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Adjacent Residues in the E1 Initiator β-Hairpin Define Different Roles of the β-Hairpin in Ori Melting, Helicase Loading, and Helicase Activity

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SUMMARY

We have analyzed two residues in the helicase domain of the E1 initiator protein. These residues are part of a highly conserved structural motif, the β-hairpin, which is present in the helicase domain of all papovavirus initiator proteins. These proteins are unique in their ability to transition from local template melting activity to unwinding. We demonstrate that the β-hairpin has two functions. First, it is the tool used by the E1 double trimer (DT) to pry open and melt double-stranded DNA. Second, it is required for the unwinding activity of the hexameric E1 helicase. The fact that the same structural element, but not the same residues, contacts both dsDNA in the DT for melting and ssDNA in the double hexamer (DH) for helicase activity provides a link between local origin melting and DNA helicase activity and suggests how the transition between these two states comes about.

INTRODUCTION

The preparation of template DNA for replication involves several basic steps that are obligatory in all organisms. One step is the initial separation of the two DNA strands at the origin of DNA replication (ori), local melting, to generate a single-stranded region of DNA. This step is essential because most known DNA helicases, which are required for unwinding of the template, require a single-stranded region to initiate unwinding (for a review, see Patel and Donmez [2006]). In spite of the obvious importance of local melting for the replication process, only a few activities are known that can perform this task. In eukaryotes, no such activity has been identified. In bacteria, DnaA is responsible for local ori melting, but the mechanism involved is unknown except that the process involves a large complex of DnaA and is dependent on bound ATP (Bramhill and Kornberg, 1988; Sekimizu et al., 1987). Viral initiator proteins such as T-ag and E1 from the papovavirus family (papillomaviruses, polyomavirus, and SV40) are the only other proteins known to melt double-stranded ori DNA, although the mechanism employed by these proteins is also unknown (Borowiec et al., 1991; Borowiec and Hurwitz, 1988; Gillette et al., 1994; Sanders and Stenlund, 1998).

From recent studies, there is evidence that the local melting of the ori is initiated by a specific form of the E1 protein, a double trimer (DT), which forms on the E1 binding sites present in the ori. Formation of the DT is dependent on ATP binding, but not hydrolysis. The DT is a required precursor for the formation of the active DH helicase, and correct melting appears to be a prerequisite for the formation of a DH that can unwind the ori (Schuck and Stenlund, 2005).

Recent crystal structure determination of fragments of both SV40 T-ag and papillomavirus E1 has identified a highly conserved structural element, a β-hairpin, in the helicase domain of these proteins (Abbate et al., 2004; Enemark and Joshua-Tor, 2006; Gai et al., 2004; Li et al., 2003). In both the E1 and T-ag structures, this β-hairpin is oriented toward the central channel of the hexamer (Figures 1A and 1B) and in the E1 structure, which also includes ssDNA, the hairpin is making contacts with the ssDNA. Mutational analysis of T-ag has suggested that this β-hairpin is required for its DNA helicase activity (Shen et al., 2005). Biochemical and mutational analysis of the corresponding β-hairpin in the bovine papillomavirus E1 protein has indicated that the β-hairpin instead may be required for local ori melting activity. Mutation of the highly conserved H507 at the tip of the β-hairpin results in a protein that fails to melt ori DNA in the presence of ADP and fails to form a DT, the precursor form of E1 required for assembly of the DNA helicase (Schuck and Stenlund, 2005).

Here we demonstrate that the E1 β-hairpin is required for both local ori melting and DNA helicase activity but that these activities are associated with different residues in the β-hairpin (Figure 1B). K506 is required for both local ori melting and DNA helicase activities, but H507 is only required for local ori melting. We also show that the β-hairpin likely interacts with dsDNA directly and that, although either of the aromatic side chains H, Y, or F at position 507 is functional for DT formation, melting of the ori requires

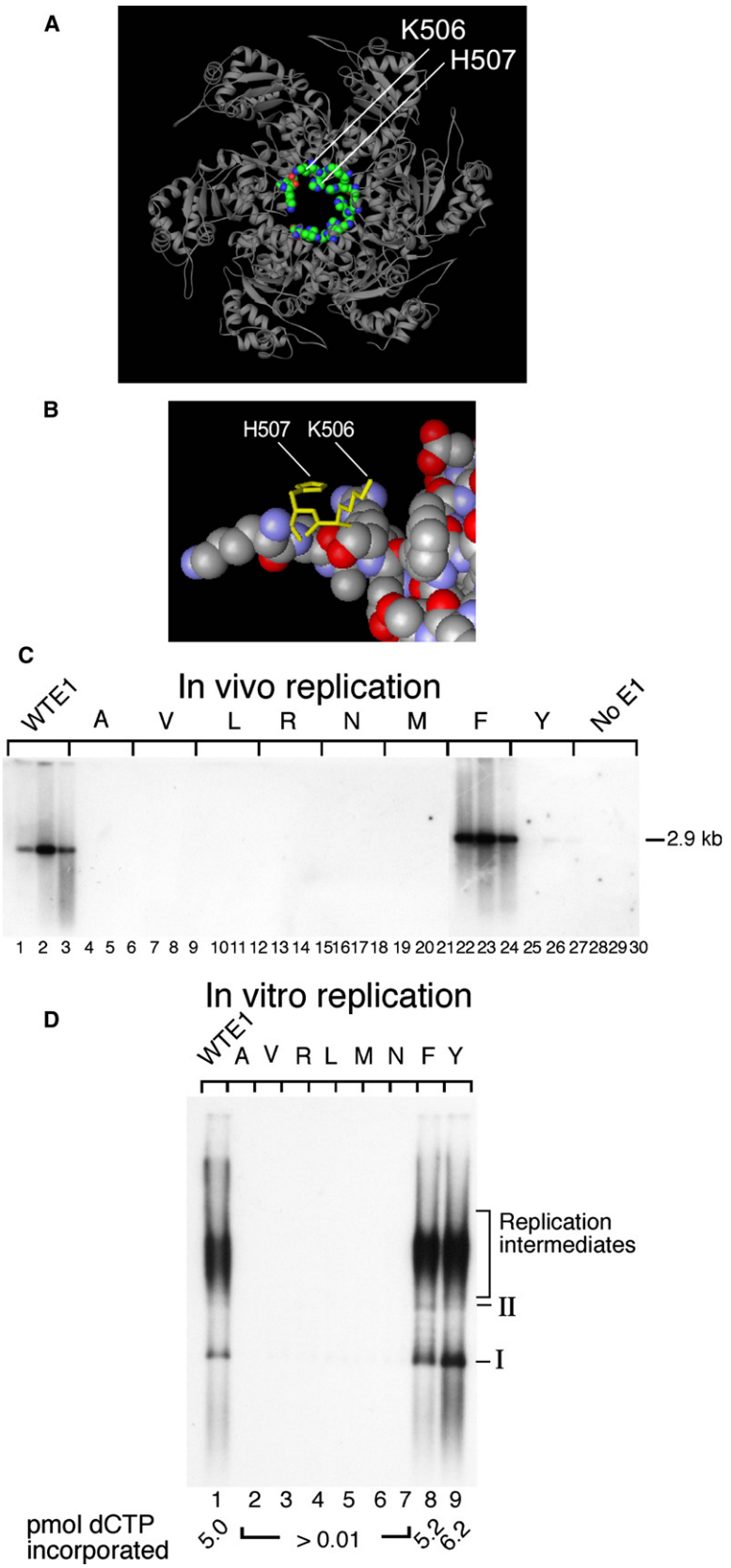

Figure 1. DNA Replication by Substitution Mutants in the β-Hairpin

(A) Image representing the structure of a hexamer of the BPV E1 helicase domain highlighting the positions of two residues, K506 and H507, which form the tip of the β-hairpin and generate an inner constriction in the hexameric ring (Enemark and Joshua-Tor, 2006).

(B) Image of the β-hairpin structure in BPV E1 showing the highly conserved residues K506 and H507.

