amounts of radioactivity were loaded in the three lanes. The positions of the OBP binding sites boxes I, II, and III as well as the AT-rich spacer sequence are indicated. B, HSV-1 ICP8 binds oriS*. An autoradiograph of a gel retardation experiment is shown. The reaction mixture contained 2 fmol oriS or oriS* from a SphI-EcoRI fragment from pORI(wt) radiolabeled at the EcoRI site of the upper strand. Increasing amounts of ICP8 (0.5, 5, 50, and 500 ng) were added to oriS* and 500 ng of ICP-8 to oriS. C, E. coli SSB binds oriS*. Reaction conditions were as above. Increasing amounts of SSB (1, 10, 100, and 1 μg) were added to oriS*, and 1 μg of SSB was added to oriS.
also found that TT to GG transversions in box III alone contributed to the formation of an OBP-oriS* complex. Surprisingly, the ability to form an OBP-oriS* complex was restored in the double mutant oriS(--box I, --box III). A different double mutant oriS(--box II, --box III) did not give rise to an OBP-oriS* complex.

The finding that OBP binds oriS* (--box I, --box III) indicates that sequences in box I and box III might interact. The point mutations that are introduced may therefore not only change the recognition sequence for OBP in the stem of the oriS* hairpin, but they may also change the conformation of the 5' tail of oriS*.

To investigate the influence of the mutations in boxes I, II, and III on the structure of oriS*, we performed a series of S1 nuclease digestion experiments (Fig. 9). We found that oriS* (wt), oriS* (--box I), oriS* (--box III), and oriS* (--box II, --box III) were all sensitive to the S1 nuclease in the AT-rich spacer sequence, suggesting that they adopted similar hairpin structures. We noted, however, that the electrophoretic mobilities of the full-length fragments varied, suggesting the existence of a stable secondary structure in the box III region of oriS*.

We further investigated this phenomenon by subjecting the same oriS* variants labeled at the 3' end of the upper strand to depurination by formic acid followed by cleavage induced by piperidine. The samples were analyzed on sequencing gels (Fig. 10A). The results show that the electrophoretic mobilities of the cleavage products from oriS* (wt), oriS* (--box I), oriS* (--box III), and oriS* (--box II, --box III) were identical for fragments containing box II, the AT-rich sequence, and box I. In contrast, long fragments that contained sequences from the box III region behaved anomalously (indicated by the arrow in Fig. 10A). Fragments from oriS*(wt) and oriS* (--box I, --box III) had similar electrophoretic mobilities. In contrast, the corresponding long fragments obtained from oriS(--box I) and oriS(--box III) migrated differently. DNA sequencing with the upper strand as a template ensured that the unusual electrophoretic mobilities were not caused by differences in nucleotide sequence (Fig. 10B). Together our results indicate that the box III region adopts a secondary structure in oriS* that may involve direct base pairing between complementary sequences of boxes I and III.

**DISCUSSION**

We have in this work presented results demonstrating that the Herpes simplex virus type I origin of DNA replication, oriS, can be converted in vitro by heating and cooling to a novel conformation referred to as oriS*. oriS* is a DNA hairpin with 5' and 3' tails that are partially single-stranded. The HSV-1 initiator protein, OBP or UL9 protein, binds specifically to oriS*, giving rise to a unique complex that by several criteria can be distinguished from the complexes that are formed between OBP and oriS. The OBP-oriS* complex has a significantly increased stability. It requires binding both to the hairpin and to sequences in the 5' tail. The formation of the OBP-oriS* complex is influenced by mutations that affect the structure of the loop in the oriS* hairpin as well as mutations in boxes I and III of oriS. Two observations argue that the OBP-oriS* complex plays a role also in vivo. First, we have demonstrated that mutations that reduce DNA replication also inhibit the formation of the OBP-oriS* complex. Second, a study by electron microscopy has demonstrated that OBP will

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**Fig. 5.** OBP binds preferentially to oriS* formed by the upper strand of oriS. An autoradiograph of a gel retardation experiment. The reaction mixture contained 0.4 pmol of OBP or oriS* either from a HindIII-EcoRI fragment of pORI(wt) radiolabeled at the HindIII site of the lower strand (first three lanes) or from a SphI-EcoRI fragment radiolabeled at the EcoRI site of the upper strand (last two lanes).

**Fig. 6.** Structural elements in the 5' tail of oriS contribute to complex formation. A, an autoradiograph of a gel retardation experiment. The reaction mixture contained 0.4 pmol of OBP and 2 fmol of either oriS* or single-stranded oligonucleotides derived from the upper strand of oriS. The following oligonucleotides were used: oriS136 corresponds to the upper strand of oriS; oriS93 lacks the 5' strand of oriS.

**Fig. 7.** An oligonucleotide complementary to the 5' tail inhibits the formation of an OBP-oriS* complex. Autoradiographs of agarose gels are shown. The reaction mixtures contained 2 fmol of oriS* and 0.4 pmol of OBP. A, 0.3 μg of the oligonucleotides PE45 and PE46 were added to the reaction mixture either before or after the addition of OBP. PE45 is co-linear with the 5' tail of oriS*, and PE46 is complementary to the to the 5' tail. B, 0.03 and 0.3 μg of the oligonucleotide PE146 was added to the reaction mixtures either before or after the addition of OBP. PE146 is complementary to the 3' tail of oriS*.
produce unwound stem-loop structures at oriS that look like extended versions of oriS* (19).

In what follows we will describe a comprehensive model for the activation of HSV-1 oriS by OBP and the assembly of the HSV-1 replisome. We will also discuss the modular structure of oriS and a role for alternative DNA conformations during the activation of an origin of DNA replication.

