Herpes Simplex Virus Origin-Binding Protein (UL9) Loops and Distorts the Viral Replication Origin

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To investigate the role of the herpes simplex virus origin-binding protein (UL9) in the initiation of DNA replication, we have examined the effect of UL9 binding on the structure of the viral origin of replication. UL9 loops and alters the DNA helix of the origin regardless of the phasing of the binding sites. DNase I and micrococcal nuclease footprinting show that UL9 binds two sites in the origin and loops the AT-rich DNA between them independent of the topology of the DNA. KMnO₄ and dimethyl sulfate footprinting further show that UL9 alters the DNA helix in the AT region. In contrast to the looping reaction, however, helical distortion requires the free energy of supercoiled DNA. UL9 also loops and distorts the origin DNA of a replication-defective mutant with a 6-bp insertion in the AT region. Because the helical distortion of this mutant DNA is different from that of functional origins, we conclude that an imperfect tertiary structure of the mutant DNA may contribute to its loss of replication function.

Herpes simplex virus (HSV) is an excellent model system for the study of protein-protein and protein-DNA interactions at a eukaryotic origin of replication. Genetic and biochemical analyses are simplified, since most of the components necessary for viral DNA metabolism are encoded by the virus. HSV may share some of the initiation events of the well-characterized Cairn's type DNA replication origins even though studies of the replicative intermediates of HSV suggest that viral DNA replication occurs by a rolling circle mechanism (2, 16, 22-24, 49).

HSV contains two types of replication origins. One origin, *ori_L*, is in the long unique segment of the virus (15, 18, 27, 51), and the other, *ori_S*, is in the repeat region flanking the short unique segment (15, 41, 49). These origins are highly homologous, and both are functional *in vivo*. The optimal origin sequences have been defined for *ori_S* (10, 28, 43). This origin contains a large 45-bp palindrome centered on an alternating AT motif and an additional 30 bp leftward of the palindrome (28). Two UL9-binding sites overlap the ends of each arm of the palindrome (13, 35). A third, weaker, UL9-binding site has recently been identified (12, 50). Insertions of increasing numbers of AT dinucleotides into the center of the palindrome have an oscillating effect on origin function (28). Substitution of the AT region in the center of the palindrome with GC-rich sequences impairs replication (28, 43). Therefore, the AT region has both a spacing role, presumably between the UL9 binding sites, and an unknown functional role in replication.

HSV encodes proteins both directly and indirectly involved in viral DNA replication. Seven viral genes are sufficient for replication of the virus (32, 52). These encode a DNA polymerase, polymerase accessory protein, helicase, primase, a single-stranded DNA-binding protein, the UL8 protein of unknown function, and an origin recognition protein, UL9 (reviewed in reference 7). Genetic and biochemical studies indicate that some of these proteins can form complexes with multiple functional activities (6, 8, 20, 34). It is UL9 that imparts specificity to the HSV replication complex (19, 39). UL9 appears to bind as a dimer to a pair of

overlapping inverted pentanucleotide repeats (25) at sites I, II, and III in the origin (13, 25, 35). Site I on the left side of the palindrome is a stronger binding site than site II on the right side (12, 13). Site III is the weakest binding site and binds UL9 only in the presence of sites I and II (12). Differences in binding affinity probably reflect base variations in the recognition elements of the binding sites.

In vitro replication systems for simian virus 40 (SV40) (40), *Escherichia coli* (1, 33), and bacteriophage λ (33) have identified common events in the initiation of replication of Cairns-type origins. In the first stage of initiation, an origin recognition protein specifically binds to multiple recognition sites and unwinds the DNA strands at the origin of replication. The second stage starts with the assembly of the replication machinery at the partially unwound origin. A helicase contained in this replication complex extends the partially unwound origin into a replication bubble. Finally, primase and DNA polymerases initiate the polymerization reaction in the replication bubble. The *E. coli* and bacteriophage λ origin recognition and helicase activities are on separate proteins, whereas the SV40 origin-binding protein, T antigen, has both activities. However, the origin-binding protein in all cases has the capacity to catalyze the first event in the initiation of replication, the unwinding of the duplex origin DNA. We wanted to determine whether UL9 could alter the structure of the HSV origin of replication.

