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PriA-directed Assembly of a Primosome on D Loop DNA*

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Escherichia coli **strains carrying null mutations in *priA* are chronically induced for the SOS response and are defective in homologous recombination, repair of UV damaged DNA, double-strand break repair, and both induced and constitutive stable DNA replication. This led to the proposal that PriA directed replication fork assembly at D loops formed by the homologous recombination machinery. The demonstration that PriA specifically recognized and bound D loop DNA supported this hypothesis. Using DNA footprinting as an assay, we show here that PriA also directs the assembly of a φX174-type primosome on D loop DNA. The ability to load a complete primosome on D loop DNA is a step necessary for replication fork assembly.**

Escherichia coli strains carrying null mutations in *priA* exhibit a complex phenotype that includes constitutive induction of the SOS response (1) and defective homologous recombination (2, 3), repair of UV-damaged DNA (2, 3), double-strand break repair (3), and both induced and constitutive stable DNA replication (4). The dependence of stable DNA replication on recombination proteins and the likelihood that replication initiation during stable DNA replication occurred at either D loops or R loops (5) led to the proposal that all of the phenotypes elaborated in *priA* null mutant strains resulted from a failure of replication forks to assemble at these structures (2, 3).

This proposal was consistent with the ability of PriA to direct the assembly of a φX174-type primosome at specialized DNA sequences called primosome assembly sites (PAS)[1] (6). Primosomes are an essential part of the replication fork apparatus, providing both the DNA unwinding function and the Okazaki fragment-priming function (7). McGlynn *et al*. (8) demonstrated that PriA could indeed recognize and bind specifically to D loops. We showed in the accompanying report (9) that PriA exhibited two modes of specific DNA recognition. Binding to double-stranded (ds) DNAs carrying 3′-single-stranded (ss) extensions of at least 12 nucleotides (nt) presumably reflected the 3′ → 5′ DNA helicase activity of the protein (10, 11), whereas binding to D loops reflected the ability of PriA to bind to bent DNA at three strand junctions.

Replication fork assembly in simple terms requires that all the necessary catalytic activities, DNA helicase, primase, and DNA polymerase, have access to a specific point on the DNA. Generally, this occurs at an origin of replication, such as *oriC* in *E. coli* (7). A temporal imperative is imposed as well because a protein-protein interaction between DnaB, the replication fork helicase, and the τ subunit of the DNA polymerase III holoenzyme must be established for proper replication fork formation (12). This, in turn, requires that binding of the polymerase to the primer for the nascent leading strand occur at roughly the same time that the primosome assembles. If these events are not coordinated properly, the primosome may move away from the polymerase before the required protein-protein interaction is established. If replication forks do form at D loops, clearly primosome assembly must proceed efficiently and rapidly so that the DnaB-τ interaction is established with holoenzyme that binds to the 3′-end of the invading strand.

Primosome assembly occurs in discrete steps at a PAS sequence (13): (i) PriA recognizes and binds to the PAS, (ii) PriB joins PriA to form a PriA-PriB-PAS DNA complex, (iii) DnaT then joins this complex to form a triprotein complex on the PAS DNA, and (iv) DnaB is then transferred from a DnaB-DnaC complex in solution to the PriA-PriB-DnaT-PAS DNA complex to form a preprimosome consisting of PriA, PriB, DnaT, and DnaB on the DNA. Complete primosome assembly occurs when DnaG adds to this complex by virtue of a protein-protein interaction with DnaB (14). We know that PriC is also present in the preprimosome and primosome when they are formed on large DNAs, but we have not been able to define the step at which it is added to the complex (15).

We have investigated primosome formation on D loops composed of synthetic oligonucleotides. We were able to detect formation of a stable PriA-PriB-DnaT complex by gel mobility shift analysis. However, unlike the case where a 300-nt long ssDNA carrying the φX174 PAS was used as the substrate (13), we could not detect any higher order complexes when DnaB and DnaC were added to the reaction. We therefore used DNA footprinting with both deoxyribonuclease I (DNase I) and S1 nuclease as probes. A specific complex could be detected between PriA and the D loop DNA, but no evidence of higher order complexes was obtained. Unwinding of one end of the D loop, which required all the primosomal proteins, however, could be detected as a result of changes in the sensitivity of the DNA substrate to S1 nuclease in the presence of ATP. Thus, we conclude that a complete preprimosome could be assembled on D loop DNA. This is consistent with our demonstration that replication forks can assemble and extend the invading strand in a D loop in a primosomal protein-, single-stranded DNA-binding protein (SSB)-, and DNA polymerase III holoenzyme-dependent manner (16).