(C) In vivo DNA replication. The eight substitutions at residue H507 were introduced into the mammalian E1 expression vector pCGE1 by site-directed mutagenesis and tested for activity in transient DNA replication assays. Ori plasmid (100 ng), 0.5 μg of E2 expression vector (pCGE2), and 2 μg of expression vector for the WT or mutant E1 proteins were cotransfected into CHO cells using electroporation, and low molecular weight DNA was harvested 48, 72, and 96 hr after transfection. The DNA was digested with HindIII, which linearizes the plasmid, and DpnI, which digests unreplicated, methylated DNA, and analyzed by Southern blotting.

(D) Cell-free DNA replication. The ability of WT E1 and the eight H507 substitutions to support DNA replication in a cell-free replication system were compared. Each substitution mutant protein (6 pmol), purified from *E. coli*, was incubated with 50 ng of ori plasmid in replication mix (see Experimental Procedures) in the presence of radiolabeled dCTP. After 60 min at 37°C, the replication products were isolated and analyzed by agarose gel electrophoresis and quantitated.

either H or F at this position in the β-hairpin. Together, these results identify the β-hairpin as the instrument that E1 uses for both the local melting of dsDNA and for DNA helicase activity, although these activities reside in different E1 complexes.

RESULTS

DNA Replication Activity of Substitution Mutants at H507

To define the function of the tip of the β-hairpin, we substituted H507 with a range of amino acids with different properties, including A, V, L, R, N, M, F, and Y. We generated these substitutions in the *E. coli* expression vector pET E1 and expressed and purified full-length E1 with the respective substitutions (see Figure S1 in the Supplemental Data available with this article online). We also generated the same substitutions in the context of the mammalian expression vector pCGE1. We first tested these substitutions in transient DNA replication assays in vivo to determine whether they affected DNA replication.

We transfected an expression vector encoding the viral E2 protein, which is required for DNA replication in vivo, together with an ori plasmid and expression vectors for either WT E1 or the individual 507 substitutions into CHO cells using electroporation. Two, three, and four days after transfection, low molecular weight DNA was harvested and analyzed by Southern blotting after digestion with DpnI, which digests unreplicated (methylated) DNA, and HindIII, which linearizes the plasmid (Figure 1C). We observed robust replication, detectable as a prominent 2.9 kb band, in the presence of the WT E1 and H507F expression vectors (lanes 1–3 and 22–24, respectively) as well as a faint trace of replication (>10-fold reduced) with the H507Y vector (lanes 25–27). The remaining H507 substitutions did not support detectable DNA replication.

We next tested the substitutions for their ability to support DNA replication in vitro (Figure 1D). In such an in vitro DNA replication assay, a plasmid containing the ori is incubated in the presence of E1 in a H293 cell extract in the presence of radiolabeled nucleotide. After separation of the replication products by agarose gel electrophoresis, the level of DNA synthesis can be quantitated. The only substitutions that had detectable in vitro DNA replication activity were H507Y and H507F (Figure 1D, compare lanes 1, 8, and 9). The other substitutions generated no detectable replication product (lanes 2–7). These results demonstrate that H507 is important for DNA replication and that F and Y, which have side chains similar to that of H, are also active for replication.

Complex Formation by Substitution Mutants at H507

Preparation of a template for initiation of DNA replication can be divided into several steps. The first step, sequence-specific binding of the initiator, is followed by local melting of the ori, which can be detected by treatment with permanganate, which reacts with unbasepaired T residues. After melting, the DNA helicase activity unwinds the template. A defect in any of these steps would result in a defect in DNA replication. To determine whether the H507 substitutions could generate the appropriate E1 DNA complexes required for these different activities, we first tested them for their ability to form a DH on a short (32 bp) probe (Figure 2A). This complex, which is nonfunctional and does not unwind the template, provides a control for the ability of E1 to oligomerize into a DH and to bind and hydrolyze ATP (Schuck and Stenlund, 2005). All mutants with the exception of H507V (lanes 7–9) were capable of DH formation on the 32 bp probe, albeit at different levels. This result demonstrates that the β-hairpin is not directly involved in E1 oligomerization or ATP binding and hydrolysis. The H507V protein (lanes 7–9) was prone to aggregation, which is the likely cause for its failure to function in EMSA.

We have previously demonstrated that E1 has the intrinsic propensity to form E1 BS-independent trimers on dsDNA in the presence of ADP and that formation of the trimer relies on the E1 β-hairpin. (Schuck and Stenlund, 2005). We next tested the mutant proteins for their ability to form this trimer. Using a short probe where the E1 BS had been mutated, the WT E1 (Figure 2B, lanes 1–3) and two of the mutants, H507F (lanes 10–12) and H507Y (lanes 19–21), formed the trimer while the other substitutions failed to do so. Clearly, the failure to form the trimer did not result from a general DNA binding defect, since all of the substitutions formed other complexes such as dimers on this probe. H507V (lanes 25–27) was the only mutant with a general DNA binding defect, likely due to the aggregation problem mentioned above.

We next determined whether the H507 substitutions could form the functional DT that melts the ori and the functional DH that unwinds the ori. Formation of these complexes requires an 84 bp ori probe with four E1 BS (Figure 2C). In the presence of ADP, the progression from DT to DH is arrested at the DT stage because DH formation requires ATP hydrolysis. We can therefore analyze DT formation in the presence of ADP and analyze DH formation in the presence of ATP for each mutant protein. Our expectation was that the same mutants that were unable to form the trimer would also be unable to form the DT. Furthermore, since the DT is a precursor for the DH, the mutants defective for trimer and DT formation are also expected to be defective for DH formation on the 84 bp probe. Only the two mutants H507Y (lanes 8–10) and H507F (lanes 14–16), which could form the trimer, were also capable of forming the DT, while H507L (lanes 20–22) as well as the rest of the H507 substitutions failed to do so (see Figure S2). H507F also formed a DH (lanes 17–19), while H507Y and H507L (lanes 11–13 and 23–25, respectively) as well as the remaining mutants (see Figure S2), failed to do so. We can therefore distinguish three types of mutants. The majority of the substitutions are defective for DNA replication as well as for trimer, DT, and DH formation. The two substitutions H507F and Y are exceptions in that they have replication activity