In this study, we show that UL9 alters the structure of the origin of replication in two ways. Neither of these structural changes requires the addition of exogenous ATP. Nuclease footprinting demonstrates that UL9 loops the AT-rich DNA between UL9-binding sites I and II. Chemical footprinting shows that the helix of the looped DNA can be distorted by UL9 when free energy is provided by supercoiling. Footprinting of mutant origins with insertions in the AT-rich DNA indicates that UL9 also loops and distorts the mutant origins. The helical distortion of a replication-defective origin, however, is different from that of functional origins.

MATERIALS AND METHODS

Cells and virus. Sf9 cells and baculovirus were maintained as described by Summers and Smith (45). Recombinant

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baculovirus expressing the HSV type 1 (HSV-1) origin-binding protein UL9 (*Autographa californica* nuclear polyhedrosis virus [AcNPV]-UL9) was a generous gift of M. Challenge (36).

Cloning of HSV origins. pS201, the wild-type HSV-2 origin, and two AT insertion mutants, pS201AT3 and pS201AT23, were kindly provided by D. Galloway (28). Origin-containing *Apa*I restriction fragments were released from these plasmids and purified by low-melting-point agarose gel electrophoresis as described by Maniatis et al. (29). These restriction fragments were subcloned into an *Apa*I site inserted between the polylinker and the forward primer-binding site of pUC19. The transformants were sequenced, and clones with identical orientations were selected. We defined the clone containing the wild-type origin derived from pS201 as WT. The replication-competent, 46-bp insertion mutant was redefined as [AT]₂₃, and the replication-defective, 6-bp insertion mutant was redefined as [AT]₃. DNA was isolated from these clones by the alkaline lysis method (3) and purified in a CsCl gradient as described by Maniatis et al. (29).

Purification of recombinant UL9. Sf9 cells were infected with AcNPV-UL9 at a multiplicity of 10 to 20 viruses per cell and incubated at 27°C for 68 to 72 h. All purification procedures were carried out at 4°C. Infected cells were gently shaken off flasks and collected by centrifugation at 1,000 × *g*. The cell pellet was washed with 8.33 ml of phosphate-buffered saline per g of wet weight of cells and collected by centrifugation at 1,000 × *g*. Hypotonic lysis and high salt nuclear protein extraction were carried out as described previously by Elias et al. (14). The clarified extracts were adjusted to 0.5 M NaCl by dilution with 2.4 volumes of buffer B (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.6], 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM Na₂EDTA [pH 8.0], 10% glycerol) containing 2 µg of leupeptin per ml. The adjusted extract was loaded onto a DEAE-Sephadex column (12 ml/h, 2.5 cm by 3.5 cm) equilibrated in buffer B with 0.5 M NaCl. The flow-through fraction was collected, adjusted to 0.2 M NaCl with 1.5 volumes of buffer B, and applied to a Whatman P11 column (5 ml/h, 1 cm by 7 cm) equilibrated in buffer B with 0.2 M NaCl. The column was washed with 10 volumes of buffer B and with 0.3 M NaCl in buffer B until the optical density at 280 nm was zero. UL9 was eluted in 10 volumes of 0.4 M NaCl in buffer B and collected in half-column-volume fractions. Fractions containing UL9 were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining. The UL9 fractions were dialyzed against buffer C (20 mM HEPES-NaOH [pH 7.6], 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM Na₂EDTA [pH 8.0], 0.3 M NaCl, 50% glycerol) and stored at -20°C. UL9 protein purified by this scheme was approximately 80% pure by Coomassie staining and had stable DNA-binding properties for at least 2 months.