MATERIALS AND METHODS

Replication Proteins—PriA, PriB, DnaT, DnaB, and DnaC were prepared as described by Marians (17).

DNA Substrates—DNA substrates were prepared as described in the accompanying article (9).

Gel Mobility Shift Analysis—Gel mobility shift analysis was as described in Liu *et al.* (18).

DNA Footprinting—Reaction mixtures (15 μl) containing 50 mM Tris-HCl (pH 8.3 at 4 °C), 10 mM $MgCl_2$, 10 mM dithiothreitol, 500 μg/ml bovine serum albumin, 1 nM 5′-^{32}P-end-labeled DNA substrate, either 200 μM $CaCl_2$ (for DNase I cleavage) or 200 μM $ZnCl_2$ (for S1 nuclease

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[1] The abbreviations used are: PAS, primosome assembly site; ss, single-stranded; ds, double-stranded; SSB, single-stranded DNA-binding protein; nt, nucleotide; DNase I, deoxyribonuclease I.

cleavage), or no, 5 μM, or 1 mM ATP as indicated, and the primosomal proteins as indicated, were incubated at 30 °C for 10 min. Either DNase I (0.4 unit, Roche Molecular Biochemicals) or S1 nuclease (1 unit, Amersham Pharmacia Biotech) was then added and the incubation continued for an additional 1 min. Reactions were terminated by the addition of EDTA to 30 mM and 1 μg of salmon sperm DNA. DNA products were ethanol precipitated in the presence of 0.3 M NaOAc and resuspended in 98% formamide, 10 mM EDTA. The cleavage products were analyzed by electrophoresis at 20 watts for 2 h through 12% polyacrylamide gels (30 cm × 16 cm × 0.45 mm) (29:1, acrylamide: bisacrylamide) containing 50% (w/v) urea using 50 mM Tris, 40 mM boric acid, 1 mM EDTA as the electrophoresis buffer. The lower chamber was made 250 mM in NaOAc to compress the spacing between bands in the lower part of the gel. Gels were dried, exposed to PhosphorImager screens, and autoradiographed. Maxam-Gilbert sequence ladders were prepared as described (19).

RESULTS

Formation of Primosomal Protein Complexes on D Loops Detected by Gel Mobility Shift Analysis—We have been able to analyze each step of primosome assembly on the φX174 PAS sequence using gel mobility shift analysis (13). We therefore used the same approach initially in an attempt to detect a similar ordered assembly of the primosome on D loop DNA.

The bubble and D loop used here have been described in detail in the accompanying report (9). The top and bottom strands are composed of 82-nt long oligonucleotides (oligos) that have a central noncomplementary region of 42 nt. When annealed together, these two oligos form the bubble DNA. To form the D loop DNA, a 42-nt long invading strand is annealed to the top strand of the bubble. As elaborated in the accompanying report (9), the bubble is composed of oligonucleotides 1 and 2L, with the top strand being oligo 1. The invading strand in the D loop is oligo 5S.

As demonstrated previously (8) and in the accompanying report (9), PriA was capable of forming a stable complex with D loop, but not bubble, DNA (Fig. 1*A, lane 2*). No change was detected in the mobility of the protein-DNA complex formed when PriB was also included in the reaction mixture (Fig. 1*A, lane 3*). Similar results were observed in our analysis of primosome assembly on the φX174 PAS sequence (13). In that investigation, the presence of the PriA-PriB-PAS DNA complex was only revealed by Western analysis. Because PriB is a small protein (20), the mass added to the complex is insufficient to cause a detectable change in mobility. Although we have not performed a similar Western analysis here, the fact that we can detect a PriA-PriB-DnaT complex specific for D loop DNA (Fig. 1*A, lane 3*) argues that the PriA-PriB-D loop DNA complex does form. As in the case where PAS DNA was used as a substrate (13), formation of the PriA-PriB-DnaT-D loop DNA complex did not require ATP (data not shown).

With PAS DNA as a substrate, addition of DnaB and DnaC to the PriA-PriB-DnaT-PAS DNA complex in the presence of 10 μM ATP resulted in the appearance of a new complex that contained the later three proteins as well as DnaB (13). However, this was not observed with D loop DNA (Fig. 1*B, lane 8*). Whereas this could be taken as indicating that a preprimosome did not assemble on the D loop DNA, other interpretations were possible. The PAS and D loop DNA substrates are distinct in that the PAS DNA is nominally single-stranded, with no duplex region that can be unwound, whereas the D loop DNA has two duplex regions. Thus, even though the experiment shown in Fig. 1*B* was not performed in the presence of an ATP concentration high enough to effect primosome-catalyzed DNA unwinding (21), we considered that the structure itself might be destabilizing, preventing detection of primosome complex formation by an assay that has slow time resolution such as gel mobility shift analysis. We therefore turned to DNA footprinting techniques to assess the possibility of primosome assembly at D loops.