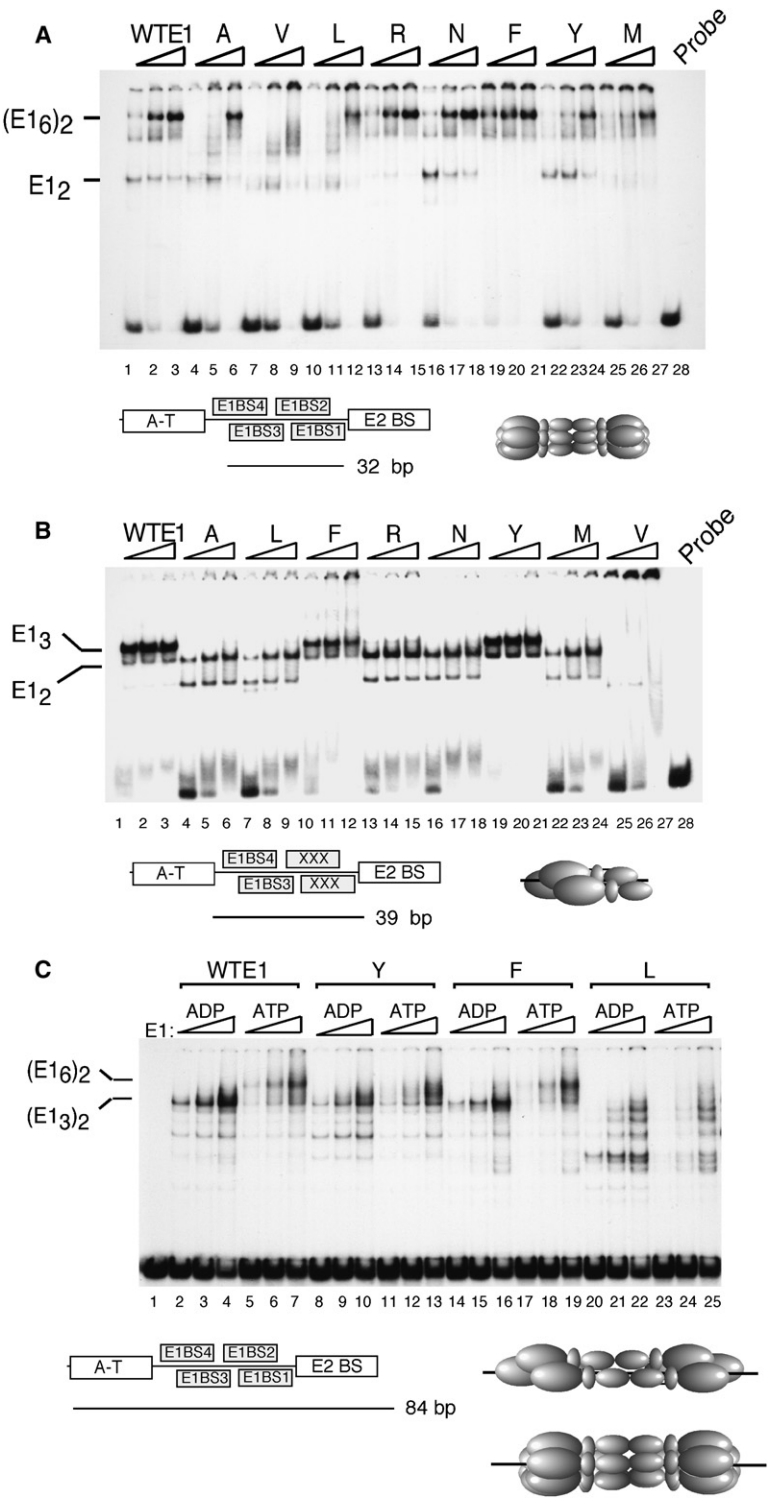

Figure 2. Complex Formation by Substitution Mutants at H507

(A) Formation of the nonproductive DH by H507 substitutions. EMSA was performed using the 32 bp ori probe shown below the panel. Three quantities of the WT and mutant E1 (60, 120, and 240 fmol) were used in the presence of ATP as indicated in the figure. Lane 28 contained probe alone.

(B) Trimer formation by H507 substitutions. EMSA was performed using a 39 bp probe with a mutated E1 BS as shown below. Three quantities (15, 30, and 60 fmol) of the WT E1 (lanes 1–3) and the respective mutant proteins were used in the presence of ADP. Lane 28 contained probe alone.

(C) DT and DH formation by H507 substitutions. EMSA was performed using the 84 bp ori probe shown below. Three quantities (30, 60, and 120 fmol) of the WT E1 (lanes 2–7), H507Y (lanes 8–13), H507F (lanes 14–19), and H507L (lanes 20–25) were used in the presence of ADP or ATP as indicated in the figure. Lane 1 contained probe alone.

and also are able to form a trimer and DT; however, only H507F forms the DH on the ori.

Template Melting by H507 Substitutions

The results presented above demonstrated that the majority of the H507 substitutions (H507A, V, L, R, N, and M) are defective for DT formation, which may account for their replication defect. Since the DT melts the ori, we would expect that the mutants defective for DT formation would also have melting defects. We therefore performed permanganate reactivity assays, which detect melted DNA (Figure 3). We incubated an ori probe labeled on

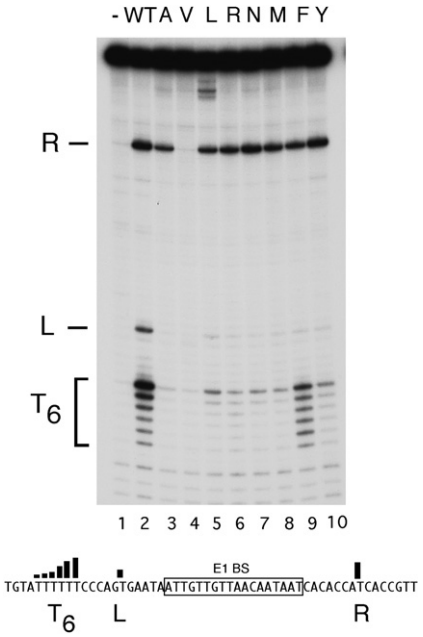

Figure 3. Permangante Reactivity of Substitutions at Residue 507

Permanganate reactivity assays were performed by incubating the 84 bp ori probe with 1 pmol of WT E1 (lane 2) or the respective H507 substitutions (lanes 3–10) in the presence of ADP. Following treatment with $KMnO_4$, modified DNA was cleaved with piperidine and analyzed by denaturing PAGE. Lane 1 shows the permanganate reactivity of the probe in the absence of E1. Positions of prominent permanganate reactivity (R, L, and T6) are indicated. Below is a schematic for the positions of permanganate reactivity for WT E1 relative to the ori sequence.

the top strand with the WT or mutant E1 proteins in the presence of ADP and treated with $KMnO_4$ for 2 min at room temperature. WT E1 generates a characteristic melting pattern consisting of melting at three positions, one T residue to the left of the E1 BS (L), one T residue to the right of the E1 BS (R), and the T6 stretch to the left of the E1 BS (Figure 3, lane 2). The right-hand reactivity was observed with all the mutants with the exception of H507V. The left-hand reactivity and the reactivity in the T6 stretch were only observed with the mutant H507F, albeit at lower levels than for WT E1 (lane 9). These results demonstrate that all substitutions at H507 with the exception of H507F show melting defects and that although H507Y can form a DT, this DT does not melt the DNA correctly. This was a surprising result in light of the activity of H507Y for DNA replication in vitro (Figure 1D). We have provided an explanation for the activity of H507Y in in vitro replication later in the paper.

H507 Is Not Required for DNA Helicase Activity

An ori fragment unwinding assay is a comprehensive assay that measures the ability of E1 to bind DNA correctly, to locally melt the ori, to provide DNA helicase activity, and, most importantly, to couple these activities to each other. In such an assay, E1 is incubated with an ori fragment in the presence of *E. coli* SSB, and generation of ssDNA can be detected by the appearance of ssDNA as an ssDNA/SSB complex by EMSA (Figure 4A). The H507F substitution, which had near-WT activity for DNA replication in vivo, showed significant ori fragment unwinding activity, indicating that it can bind, melt, and unwind the ori fragment nearly as well as the WT protein (compare lanes 3 and 4 to lanes 17 and 18), consistent with the in vivo and in vitro replication results. In contrast, H507Y (lanes 19 and 20) showed only trace amounts of activity in this assay (~10% of WT), similar to H507L and H507N (lanes 9 and 10 and 13 and 14), while the rest of the substitutions lacked detectable activity. These results are completely consistent with the melting results above and indicate that the lack of melting observed for all mutants except H507F in the permanganate reactivity assays also results in a defect for ori fragment unwinding.

To ascertain that defects in helicase activity were not the cause of the replication defect, we measured DNA helicase activity of the H507 substitutions using several different helicase assays, one of which is shown in Figures 4B and 4C, where we used a time-resolved fluorescence helicase assay. In this assay, E1 is incubated with a fluorescently labeled oligonucleotide substrate in which the fluorescence is quenched by the presence of the complementary strand. As the substrate is unwound, the quencher is removed, resulting in a dramatic increase in fluorescence. Thus, the fluorescence and hence the unwinding in a single sample can be monitored in real time. We chose a very low ratio (8:1) of E1 to substrate, approximately one 1 E1 hexamer per helicase substrate, to reveal differences in all steps of the unwinding process, including the binding of E1 to the substrate and formation of the hexameric helicase on ssDNA (Sedman and Stenlund, 1998). We then measured unwinding every 2 min for 32 min (Figure 4B). Interestingly, the five substitutions (A, M, F, Y, and R) that showed the lowest levels of unwinding compared to WT E1 had only a 2-fold reduction in the level of unwound substrate after 30 min. H507N and -L showed levels of unwinding similar to that of WT E1 after 30 min. We also in a separate experiment tested WT E1, H507N, and H507F in triplicate and obtained virtually identical results (Figure 4C). Clearly, these modest helicase defects are not responsible for the replication defects of the H507 substitutions. H507F, which has among the lowest activities in the helicase assay, is the only substitution that has close to WT activity for DNA replication in vivo and in vitro. Similarly, H507N, which shows no defect for helicase activity, has no activity for DNA replication. A position in which such a wide range of substitutions only has modest effects on the helicase activity is clearly not a critical residue for the helicase activity of the E1 protein.