Footprinting with DNase I. Plasmid DNA was cut with *Eae*I and 3' end labeled with the Klenow fragment of DNA polymerase as described previously (25). The end-labeled DNA was extracted with phenol-chloroform-isoamyl alcohol (ΦCHISAM) and purified on a Bio-Gel P6 (100 to 200 mesh) spin column equilibrated with H₂O. The eluate was adjusted to conditions recommended by New England BioLabs for digestion by *Eco*RI and *Pvu*II. After digestion, the origin-containing restriction fragments were isolated on a non-denaturing 5% acrylamide (29:1) gel and electroeluted. DNA was precipitated with ethanol and resuspended at 0.1 ng/µl.

Variable amounts of protein, as indicated in the figure legends, were incubated with 0.1 ng of probe in the presence of 5.5 µg of poly(dI-dC) · poly(dI-dC)-25% glycerol-0.5 mM DTT-0.2 M NaCl-0.5 mM Na₂EDTA-4 mM ATP-5 mM MgCl₂ in a total volume of 60 µl. To accurately reflect *in vivo* temperature, the binding reactions were incubated at 37°C for 15 min. Then 0.025 U of DNase I was added in a total volume of 1 µl of nuclease dilution buffer {20 mM [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (PIPES)-NaOH (pH 7.0), 0.1 M MgCl₂, 0.05 M CaCl₂, 1 mM NaCl, 0.1 mM Na₂EDTA (pH 8.0)}, and the reaction was continued for 1 min. Reactions were stopped by the addition of 6 µl of 0.5 M Na₂EDTA (pH 8.0) and an equal volume of ΦCHISAM. DNA was isolated from the aqueous phase by elution through a P6 spin column equilibrated with H₂O. The eluate was lyophilized and resuspended in 10 µl of 95% formamide sample buffer and electrophoresed on a 10% acrylamide-8.3 M urea sequencing gel. Gels were dried and exposed to X-ray film at -70°C with an intensifier.

Footprinting with MNase. The procedures and buffers were identical to those used for DNase I footprinting except that 0.4 U of micrococcal nuclease (MNase) was used instead of DNase I.

Footprinting with KMnO₄. The procedure used is identical to that described by Parsons et al. (37). Briefly, 0.2 µg of supercoiled plasmid DNA containing the HSV origin of interest was incubated with UL9 under the conditions described above except that poly(dI-dC) · poly(dI-dC) was not included. KMnO₄ was added to 30 mM, and incubation continued for 4 min. Reactions were stopped by the addition of 1/10 volume of β-mercaptoethanol, and DNA was extracted by ΦCHISAM and recovered from the aqueous phase by using a Sephadex G50 (fine) spin column equilibrated with H₂O. DNA was linearized with *Nde*I and precipitated with ethanol. Linearized DNA was annealed to either a 5' end-labeled M13 forward or reverse primer (as described in the figure legends) and extended. Electrophoresis was carried out on 8% acrylamide-7 M urea sequencing gels. Gels were dried and exposed to X-ray film at -70°C with an intensifier.

Footprinting with DMS. The procedures and buffers were identical to those used for KMnO₄ footprinting except that 4 µl of dimethyl sulfate (DMS) diluted 1:20 in H₂O was used instead of KMnO₄. Incubation with DMS was carried out for 3 min before the modification reaction was stopped with 14 µl of 1.5% sodium dodecyl sulfate-0.1 M Na₂EDTA-7.15 M β-mercaptoethanol.