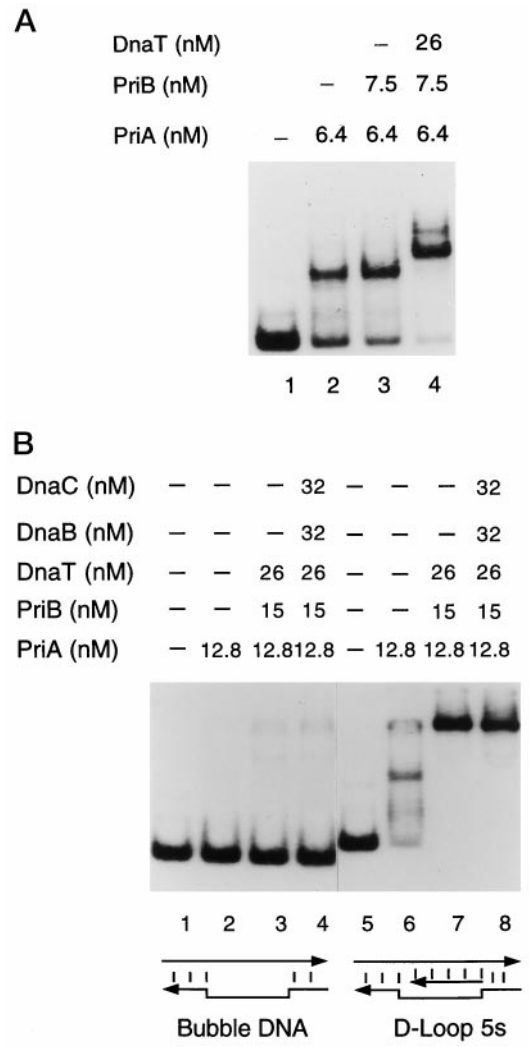

FIG. 1. **Formation of primosomal protein complexes on D loop DNA analyzed by gel mobility shift analysis.** Standard DNA binding reaction mixtures containing the indicated primosomal proteins were analyzed by gel mobility shift analysis as described under "Materials and Methods." *A*, formation of a PriA-PriB-DnaT complex on D loop DNA. *B*, the addition of DnaB and DnaC to the PriA-PriB-DnaT complex does not yield a stable preprimosomal complex.

Distinct Modes of Interaction of PriA with D Loop DNA and Duplex DNA with a 3′-Single-stranded Extension—Because the bubble and D loop DNA contained regions of both duplex and single-stranded DNA, we used two different probes in the footprinting reactions, DNase I, to detect interactions with dsDNA, and S1 nuclease, to detect interactions with ssDNA. As shown, for example, in Figs. 2 and 6, the expected specificity was observed: DNase I only digested regions of the D loop and bubble that, based on the nucleotide sequence (9), should have been duplex, whereas S1 nuclease only digested regions of these DNAs that were predicted to be single-stranded based on the nucleotide sequence (9).

DNA substrates were used that were 5′-end-labeled on either the top or bottom strand. As predicted from the gel mobility shift analysis, no interaction could be detected between PriA and the bubble DNA (Fig. 2, *A* and *B, lanes 5*, and Fig. 5). On the other hand, PriA gave a complicated footprint on the D loop DNA (Figs. 2, *A* and *B*, *lanes 8*, and 6). Both the top and bottom strand of the left-hand flanking duplex region were protected from DNase I digestion. Protection on the top strand started 14