An interesting aspect of these helicase assays is the slow rate of unwinding that is observed in all cases. This effect is observed in all of our helicase assays and is not related to the fluorescent substrate (data not shown). Since the substrate is very short, it is unlikely that this

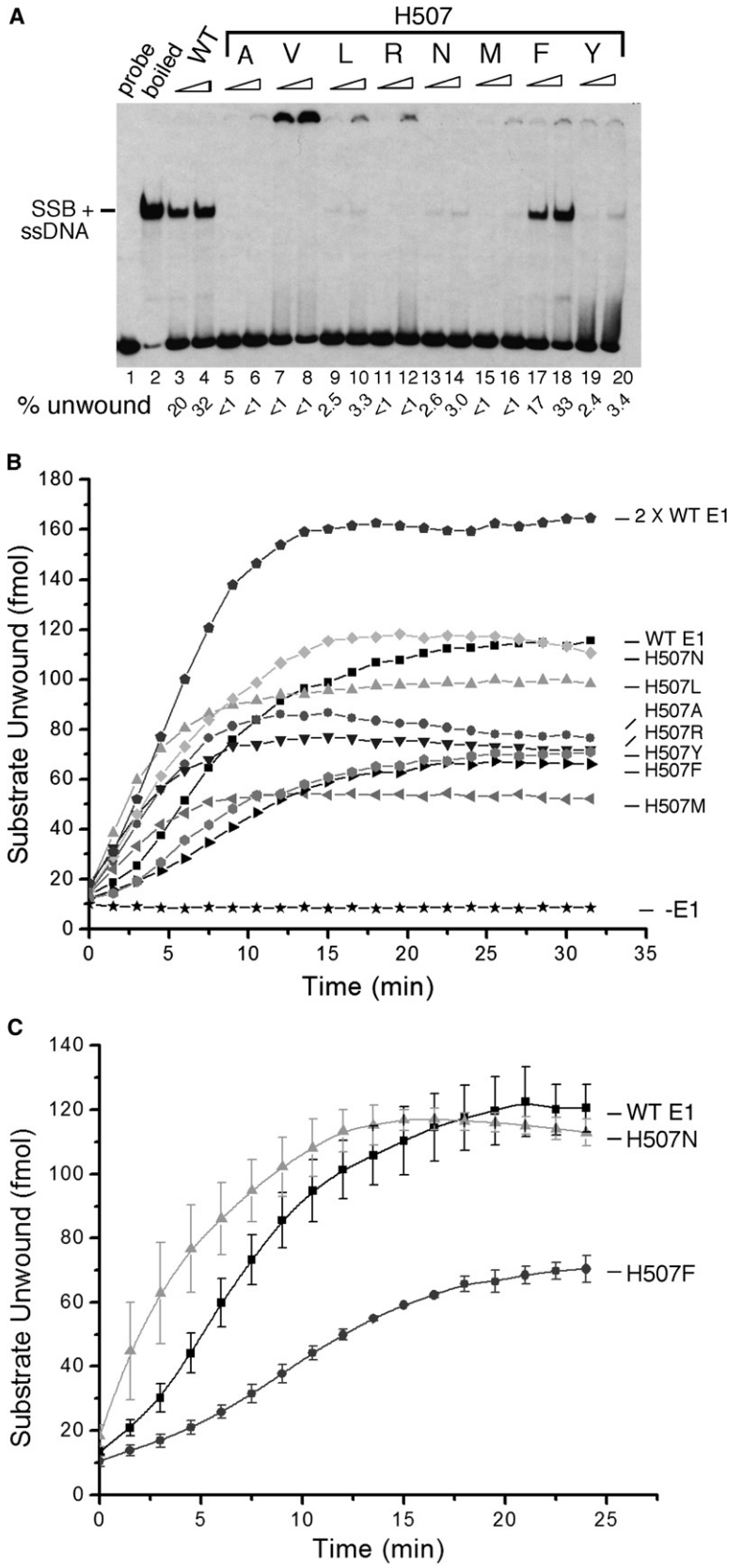

Figure 4. Ori Fragment Unwinding and Helicase Activity of Substitution Mutants at H507

(A) Ori fragment unwinding assays were performed by incubating an ori probe with 80 or 160 fmol of WT E1 (lanes 3 and 4), or of the respective H507 substitutions (lanes 5–20) in the presence of *E. coli* SSB followed by analysis by PAGE. Lane 1 contained probe alone; in lane 2, SSB was added to denatured probe, providing a marker for the ssDNA-SSB complex.

(B) DNA helicase activity of substitution mutants at H507. The substitutions at H507 were tested for DNA helicase activity using a time-resolved fluorescence based oligonucleotide displacement assay. WT E1 (1.5 pmol) or the respective E1 mutants were incubated with 200 fmol of fluorescent substrate at 37°C, and fluorescence was measured every 2 min for 32 min. In one sample (2 × WT E1) 3 pmol of WT E1 was used.

(C) WT E1, H507F, and H507N were tested for DNA helicase activity in triplicate using the same procedure as in (B). The error bars represent the standard deviation for each point.

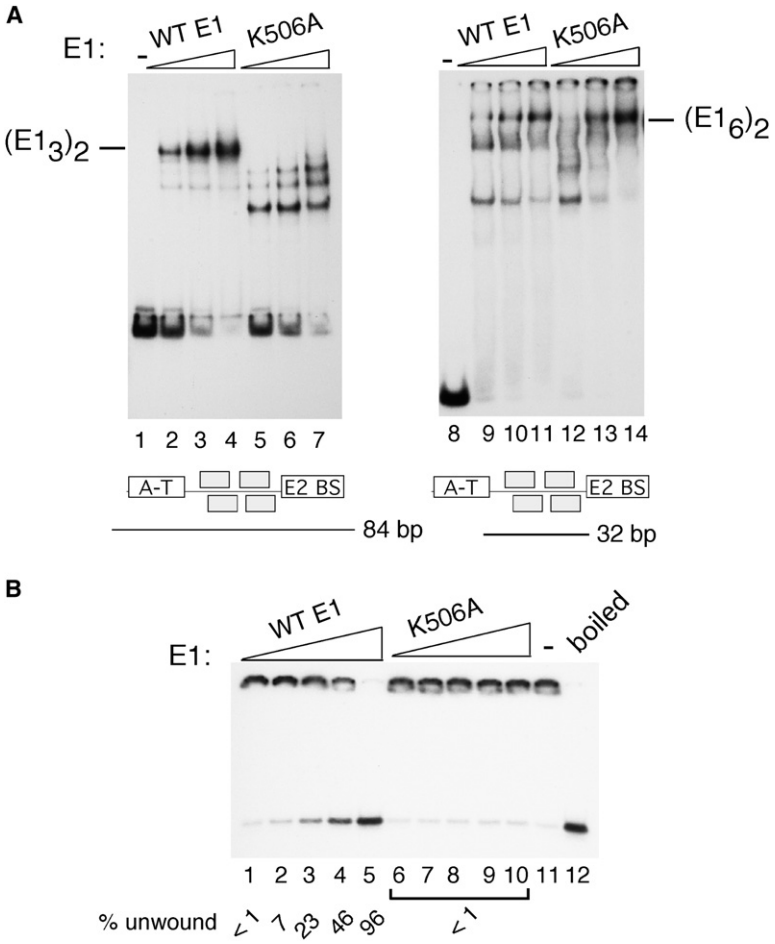

Figure 5. K506A Is Defective for Double Trimer Formation and for DNA Helicase Activity

(A) The β-hairpin substitution mutant K506A was tested by EMSA for the ability to form the DT (left panel) and the inactive DH (right panel) in parallel with WT E1. In the left panel, three quantities (30, 60, and 120 fmol) of WT E1 (lanes 1–3) and K506A (lanes 4–6) were used in the presence of ADP and the long 84 bp probe. In the right panel, 60, 120, and 240 fmol of WT E1 (lanes 1–3) or K506A (lanes 4–6) were used with the short 32 bp ori probe shown below.