RESULTS

UL9 loops the DNA between origin-binding sites. DNase I and MNase footprinting analyses were used to determine the effects of UL9 binding to various replication origins. DNA is resistant to DNase I cleavage if protein binding blocks access of DNase I to the minor groove or alters the geometry of the minor groove (17). MNase cuts the phosphate backbone of distorted DNA but is inhibited by guanine and cytosine residues around the site of cleavage (11, 53). Both functional (WT and [AT]₂₃) and nonfunctional ([AT]₃) replication origins (Fig. 1) were used to determine whether the replication defect of [AT]₃ was caused by changes in UL9 binding to this origin. Increasing amounts of recombinant UL9 were incubated with singly end-labeled, origin-containing restriction fragments. Protein-DNA complexes were allowed to form at 37°C and were treated with either DNase

FIG. 2. Nuclease footprinting of the WT and mutant replication origins. (A) DNase I and MNase footprints of the bottom strand of linear origin DNA. Increasing amounts of UL9 were incubated with 0.1 ng of singly end-labeled probes from WT and mutant origins at 37°C. The numbers above each lane represent the relative amounts of UL9. The number 1 above the gel lanes indicates 0.3 μ g of UL9, and 10 indicates 3 μ g of UL9. Protein-DNA complexes were treated with either DNase I or MNase as indicated above each set of lanes. Nucleotide positions 48 and 50 are indicated for each origin. The vertical black lines between these nucleotides represent the inserted AT DNA. Sites I and II are indicated to the left of the WT footprint. Site II remains at a fixed position at the bottom of the origins, whereas site I is moved toward the top of the panels in the mutant origins. (B) Summary of the nuclease footprinting data on the bottom strand. Nucleotides 48 and 50 are identified to orient the sequences with the data above. Bars represent DNase I protection caused by UL9 binding; the black regions were strongly protected, the stippled regions were partially protected, and open regions were not protected. Triangles represent the nucleotides sensitive to MNase cleavage in the presence of UL9. Filled triangles were more sensitive than open triangles. The bottom strand of the inserted AT region and flanking sequences of the mutants are shown below the WT origin. Nucleotide positions 48 and 50 are shown, and the lines above the sequence indicate the inserted nucleotides.

annealed to the DNA and extended by Klenow polymerase. Figure 3A shows the experimental data for the bottom strand; in Fig. 3B, we illustrate the data for both strands schematically.

UL9 binding induced dramatic KMnO_4 sensitivity in both functional and nonfunctional origins. However, both quantitative and qualitative differences in the patterns of KMnO_4 sensitivity were observed when functional and nonfunctional

FIG. 3. KMnO_4 footprinting of the various replication origins. (A) KMnO_4 footprints of the bottom strand of circular origin DNA. Increasing amounts of UL9 were incubated with supercoiled plasmid DNA containing the origins of replication indicated above each panel. The numbers above each lane represent the relative increase in UL9 as described in the legend to Fig. 2. The AT regions of the various origins are shown to the left of the footprints. Nucleotides most reactive to modification by KMnO_4 are indicated by black boxes, and their locations are identified by nucleotide position on the left of each panel. The open rectangle indicates the location of a KMnO_4 -sensitive position outside of the AT region in the WT origin. (B) Schematic representation of KMnO_4 footprints. Both strands of the various origins are shown and numbered above the top strand. UL9-binding sites are shown as stippled boxes. The central palindrome is shown between the strands of DNA in the WT origin. UL9-binding site II, in all the origins, is aligned on the right side of the figure, and AT inserts are enclosed in open boxes. KMnO_4 signals are represented by vertical bars between the DNA strands. The solid bars represent the most reactive nucleotides, and the stippled bars represent less reactive nucleotides. The open rectangles indicate the location of a KMnO_4 -sensitive position outside of the AT region in the WT origin.

origins were compared (Fig. 3). In the functional wild-type and $[\text{AT}]_{23}$ origins, UL9 binding increased the KMnO_4 sensitivity to the greatest extent at nucleotides 53 to 54 and 56 and 57 of the bottom strand. In contrast, protein binding to the nonfunctional $[\text{AT}]_3$ origin induced the greatest sensitivity to KMnO_4 in the inserted DNA. Similar differences between functional and nonfunctional origins were observed in the top origin strand; in the presence of UL9, nucleotides

40 and 41 were the most reactive positions in the functional origins, whereas the insert region was most reactive in the defective origin (Fig. 3B; data not shown). We conclude that UL9 distorts the DNA helix in the AT region regardless of its length. Nevertheless, differences in the phasing of the flanking UL9-binding sites lead to different patterns of helical distortion in the AT DNA.