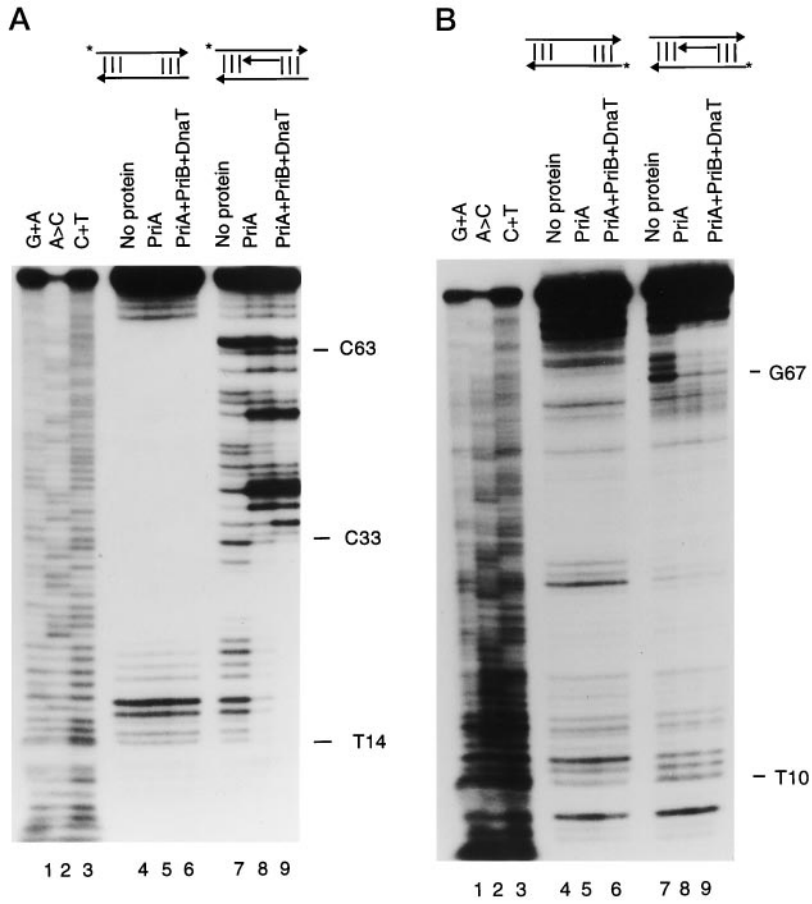

FIG. 2. **DNase I footprints of PriA and PriA-PriB-DnaT complexes on bubble and D loop DNA.** Standard DNA footprinting reaction mixtures containing either the bubble or D loop substrate labeled on either the top (*panel A*) or bottom (*panel B*) strand as indicated by an *asterisk*, and 26 nM PriA, 15 nM PriB, and 28 nM DnaT, as indicated, were analyzed as described under "Materials and Methods." Maxam-Gilbert sequence ladders in *lanes 1–3* are of the respective labeled oligonucleotide. Correspondence of the Maxam-Gilbert sequence ladder to the sequence of oligos dlp 1 and 2L are indicated on the *right-hand side* of *panels A* and *B*, respectively.

nt from the 5′-end and extended into the duplex region formed by the top strand and the invading strand. Protection on the bottom strand started 6 nt from the 3′-end and extended for about 10 nt, but did not enter the single-stranded region of the displaced strand. This positioned PriA at the left-hand three-strand junction of the D loop. This is consistent with the preference observed by gel mobility shift analysis for PriA binding to three-strand junctions with 5′-single-stranded tails (9). In the duplex DNA formed by the top and invading strands, alternating regions of protection and enhancement of cleavage could be observed. Although not perfectly so, these alternating regions showed a periodicity of about one turn of the helix (Fig. 2, *A, lane 5*, and Fig. 6).

The addition of PriB and DnaT improved the protection of the left-hand flanking region of the top strand and altered subtly the pattern observed in the duplex formed by the top strand and the invading strand. Several enhanced protections (nt T^{44}, G^{45}, and G^{54}) and a new enhanced cleavage site (nt G^{35}) could be observed (Fig. 2, *A* and *B, lanes 9*). This is consistent with the ability to observe a stable PriA-PriB-DnaT-D loop DNA complex by gel mobility shift analysis.

A similar pattern of alternating protection and enhanced cleavage was observed when DNase I was used to probe the interaction of PriA with the invading strand (Fig. 3, *lane 2*). Here the affected region was smaller (32 nt), but the regions of protection and enhancement were reflected directly across the helical axis from the top strand (Fig. 5). As was observed with labeled top strand, the addition of PriB and DnaT only altered the observed pattern slightly, resulting in increased protection at the 3′-end of the invading strand (Fig. 3, *lane 3*).

In the accompanying report (9) we demonstrated two modes of PriA binding to DNA, one in which the protein bound to duplex DNA with 3′-single-stranded extensions and one that presumably reflected binding to a D loop where the protein bound to bent DNA at three strand junctions. In order to determine whether the striking pattern of alternating protection and enhancement of cleavage observed when PriA bound the D loop was particular to one or the other mode of PriA binding to DNA, we examined the footprint of PriA on dsDNA with a 3′-single-stranded extension (Fig. 4).