(B) K506A was tested for DNA helicase activity in parallel with WT E1. Five quantities (15, 30, 60, 120, 240 fmol) of WT E1 (lanes 1–5) or K506A (lanes 6–10) were incubated with helicase substrate and analyzed by PAGE. In lane 11, no E1 was added, and lane 12 shows a boiled sample.

reflects the actual unwinding step. Instead, this slow step likely reflects the formation of the hexameric helicase on the substrate. It is interesting that some of the mutants, e.g., H507R, H507A, and H507M, plateau significantly earlier (7–10 min) than the rest of the mutants (~15 min), possibly reflecting a more rapid formation of the active helicase on the substrate. This may indicate that binding to these artificial helicase substrates is affected by the mutations at H507. Nevertheless, these results demonstrate that a defect for helicase activity is not likely to account for the DNA replication defect of substitutions at H507.

Another Residue in the β-Hairpin, K506, Is Required for DNA Helicase Activity

These results demonstrate that the function of the conserved histidine at the tip of the β-hairpin is to melt dsDNA and that clearly this residue is not specifically required for helicase activity of E1. We have previously generated a mutation in the residue adjacent to H507 (K506A) (Schuck and Stenlund, 2005). This mutant has similar defects as H507A and fails to form a trimer and a DT (Figure 5A, compare lanes 2–4 and 5–7) and also fails to melt the template (data not shown), although this mutant can form the nonfunctional DH on a short probe as well as WT E1 (Figure 5A, compare lanes 9–11 and 12–14) and therefore is capable of oligomerization and ATP hydrolysis. However, in contrast to the substitutions at position H507, which maintained significant helicase activity, K506A has no detectable helicase activity compared to WT E1 (Figure 5B, compare lanes 1–5 and 6–10). Thus, template melting is affected by substitution of either K506 or H507, but the helicase activity is significantly affected only by the K506A substitution. This result demonstrates that the β-hairpin is involved in at least two different biochemical activities, template melting and helicase activity, and that these two functions can be distinguished by substitutions at these two adjacent residues.

H507Y Shows Altered Specificity for Initiation of DNA Replication In Vitro

The lack of melting and unwinding activity of H507Y was curious, given the high levels of in vitro DNA replication for this mutant (Figure 1D). To understand this discrepancy, we performed a form-U assay, which detects unwinding as a fast-migrating underwound form of an input plasmid (Dean et al., 1987) (Figure 6A). The substrate for this assay is generated by incubating an ori plasmid with

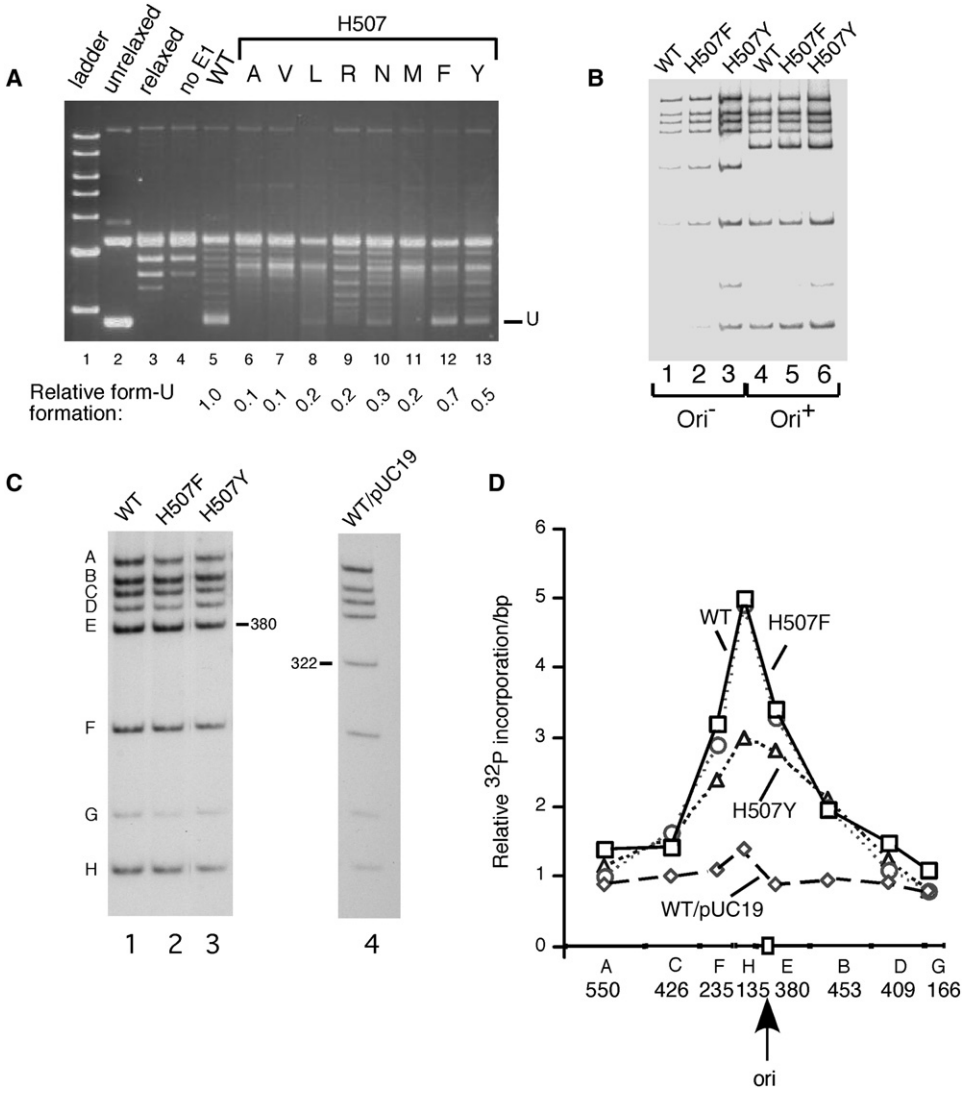

Figure 6. Specificity of Initiation of DNA Replication In Vitro

(A) Plasmid unwinding by substitution mutants at H507. WT E1 and H507 substitutions were tested for the ability to unwind an ori plasmid using a form-U assay. The ori plasmid DNA (lane 2) was relaxed by incubation with topoisomerase I (lane 3) and then incubated in the absence of E1 (lane 4) or in the presence of 6 pmol of WT E1 (lane 5) or of the indicated H507 substitutions, respectively (lanes 6–13). The samples were analyzed by agarose gel electrophoresis and stained with ethidium bromide, and the level of form-U DNA was quantitated. Lane 1 contains a DNA ladder.

(B) In vitro DNA replication assays were performed using either pUC 19 (lanes 1–3) or an ori plasmid containing a 60 bp ori fragment cloned into the polylinker of pUC 19 (lanes 4–6). The plasmids were incubated in the presence of 6 pmol of WT E1, (lanes 1 and 3), H507F (lanes 2 and 4), or H507Y (lanes 3 and 6). The replication products for each template were digested with PvuII and DdeI and analyzed by PAGE. The level of incorporation of ^{32}P dCTP was determined for each sample and compared.

(C) In vitro DNA replication assays were performed using the ori^{+} template and either WT E1, H507F, or H507Y (lanes 1–3), respectively, and terminated after 8 min. As a control, WT E1 was used to replicate the ori^{-} plasmid pUC 19 under the same conditions (lane 4). The replicated material was digested with PvuII and DdeI and analyzed by PAGE.