Most UL9-induced KMnO_4 modifications were present

FIG. 4. KMnO_4 and DMS footprinting of the top strand of the WT origin. Protein (1.0 μg) was incubated with supercoiled DNA containing the WT origin of replication and treated with the reagents indicated above each panel. The presence or absence of UL9 is shown above each lane. After modification the DNA was recovered, linearized, and annealed to a sequencing primer. Stops in the extension of this primer identified modifications on the top strand of the DNA. The cytosine residue at nucleotide 44 is shown on the right of the DMS footprint. On the left of the figure, we indicate the positions of nucleotides 20 and 21. A schematic representation of the data is illustrated below the gel. The top strand of DNA from nucleotides 38 to 60 is shown. Bars represent the KMnO_4 stops, and carats represent the DMS stops.

between UL9-binding sites I and II (Fig. 3 and 4). Many of these sites were located at the same positions relative to sites I or II in both WT and mutant origins. For examples, see modified nucleotides 40 and 41 on the top strand and 53 to 54 and 56 and 57 on the bottom strand. These observations suggest that most structural changes are induced by the binding of UL9 to sites I and II. Surprisingly, UL9 altered the KMnO_4 modification of several nucleotides to the left of site I in the WT origin. UL9 induced modification of nucleotide 19 in the bottom strand of the origin and reduced modification of nucleotides 20 and 21 in the top strand (Fig. 4). DNA in this region may have an atypical helical structure that is changed by UL9 binding. Interestingly, these positions are located between UL9-binding sites I and III in the WT origin. These findings suggest that UL9 may bind to site III, in agreement with the findings of Weir et al. (50) and Elias et al. (12), even though our DNase footprints did not detect stable protein binding at this location. We do not know why the UL9-induced modification at nucleotide 19 appears to maintain a fixed position relative to site II in WT and mutant origins.

To complement the KMnO_4 footprinting, we used DMS footprinting analysis (Fig. 4). DMS modifies both the N1 and N3 positions of adenine and the N1 position of cytosine in single-stranded DNA, and it modifies the N3 and N7 positions of adenine and guanine, respectively, in either double-stranded or single-stranded DNA (30). Klenow polymerase pauses at the DMS-modified nucleotides, and thus the modified base can be detected by primer extension analysis (37). The HSV-2 wild-type and mutant origins contain a single

FIG. 5. Energy requirements of the looping and distortion of origin DNA. UL9 (1.0 μg) was incubated with WT origin DNA, and the protein-DNA complexes were treated with KMnO_4 . The topological forms of the DNA substrates are indicated above each pair of lanes. Form I represents covalently closed supercoiled DNA, and form III is DNA linearized at an *NdeI* site before incubation with UL9. The presence or absence of ATP and of UL9 is indicated. The AT region and nucleotide 19 are aligned and indicated in the various footprints. The KMnO_4 footprinting data are illustrated schematically below the gel. The bottom strand of DNA is shown from nucleotides 40 to 60, and the stops caused by KMnO_4 modification are represented by bars.

cytosine residue at position 44 embedded in the alternating AT-rich DNA. This nucleotide would be resistant to DMS in double-stranded or distorted DNA but would be sensitive in unwound DNA.

Although UL9 protected sites I and II from DMS modifications only to a limited extent, it induced sensitivity to KMnO_4 and hypersensitivity to DMS throughout the AT region of the WT origin (Fig. 4). Similar findings were made in the case of both insertion mutants (data not shown). These changes confirm that the DNA helix is altered in this region. We cannot, however, determine whether pausing of Klenow at the cytosine was caused by methylation of this residue in a region of single-stranded DNA or by methylation of the adjacent adenine. In preliminary experiments using DMS modification in conjunction with single-strand specific nucleases, we have not been able to detect single-stranded DNA in the AT region (data not shown).