In order to facilitate analysis of interactions at the 3′-end of the top strand, we included a 5′-single-stranded extension past the duplex region so that DNase I cleavages at the intersection of the 3′-single-stranded tail and the dsDNA would fall in an easily readable section of the gel. The pattern of alternating protection and enhancement observed on both the top and invading strand of the D loop was not evident (Fig. 4, *A* and *B, lanes 5*). Protection on the top strand of the duplex extended back about 14 nt from the single-stranded tail. Protection on the bottom strand, although weaker, appeared to extend back about 25 nt from the 3′-end. No enhanced cleavages were observed at all. Probing with S1 nuclease indicated that about 7 nt of the 3′-single-stranded tail abutting the dsDNA was also protected by PriA binding (data not shown).

Thus, the DNA footprinting reported here supports the conclusion of the accompanying report (9) that there are two modes of PriA binding to DNA. Modification of the pattern of DNase I cleavages as a result of PriA binding to the D loop was observed along 58 base pairs of dsDNA formed by the co-helical segments of the duplex consisting of the top and bottom strands with that of the duplex consisting of the top and invading strands (Fig. 5). This is roughly 200 Å, a very large distance when compared with the 36-Å Stokes radius of PriA (21). This suggests, based on the assumption that one PriA molecule could protect about 70 Å, that there are either multiple mole-

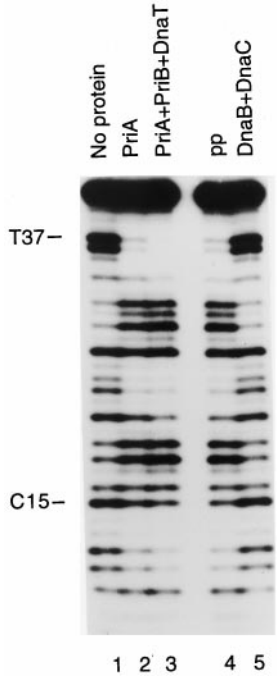

FIG. 3. **DNase I footprints of primosomal protein complexes on D loop DNA labeled on the invading strand.** Standard DNA footprinting reaction mixtures containing 5 μM ATP and 26 nM PriA, 15 nM PriB, 28 nM DnaT, 32 nM DnaB, and 32 nM DnaC, as indicated, were analyzed as described under "Materials and Methods." *pp*, this reaction mixture contained PriA, PriB, DnaT, DnaB, and DnaC. Correspondence of the Maxam-Gilbert sequence ladder to the sequence of oligo 5S is indicated on the *left-hand* side of the figure.

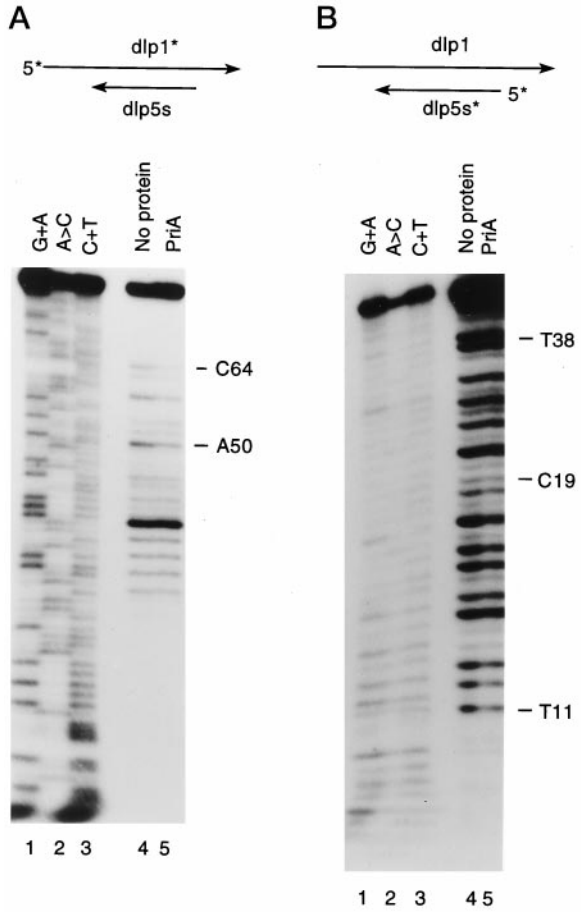

FIG. 4. **PriA binding to a duplex DNA with a 3′-single-stranded extension does not elicit the alternating pattern of enhancement and protection from DNase I cleavage observed on D loop DNA.** DNA footprinting reactions containing DNA duplexes composed of the top and invading strand of the D loop labeled on either the top (*panel A*) or invading (*panel B*) strand and 26 nM PriA were analyzed as described under "Materials and Methods." Correspondence of the Maxam-Gilbert sequence ladder to the sequences of oligos dlp1 and dlp5s is indicated on the *right-hand side* of *panels A* and *B*, respectively.

cules of PriA bound per D loop, or that PriA is wrapping the D loop DNA about itself in some manner.