(D) Each band in (C) was quantitated, and the incorporation was divided by fragment size to derive the relative labeling/nucleotide. The relative labeling efficiency for each restriction fragment from the plasmid is plotted in the graph.

topoisomerase to generate a relaxed form of the plasmid (compare lanes 2 and 3). In the absence of added E1, incubation of this substrate in the presence of SSB, ATP, and topoisomerase I results in loss of some topoisomers due to further relaxation (compare lanes 3 and 4). In the presence of E1, SSB, ATP, and topoisomerase I, a new, faster-migrating form is generated (form-U) which migrates at a similar position as form I DNA (lane 5). This represents an underwound form of the plasmid due to the action of the E1 helicase (Dean et al., 1987). In this assay, we observed significant unwinding with H507F (lane 12) and importantly now also with H507Y (lane 13), which

has ~2-fold reduced activity compared to WT, consistent with the in vitro DNA replication result. The remaining mutants showed unwinding levels 3- to 10-fold lower than those of WT E1 (lanes 6–11).

These data demonstrate that H507Y is unable to unwind the ori fragment but is active for DNA synthesis in vitro and for unwinding in the form-U assay. A distinction between these assays is that the fragment unwinding assay uses a small (<100 bp) ori fragment, while both the in vitro DNA replication assay and the form-U assay use the whole ori plasmid. An interesting possibility is that H507Y can use other sequences present in the plasmid backbone for unwinding in the form-U assay and for replication in vitro.

To determine whether the H507Y mutant showed an "altered specificity" phenotype, we compared WT E1, H507F, and H507Y for the ability to initiate replication on plasmids with and without an ori (Figure 6B). Significant levels of replication can be observed on plasmids lacking an ori due to the low ori specificity of the E1 protein in the absence of E2 (Sedman and Stenlund, 1995; Yang et al., 1993). We compared the ability of WT E1, H507F, and H507Y to replicate pUC 19 and the ori plasmid, which contains a 60 bp ori fragments inserted into the polylinker of pUC 19. The replication reactions were digested with PvuII and DdeI and analyzed by PAGE. WT E1 showed ~3-fold reduced levels of replication with the pUC 19 template compared to the ori template, as expected (Figure 6B, compare lanes 1 and 4). H507F showed ~2-fold reduction with the pUC 19 template compared to the ori template (compare lanes 2 and 5). Strikingly, H507Y showed identical levels of replication in reactions containing pUC 19 or the ori plasmid (compare lanes 3 and 6), demonstrating that H507Y has no preference for the ori sequence and uses other sequences in the plasmid backbone. This result is consistent with both the lack of activity in the fragment unwinding experiment (Figure 4A) and the substantial activity in the form-U assay (Figure 6A).

Importantly, although this replication result would be consistent with a reduction in ori specificity of H507Y, the failure to unwind the ori in the fragment unwinding assay (Figure 4A) demonstrates that the effect is more likely to result from an altered specificity. An altered specificity of H507Y also provides an explanation for the in vivo DNA replication results. Initiation of DNA replication in vivo requires cooperative binding of E1 and E2, which relies on binding sites for E2 (Stenlund, 2003). Since E2 binding sites are present only at the ori, H507Y cannot initiate at other sites in the plasmid and is confined to the suboptimal ori sequence for initiation, resulting in a severely reduced level of DNA replication in vivo compared to WT E1 and H507F (Figure 1C).

To determine whether a particular sequence in the plasmid backbone was required for initiation of DNA replication by H507Y, we performed in vitro replication assays in which we terminated the replication reaction early (at 8 min) to trap intermediates. After termination of the replication reactions, we digested the DNA with the restriction enzymes PvuII and DdeI and analyzed the samples by PAGE (Figure 6C). Incorporation into each DNA fragment was quantitated and total incorporation was divided by fragment size to yield the relative incorporation/bp, which was plotted as a function of the position in the ori plasmid (Figure 6D). WT E1 and the H507F mutant gave rise to identical patterns, with ~5-fold enrichment at the origin of DNA replication compared to sequences distal to the ori. In contrast, the level of replication at the ori was reduced significantly for H507Y, consistent with an altered sequence specificity of H507Y. The ability of H507Y to utilize a different target sequence is equivalent to restoration of the interaction by a compensatory mutation in the target. Such data are generally considered to be good evidence for a direct interaction. We have not been able to identify the sequence(s) that is used instead of ori, most likely because multiple such sequences are present.

DISCUSSION

H507 Is Required for Local Ori Melting, but Not for DNA Helicase Activity of E1

The majority (six out of eight) of the substitutions at H507 are severely defective for all the activities that we can measure, with the exception of DNA helicase activity. These defects include trimer and DT formation, melting, unwinding, and DNA replication in vivo and in vitro. None of the substitutions appear to have general defects such as defects for DNA binding, oligomerization, or ATP binding/hydrolysis, since they all can form the nonproductive DH on the short (32 bp) probe. The underlying cause for the defect for unwinding and DNA replication for these substitutions appears to be the failure to form the correct protein-DNA complex, i.e., the DT. This in turn is likely caused by the failure of the substituted side chains to interact correctly with DNA, as will be discussed below. The excellent correlation between the H507 β-hairpin substitutions that support DNA replication and also support trimer and DT formation provides good evidence that the recently characterized DT is an essential functional complex that melts the ori DNA, as has been proposed (Schuck and Stenlund, 2005). The fact that H507 and K506 play different roles in the helicase activity of E1 is interesting in light of the recent cocrystal of a hexamer of the E1 oligomerization and helicase domains bound to ssDNA (Enemark and Joshua-Tor, 2006). In this structure, K506 interacts through its side chain with ssDNA, providing an explanation for the importance of this residue for the helicase activity of E1. H507 in this structure interacts both through the main chain and through its side chain with the ssDNA. The side-chain interaction is clearly of limited importance for the helicase activity of E1 based on the mutational results presented here.

The Conservation of the β-Hairpin

The E1 proteins belong to the SF3 family of helicases, which also includes the initiator proteins from polyoma viruses (T-ag) and parvoviruses (Rep). Interestingly, when these three groups of helicases are aligned (Figure 7A),