Looping and helix distortion are separate activities of UL9. In the experiments described above, we performed nuclease assays for looping on linear DNA and chemical sensitivity assays for helix distortion on supercoiled DNA. To investigate the energy requirements for UL9-induced helix distortion, we compared the KMnO_4 modification of linear and supercoiled DNA in the presence or absence of ATP (Fig. 5). UL9 efficiently distorted the AT region of supercoiled WT DNA. Linearization of the same plasmid, however, rendered the origin completely resistant to KMnO_4 modification. An increase in the amount of UL9 protein in the binding reaction

with linearized substrates did not induce the KMnO_4 -sensitive structure (data not shown). ATP had no effect on the UL9-induced modifications of the origin DNA. These results indicate that UL9 requires the free energy available in supercoiled DNA to distort the helix of HSV origin DNA in an ATP-independent fashion. UL9 binding leads to DNase I protection of sites I and II of WT origins at similar protein concentrations, using either linear or supercoiled DNA substrates (data not shown). Thus, UL9 does not bind more strongly to supercoiled than to linear DNA templates, and it is not better UL9 binding that leads to helix distortion. We conclude that looping and helix distortion are separate activities of UL9.

DISCUSSION

UL9, the origin recognition protein of HSV, binds to at least two sites in the HSV origin in a cooperative manner (12). The AT-rich sequences between UL9-binding sites I and II have a functional role as well as a spacing role (28, 42). In the present study, we investigated the function of UL9 at the viral replication origin by analyzing the DNA structures formed in both functional and nonfunctional origins in the presence of UL9. We show that binding of UL9 to the origin loops and distorts the DNA between the binding sites. These events can be separated by their requirements for free energy.

Our studies also offer insight into the effects of insertion mutations on origin function. We compared structural changes induced by UL9 binding to WT and mutant origins, using nuclease footprinting. The mutant origins, constructed by Lockshorn and Galloway (28), have insertions that extend the AT region between UL9-binding sites I and II. The shorter insertion mutant, $[\text{AT}]_3$, has a 6-bp insertion that changes the phasing of UL9-binding sites by half a turn and blocks replication. The longer insertion mutant, $[\text{AT}]_{23}$, has a 46-bp insertion that enhances replication. We found that UL9 bound to sites I and II of the WT origin and both mutant origins at similar protein concentrations. UL9 binding to $[\text{AT}]_{23}$ and to $[\text{AT}]_3$ resulted in nuclease cutting of the AT regions in oscillating patterns with a period of 10 to 11 bp. Both DNase I and MNase cut the AT regions of the insertion mutants in this periodic pattern. These nuclease-sensitivity patterns are characteristic of DNA that is looped or wrapped around protein (21). We conclude that the AT regions of both mutants are looped between the UL9-binding sites through protein-protein interactions.

A periodic nuclease-cutting pattern was not evident in the WT origin in which the span of nuclease-exposed sites in the AT region is not sufficient to exhibit periodicity. Nevertheless, we assume that the AT region of the WT origin is also looped by the same protein-protein interactions that are so evident in the case of both mutants. Thus, UL9 binds to sites I and II and loops the AT region regardless of the phasing of the UL9-binding sites. Presumably, the flexibility of the protein and DNA are sufficient to compensate for the spatial and phasing differences in the three origins. Nevertheless, the wild-type and mutant origins would have different tertiary structures in the region of looped DNA. The altered tertiary structure of the $[\text{AT}]_3$ origin might contribute to its loss of replication function.