PriA is a monomer in solution (22). We have also not observed any sigmoidicity in the binding isotherms of PriA to either PAS (13) or D loop (9) DNA that might suggest dimerization of the protein upon binding. However, we could detect two distinct complexes of PriA bound to PAS DNA that differed by a factor of two in the relative amount of PriA bound (13) and quantitative Western blotting of the protein components in primosomes formed on φX174 ss(c) DNA suggested a stoichiometry of two PriA molecules per primosome (15). On the other hand, an alternating pattern of protection from and enhancement of DNase I cleavage is characteristically observed when dsDNA lies on a surface, such as what occurs when DNA is wrapped around a protein. Distinguishing between these two possibilities will require additional investigation.

Primosome Assembly on D Loop DNA Results in Unwinding of the Left-hand Flanking Duplex Region—Replication fork assembly at a D loop requires that DnaB, the replication fork helicase (24), gain access to the DNA. The experiments described in the previous section demonstrate that an intermediate consisting of a PriA-PriB-DnaT complex could be isolated on the D loop. We therefore continued our investigation by adding DnaB and DnaC to the footprinting assays. The combination of DnaB and DnaC alone, without the other primosomal proteins, gave no visible footprint, regardless of whether the top (Fig. 6) or bottom (Fig. 7) strand was labeled and whether DNase I (Figs. 6*A, lane 7,* and 7*A, lane 4*, respectively) or S1 nuclease (Figs. 6*B* and 7*B, lanes 7*) was used as a probe. Similar results were observed if the invading strand was labeled (data not shown).

DnaB can be transferred to ssDNA by DnaC in the presence of ATP (7). The footprinting experiments described in this section contained 5 μM ATP, which should have been sufficient for these purposes (13). Yet, even though no SSB was present to prevent DnaB binding, no footprint was observed. There are several possible reasons for this. The most likely explanation is that DnaB may be binding transiently under these circumstances, leaving the displaced strand quickly because of hydrolysis of ATP. Another alternative is that the DnaB is present on the DNA, but is sliding back and forth rapidly. If this were the case, one might expect a generalized reduction in DNase I sensitivity, but this was also not observed. Finally, it is also possible that there is no binding of DnaB at all under these conditions. There is insufficient evidence to decide between these three alternatives, however, these observations are consistent with our demonstration, reported elsewhere (16), that DnaB and DnaC alone are insufficient to support DNA polymerase III holoenzyme-catalyzed elongation of the invading strand.

Footprinting also failed to reveal the presence of a primosome on the D loop. Although this was consistent with our inability to observe a stable primosomal complex by gel mobility shift analysis, it was inconsistent with our ability to observe primosomal protein-dependent replication initiating at a D loop (16). Addition of DnaB and DnaC to the PriA-PriB-DnaT-D loop DNA complex did not result in any observable modification