We propose that OBP binds to the linear duplex form of oriS and turns it into a structure closely resembling oriS*. In the context of the viral genome this structure would have short stretches of single-stranded DNA in the vicinity of the extruded hairpins formed by the upper and lower strands of oriS. The viral single-stranded DNA-binding protein, ICP8, may assist OBP to promote further unwinding (8, 9). The single-stranded regions produced by OBP may serve as loading sites for the helicase-primase complex. This model for the initiation of HSV-1 DNA replication may be relevant for five of the eight human herpesviruses that have been identified so far. HSV-1, HSV-2, VZV, HHV6, and HHV7 all have OBP homologues and the lytic origins of replication are also similar. Epstein-Barr virus, cytomegalo virus, and HHV8 lack an OBP homologue, and they have to use different mechanisms for the activation of the lytic replication origins. However, the enzymes acting at the replication fork are closely related for all herpesviruses. A conserved mechanism by which the replisome is assembled may therefore still exist.

We will here try to define some of the sequence elements that have been demonstrated to affect the function of origins of DNA replication and discuss their role in HSV-1 oriS. Sequence-specific binding of the initiator protein may be considered foremost among the entities that build an origin of DNA replication. For example, OBP will bind to double-stranded DNA in the major groove through an interaction with the sequence GTTCGCAC (11). Mutational analyses of replication origins demonstrate, however, that DNA sequences well outside the recognition sequences for the initiator proteins are essential for DNA replication (13, 14, 15, 24). It has been argued that these sequences may be thermodynamically unstable and serve as DNA unwinding elements (25). If this is true it should be possible to replace them by other DNA sequences with the similar physical properties (26). The AT-rich spacer of HSV-1 oriS may serve as a DNA unwinding element. Selection experiments have, in fact, demonstrated that it can be replaced by many other AT-rich sequences (24). It has also been noted that the phasing of structural motifs in replication origins is important. A correct phasing may be essential to allow the formation of well defined and stable nucleoprotein complexes. But it may also be needed for the initiator proteins to exert an untwisting force on DNA. Consequently, altering the phasing may affect protein-DNA interactions and DNA replication in a periodic fashion. This phenomenon is illustrated by the properties of HSV-1 oriS (13). The cooperative binding of OBP to boxes I and II as well as the replication efficiency of oriS both depend on the length of the AT-rich spacer sequence (17).

A consequence of the results presented in this paper is that the mechanism by which mutations in origins of DNA replication affect DNA synthesis may only be fully understood in the context of an alternative DNA conformation. For example, new base pairs may be established in the alternative origin structure and mutations that destabilize this conformation will thus be expected to interfere with DNA replication. It has been observed that if the sequence TTCGTCCC located between box I and the AT-rich spacer sequence in oriS is replaced by either TTTACCCC or TTCGTTTT, DNA replication is significantly reduced but sequence-specific binding of OBP to box I is not affected (27). An alternative conformation of an origin of DNA replication...
replication may also present new structural determinants for an interaction with the initiator protein. The binding of OBP to oriS* may be one example. It is an intriguing finding that OBP binds to oriS* formed from the double mutant oriS(box I, box III) but not from the single mutants oriS(box I) and oriS(box III). One explanation might be that box I and box III sequences interact directly in the oriS* structure. At least two models for oriS* should be considered (Fig. 11). In the first case oriS* is shown as a central hairpin with 5' and 3' single-stranded tails (Fig. 11A). The second model contains two elements of secondary structure: the central hairpin with the AT-rich sequence in the loop is interacting with a second hairpin formed by complementary sequences in box I and box III (Fig. 11B). The first model may describe the single mutants oriS(box I) and oriS(box III). The second alternative may reflect the conformation adopted by oriS(wt) and the double mutant oriS(box I, box III). This line of reasoning seems to imply that OBP
binds oriS in a sequence-specific way and that it binds oriS* in a structure-specific fashion.

The following experimental evidence suggest that our model of the OBP-oriS* complex is biologically significant. It has been shown that point mutations in boxes I, II, and III reduce DNA replication (12). In contrast, the oriS(box I, box III) double mutant replicates as efficiently as oriS(box II). These findings argue that a functional interaction between sequence elements in box I and box III exists. Furthermore, selection experiments have emphasized that the box III region is an essential element in oriS (24). However, the initiator protein, OBP, does not bind the box III region in duplex DNA with high affinity (20). The results described in this paper show that box III is an important element for the binding of OBP to oriS*, and they indicate that box III is a part of a stable secondary structure in oriS*.

The observations presented here suggest that a large scale conformational changes may be induced by initiator protein. The plasmid pT181 offers a good example. In this instance the dimeric RepC protein facilitates the extrusion of a cruciform in negatively supercoiled DNA followed by site-specific nicking by a topoisomerase-like mechanism in the newly formed loop (28). Structural information on alternative conformations occurring in other replication origins is scarce. Replication origins do, however, in many instances contain palindrome structures or inverted repeats that are compatible with the formation of alternative DNA structures. It is conceivable that initiator proteins possess cryptic DNA binding sites that permit the recognition of activated forms of origins of DNA replication. It has, for example, been noted that the E. coli DnaC protein and the P protein of bacteriophage λ have cryptic binding sites for single-stranded DNA (29).

Origins of DNA replication play an essential role in the evolution of chromosomes inasmuch as they provide the chromosome with an identity that can be uniquely identified by the replication machinery. In this way preferential replication of a chromosome can be obtained. The evolution of new genetic elements such as viruses and plasmids may depend on the possibility of identifying new structures that will be unique to the developing chromosome. It is an intriguing possibility that interactions of the initiator proteins with alternative conformations of the origins might provide the structural information required for preferential replication.
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Alireza Aslani, Stina Simonsson and Per Elias

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