To determine whether UL9 binding alters the DNA duplex in the functional and nonfunctional replication origins, we used chemical footprinting techniques. Figure 1 shows a free energy plot of base-pair stability in the HSV origin. The region of origin DNA in which the least amount of free

energy would be necessary to distort the DNA duplex corresponds to the AT region between the two UL9-binding sites. We found that UL9 distorts the DNA in the AT region of all three origins. Interestingly, the pattern of helix distortion in the AT segment was different in the functional and nonfunctional origins. This finding is consistent with the idea that the looped DNA of the $[\text{AT}]_3$ origin has an altered tertiary structure that may block the initiation of replication. In contrast to looping, helix distortion was absolutely dependent on the origin being supercoiled. These findings imply that differences in the preexisting structures of relaxed and supercoiled DNA determine the ultimate effects of UL9 binding and DNA looping on the helical structure of the AT region in vitro. Although circularization of viral DNA is necessary for DNA replication in vivo (38), the superhelicity of viral DNA during lytic infection has not been established.

Recently Weir et al. (50) and Elias et al. (12) have investigated the function of UL9-binding site III at the left end of the origin. They have shown that deletion or mutation of this segment reduces DNA replication and UL9 binding to the remainder of the origin severalfold. Our data are consistent with the idea that UL9 interacts with site III. We have found that UL9 alters the KMnO_4 modification of 3 nucleotides between sites I and III even though our DNase footprints did not detect stable protein binding at site III. Perhaps, UL9 binds transiently to site III and interacts with UL9 bound to sites I or II to alter DNA structure.

Replication origins from prokaryotic and eukaryotic sources all contain sequences rich in AT DNA. In *Saccharomyces cerevisiae*, the origin unwinds transiently even in the absence of protein (46). Protein-induced unwinding of the yeast origin is not a highly sequence-specific event (47) as it is in the SV40 origin (9). An elegant series of experiments using yeast origins has demonstrated that the energetics of DNA distortion may control replication initiation (48) and that the role of origin-binding proteins may be simply to lower the thermodynamic activation barrier of DNA distortion. The AT motif in the HSV origin is thermodynamically unstable (5) and highly reactive to both chemical and enzymatic probes (31, 44). However, the free energy associated with UL9 binding to the HSV origin is not sufficient to distort linear DNA. KMnO_4 modification of the HSV origin also requires the free energy available in supercoiled DNA. ATP or ATP hydrolysis cannot substitute as an energy source.

Figure 6 shows a model for formation of a functional UL9-DNA complex at the wild-type origin of replication. Step A, two dimers of UL9 bind to inverted repeats in sites I and II of the HSV origin. The AT-rich DNA between these sites consists of approximately four turns of the double helix. Step B, direct interactions between the dimers of UL9 bend the intervening DNA into a partial loop. The loop in the WT origin is probably wrapped tightly around the protein core, as illustrated, whereas the inserted DNA of the mutants $[\text{AT}]_3$ and $[\text{AT}]_{23}$ would be free from the protein and thus partly susceptible to nucleases. Step C, DNA looping, in conjunction with the free energy of supercoiled DNA, distorts the DNA helix in the AT region. The altered helical structure of DNA may serve as part of a recognition element for other proteins involved in the initiation of replication. Step D, assembly of a complete initiation complex would require interactions with both UL9 and looped DNA. We suggest that this complex would be utilized in the next step of replication. Using this model, the replication defect of the $[\text{AT}]_3$ origin may be explained in the following way. Altered phasing of the UL9-binding sites results in improper inter-

FIG. 6. Model for structure of UL9-origin complexes. Steps A through D are described in the text.

actions between UL9 molecules bound to each site, creating an inappropriately looped and untwisted structure. Differences in MNase and KMnO_4 sensitivities of looped DNA in the functional and nonfunctional origins support this idea. Interactions of additional replication factors with the UL9-origin complex may be blocked because the tertiary structure of the complex is incorrect. Further footprinting analyses, in the presence of additional replication proteins, will be important in determining subsequent events in the initiation of HSV DNA replication.

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