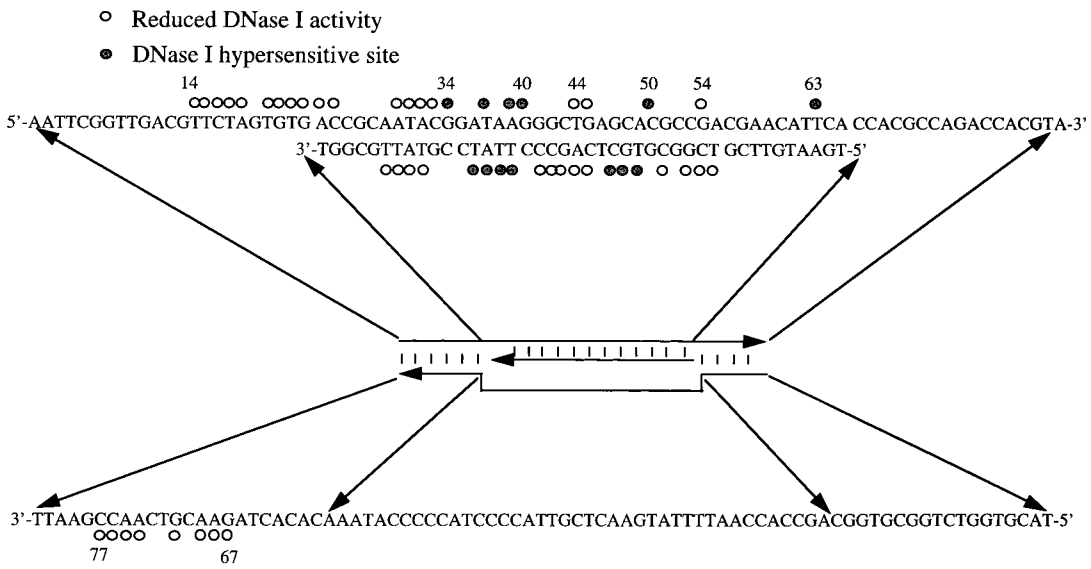

FIG. 5. **Summary of the enhancement of and protection from DNase I cleavage resulting from PriA binding to D loop DNA.**

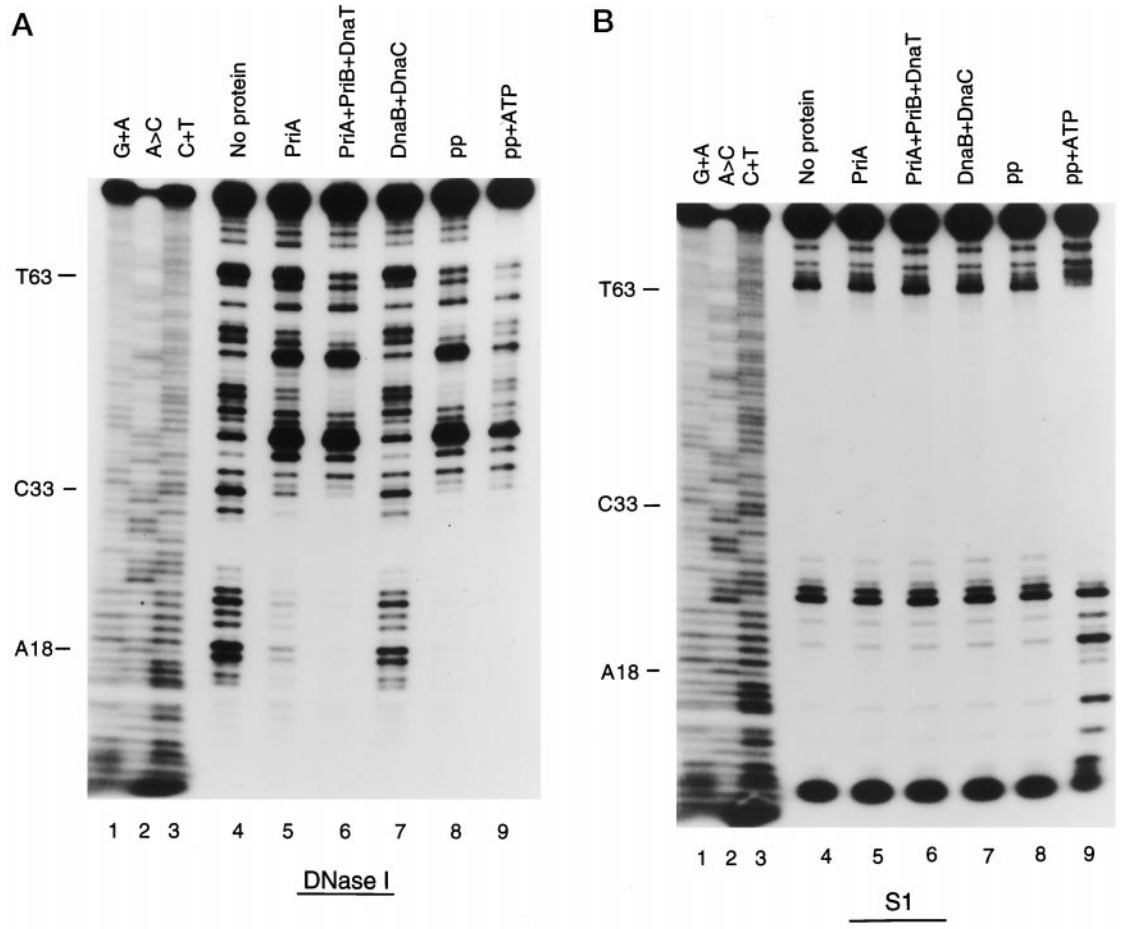

FIG. 6. **Primosomal protein-catalyzed unwinding of the left-hand side of the D loop as observed on the top strand.** Standard DNA footprinting reaction mixtures containing D loop DNA labeled on the top strand, either 5 μM ATP (*lanes 4–8*) or 1 mM ATP (*lane 9*), 26 nM PriA, 15 nM PriB, 28 nM DnaT, 32 nM DnaB, and 32 nM DnaC, as indicated, and either DNase I (*panel A*) or S1 nuclease (*panel B*) were analyzed as described under "Materials and Methods." Maxam-Gilbert (*lanes 1–3*) sequence ladders are of the top strand. *pp*, this reaction mixture contained PriA, PriB, DnaT, DnaB, and DnaC. Correspondence of the Maxam-Gilbert sequence ladder to the sequence of oligo dlp1 is indicated on the *left-hand side* of the panels.