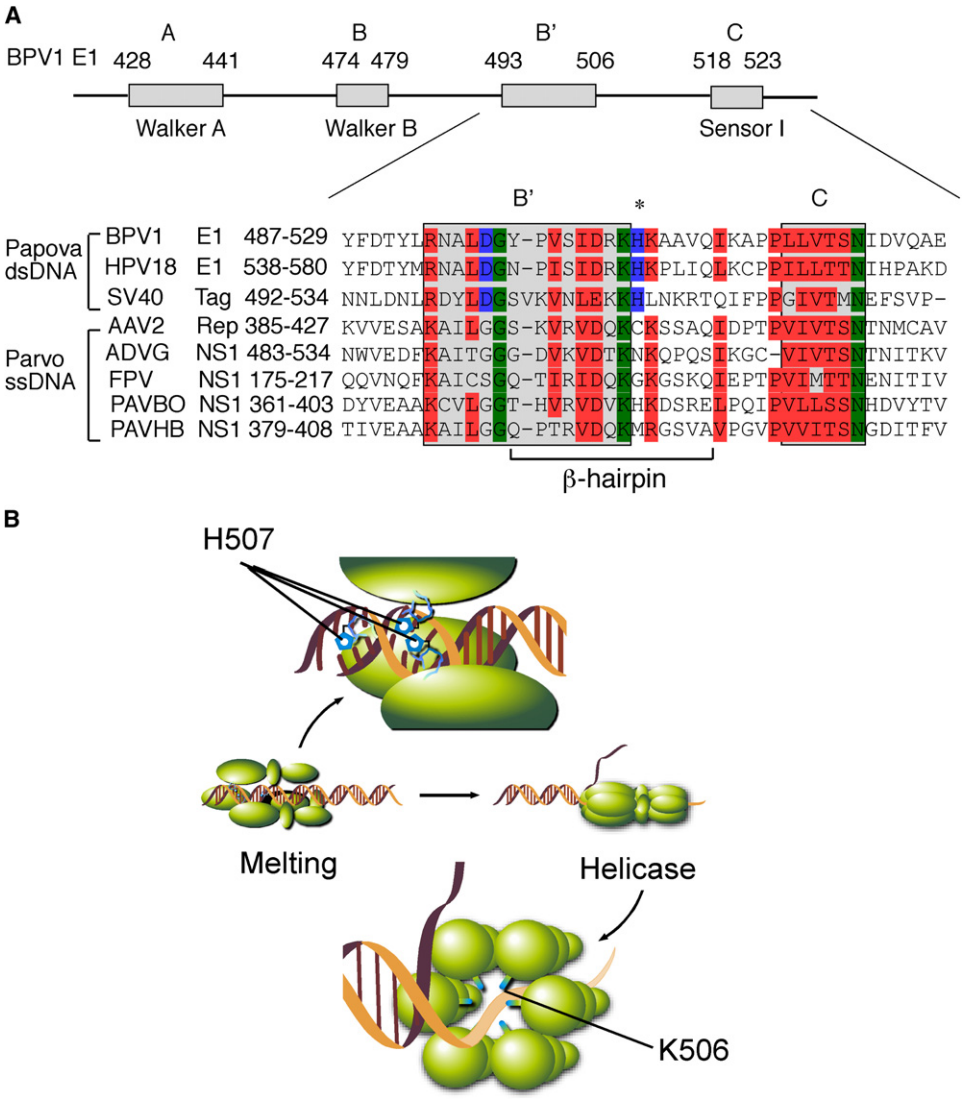

Figure 7. The β-Hairpin Histidine Melts dsDNA and Generates the Substrate for Helicase Loading

(A) The β-hairpin generates the substrate for helicase loading. Sequence alignment of the B′ and C motifs of representative members of the SF3 helicase family. Shown above the alignment is the relative location of SF3 signature motifs in BPV E1. The number above each motif corresponds to the starting and end residue number of each motif in BPV E1. The residue at the tip of the β-hairpin (H507) is marked with an asterisk. Green, invariant residues in papova and parvoviruses. Red, conserved residues in papovaviruses and parvoviruses. Blue, highly conserved residues in papova viruses only. Included in the comparison are the following: BPV1 E1, bovine papillomavirus type 1 E1 protein; HPV18 E1, human papillomavirus type 18 E1 protein; SV40 TAg, simian virus 40 large T antigen; AAV2 Rep, adeno-associated virus 2 Rep 40 protein; ADVG NS1, Aleutian mink disease parvovirus (strain G) NS 1 protein; FPV NS1, feline panleukopenia virus NS1 protein; PAVBO NS1, bovine parvovirus NS 1 protein; and PAHVB NS1, human parvovirus B19 NS1 protein.

(B) Model for the transition between the E1 DT complex, which melts DNA, and the E1 DH, which unwinds DNA. E1 forms a DT, and H507 at the tip of the β-hairpin intercalates into the DNA in the minor groove. The DT-DNA complex is the substrate for formation of the DH, which forms on one of the melted strands, and K506 at the tip of the hairpin interacts with ssDNA. For simplicity, only one trimer and one hexamer are shown.

the lysine at the position corresponding to K506 in E1 is completely conserved between these three virus groups, consistent with the importance of this residue for the helicase activity of the E1 protein. In contrast, although the papilloma and polyomaviruses have a virtually completely conserved histidine at position 507 (all of the 214 T-ag sequences have a histidine at this position, and out of 168 E1 proteins only 13 [12 tyrosine, 1 phenylalanine] do not have the conserved H507), the parvovirus group contains a variety of residues (C, N, G, H, M) at this particular position. Since it is well established that the parvovirus Rep proteins are functional helicases, this clearly demonstrates that a histidine at this particular position is not an essential feature of SF3 helicases. We believe that the reason that

the histidine is not conserved in the parvovirus group is that the parvoviruses have ssDNA genomes and therefore do not require an activity that locally melts the ori.

H507Y Shows Altered Specificity

H507Y is capable of forming the trimer and DT and is fully functional for in vitro DNA replication. However, it fails to melt and unwind the ori (Figure 3 and 4). This indicates that, while the tyrosine side chain interacts with DNA equally well as the histidine or phenylalanine to form the DT, there is a structural aspect of the tyrosine side chain that is incompatible with local ori melting and DH formation. H507Y apparently is capable of using other DNA sequences for unwinding in the form-U assay and in the in vitro DNA replication assays, indicating that the consequence of the H507Y substitution is a change in the sequence that can be melted.

The altered specificity of H507Y indicates that the histidine side chain may contact DNA directly. F and Y both have similar aromatic six-membered ring structures, and the flat ring structure is shared with the histidine (an aromatic five-membered ring). A possible mode of interaction with DNA based on these side chains is intercalation of the planar rings into DNA, as has been observed for binding of TBP, in which a phenylalanine in each stirrup intercalates into the minor groove (Kim et al., 1993a, 1993b). This intercalation results in major distortion of the DNA, including unstacking of neighboring base pairs.

The likely target for the β-hairpin is the stretch of 6 T-A bp, which is the only sequence flanking the E1 BS that is essential for DNA replication. These are the same six T residues that are melted by the E1 DT (Figure 3). Mutants affecting this T-A stretch in the template have a melting defect, but such templates can still support DT formation (Schuck and Stenlund, 2007). The dependence on the T-A stretch for melting, but not for DT formation, mirrors the distinction that we observe between H507F and H507Y. While the phenylalanine and tyrosine substitutions both can generate the DT on the ori fragment, the tyrosine substitution cannot melt this template. An interesting possibility, therefore, is that the histidine and phenylalanine at the tip of the β-hairpin both are capable of engaging the 6 T-A bp flanking the E1 BS, while the extra hydroxyl of tyrosine, although it allows DT formation, precludes the β-hairpin from interacting with DNA in a manner that is required for melting.

Implications for Helicase Loading

An activity that can melt DNA in preparation for DNA replication has not been identified in eukaryotes. Melting activity could reside in any of the proteins or protein complexes that are known to take part in initiation of DNA replication (e.g., ORC, MCM 2–7, etc.) or in some unknown complex. The current view of helicase loading, which is derived from what is believed to happen in *E. coli*, is that the replicative DNA helicase (DnaB) is loaded as a preexisting ring structure onto pre-existing ssDNA generated by DnaA (Carr and Kaguni, 2001; Fang et al., 1999; Marszalek and Kaguni, 1994). The viral initiator proteins such as E1 provide an alternative mechanism for helicase loading. Since the DT is a precursor for the formation of the DH, the DT is incorporated into the helicase in the assembly process. Because of the obvious facility of an arrangement in which local melting is an integral part of helicase assembly, it is conceivable that a similar strategy might be used also in eukaryotes in which a melting activity has not been identified. For example, a subassembly of a multimeric helicase could contain the melting activity. Archaeal MCM proteins contain a β-hairpin structure, but it is unknown whether this β-hairpin functions in the helicase, in melting, or in both processes (Fletcher et al., 2003). The precise feature that makes the E1 β-hairpin capable of melting DNA (the aromatic side chain at the tip) is not present in the β-hairpin in the MCM protein (Fletcher et al., 2003). However, the β-hairpin that is thought to intercalate into DNA and effect melting in the phage T7 RNA polymerase also lacks this particular feature.

Well-studied hexameric DNA helicases such as DnaB and T7 helicase are believed to function by binding as hexamers to ssDNA, followed by translocation on ssDNA and displacement of the complementary strand (Egelman et al., 1995; Jezewska et al., 1998; Kaplan and O'Donnell, 2002; Yu et al., 1996). A recent X-ray crystal structure of the helicase and oligomerization domains of E1 in the presence of ssDNA indicates that this is the case also for the E1 protein (Enemark and Joshua-Tor, 2006). A remaining question is how one of the DNA strands is displaced to the outside of the hexameric ring while one strand remains inside the ring. The involvement of the β-hairpin in both melting and helicase activity provides some hints (Figure 7B). The E1 DT, which forms on dsDNA, melts the dsDNA by interaction of the β-hairpin histidine with DNA, and this complex now becomes the substrate for DH formation. If, after melting, further addition of E1 molecules to the DT occurs specifically on one of the melted strands, the result would be exclusion of one strand and encirclement of the other strand by the hexamer, i.e., helicase loading. In such a model, the β-hairpin can remain in contact with the DNA throughout the melting process and the transition to the DNA helicase, consistent with the dual function of the β-hairpin.

EXPERIMENTAL PROCEDURES

E1 Protein: Expression and Purification

WT E1 and E1 mutants were expressed in *E. coli* as N-terminal GST fusions, purified by affinity chromatography, and cleaved and isolated by ion exchange chromatography as described (Sedman et al., 1997). E1 purified in this manner is monomeric, as determined by glycerol gradient sedimentation and gel filtration (Sedman and Stenlund, 1998). The concentrations of the WT and mutant E1 proteins were determined using the fluorescent dye SYPRO Red using 532 nm as the excitation wave-length in a FujiImager FLA 5000.

Plasmid Constructs

The template used for in vivo and in vitro DNA replication assays was a 60 bp minimal ori fragment (7914–7927) cloned between the XbaI and HindIII sites in pUC 19 (Ustav et al., 1991). The template for generation

of ori probes is a 110 base pair BPV-1 sequence (nucleotides 7894–7857, centered on the E1 binding site) cloned between the XbaI and HindIII sites in pUC19 generating the plasmid 11/12/X (Sanders and Stenlund, 2000).

In Vivo DNA Replication Assay

In vivo DNA replication assays were performed as described (Ustav et al., 1991). Briefly, CHO cells were transfected by electroporation with an expression vector for the E2 protein (pCGE2), an ori plasmid (7914–7927), and an expression vector for either WT E1 (pCGE1) or the respective H507 substitutions. Two, three, and four days after transfection, low molecular weight DNA was prepared using alkaline lysis, digested with DpnI and HindIII, and analyzed by Southern blotting.

In Vitro DNA Replication

In vitro DNA replication assays were performed essentially as described (Sedman and Stenlund, 1995; Yang et al., 1991). In vitro replication was performed in 25 μl reaction mixtures containing the following: 40 mM HEPES-KOH (pH 7.5); 8 mM $MgCl_2$; 0.5 mM DTT; 3 mM ATP; 0.2 mM each of GTP, UTP, and CTP; 0.1 mM each of dATP, dGTP, and dTTP; 10 μM of [^{32}P]dCTP (2 μCi; 3000 Ci/mmol); 40 mM creatine phosphate; 400 ng creatine kinase; 10 μl S100 extract; and 0.5 μl high-salt nuclear extract from H293 cells. The concentration of template in the in vitro reactions was 2 ng/μl. Reactions were incubated for 60 min at 37°C unless stated otherwise. The reactions were stopped by addition of SDS to 1% and EDTA to 10 mM and treated with proteinase K followed by phenol/chloroform extraction and precipitation with ethanol and ammonium acetate. The products were analyzed by electrophoresis on 1% agarose gels in TAE buffer.

For the in vitro replication assays shown in Figure 6, a slightly different protocol was used. To alleviate the long lag preceding initiation that is observed in these assays, the replication mix was preincubated for 20 min at 32°C in the presence of ATP but in the absence of rNTPs and dNTPs. Upon addition of rNTPs and dNTPs, initiation of DNA replication is instantaneous.

DNA Helicase Assays

Oligonucleotide displacement assays were performed using two methods. For the time course experiments in Figure 4, a helicase kit from PerkinElmer (TruPoint) was used. Briefly, an europium-labeled 44-mer annealed to a 26-mer containing a fluorescence quencher was incubated with E1 in a buffer containing 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, and 0.2 mg of BSA/ml and incubated at 37°C. Time-resolved fluorescence was measured every 2 min using 1420 Victor software in a PerkinElmer fluorometer. The substrate concentration was 4 nM and the capture strand concentration was 15 nM, and the reactions were carried out in a volume of 50 μl. WT E1was used at two concentrations, 30 nM and 60 nM (2 × E1), while the H507 substitutions were tested at 30 nM. At 30 nM of WT E1, maximally 50% of the substrate is unwound.

Oligonucleotide displacement assays in Figure 5 were performed essentially as described (Sedman and Stenlund, 1998; Seo and Hurwitz, 1993). A 50-mer oligonucleotide with partial complementarity to M13mp18 was synthesized, generating a substrate with a 28 nucleotide long double-stranded region and a 22 nucleotide long single-stranded 3′ tail. E1 was incubated with substrate in a buffer containing 50 mM Tris-HCl (pH 7.9), 3 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, and 0.2 mg of BSA/ml at 37°C for 15 min. After incubation, SDS was added to 0.1%, and the samples were analyzed by PAGE.

EMSA

Four percent acrylamide gels (39:1 acrylamide:bis) containing 0.5 × TBE, lacking EDTA, were used for all EMSA experiments. E1 was added to the probe (~2 fmol) in 10 μl binding buffer, BB (20 mM HEPES (pH 7.5), 100 mM NaCl, 0.7 mg/ml BSA, 0.1% NP40, 5% glycerol, 5 mM DTT, 5 mM $MgCl_2$, and 2 mM ATP or ADP). After incubation at room temperature for 1 hr, the samples were loaded and run for 2 hr at 9V/cm. The ability to generate discrete complexes, especially the DT and DH, was critically dependent on high-purity acrylamide, freshly prepared APS solution, overnight polymerization of the gels, and precise prerunning time (9V/cm for 4 hr).

Combined EMSA and Unwinding Assays

Unwinding assays were performed by incubating 2 fmol of probe with E1 at 32°C for 30 min under EMSA conditions (see above) but in the presence of 10 μg/ml *E. coli* SSB. Before loading samples on the EMSA gel, the concentration of NaCl was increased to 500 mM to disrupt E1 DNA complexes. The ssDNA was detected as an SSB/ssDNA complex.

Form-U Assays

Relaxed DNA substrate was prepared by incubation of plasmid DNA with human topoisomerase I (Sigma) in 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM PMSF, and 1 mM 2-mercaptoethanol. After incubation overnight at 37°C, the relaxed products were deproteinized, ethanol precipitated, and resuspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The conditions for unwinding were modified from those described previously (Dean et al., 1987). Reaction mixtures (30 μl) containing 20 mM HEPES (pH 7.5), 5% glycerol, 40 mM creatine phosphate, 7 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM ATP, 33 μg of creatine kinase/ml, 125 ng of relaxed ori plasmid DNA, 2 units of human topoisomerase I, 450 ng of *E. coli* SSB, and 400 ng of E1 were incubated for 3 hr at 37°C, and the reaction was terminated by the addition of 15 mM EDTA, 0.3% SDS, 1 μg of tRNA, and 0.3 μg of proteinase K and further incubated for 15 min at 37°C. Reactions were extracted with phenol/chloroform and ethanol precipitated. The DNA was dissolved in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and electrophoresed in 1% agarose gels at 9V per cm. Gels were stained with ethidium bromide, photographed, and quantitated under UV irradiation.

Permanganate Reactivity Assays

Permanganate reactivity assays were performed as described (Sanders and Stenlund, 1998). Briefly, binding reactions (20 mM HEPES [pH 7.9], 100 mM NaCl, 0.1% NP40, 5% glycerol, 1 mM DTT, 5 mM $MgCl_2$, and 5 mM ATP) containing ~10 fmol of end-labeled probe were assembled and incubated with E1 at room temperature. After 30 min, KMnO4 was added to a final concentration of 6 mM and reactions incubated for a further 2 min. Modification was terminated by adding β-mercaptoethanol to 80 mM, SDS to 0.3%, and EDTA to 10 mM. Reactions were then digested with proteinase K (20 μg/ml) for 60 min at 37°C, and the DNA was recovered by phenol-chloroform extraction and ethanol precipitation. Cleavage at modified bases was achieved with piperidine (30 min at 90°C).

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.molecule.org/cgi/content/full/25/6/825/DC1/.

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