to the footprint, regardless of whether the top (Fig. 6, *A* and *B, lanes 8*) or bottom strand (Fig. 7, *A, lane 5*, and *B, lane 8*) was labeled or whether DNase I (Figs. 6*A* and 7*A*) or S1 nuclease (Figs. 6*B* and 7*B*) was used as a probe.

Again, there were alternative possible explanations for this observation. We rejected the possibility that this implied that a primosome was not forming on the D loop DNA because, as mentioned above, we could observe primosomal protein-dependent replication on a D loop template DNA (16). Instead, we considered two alternatives: either the oligonucleotide sub-

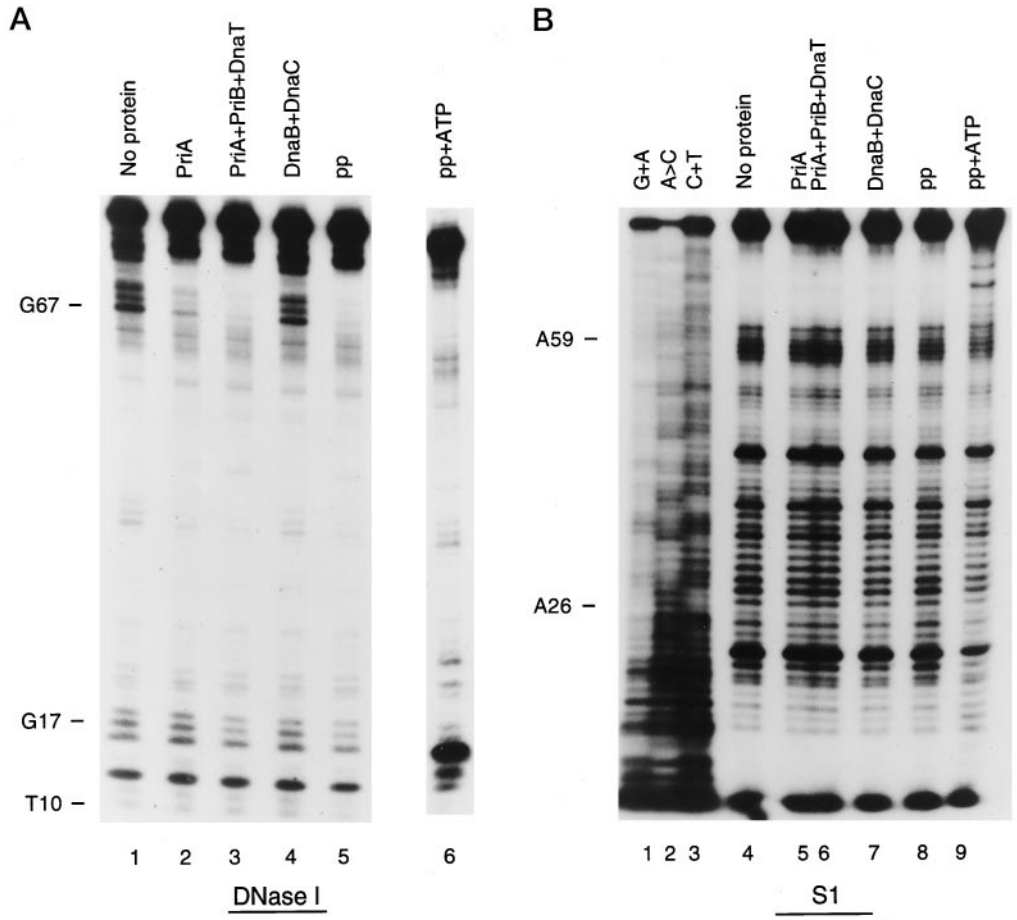

FIG. 7. **Primosomal protein-catalyzed unwinding of the *left-hand side* of the D loop as observed on the bottom strand.** Standard DNA footprinting reaction mixtures containing D loop DNA labeled on the bottom strand, either 5 μM ATP (*panel A, lanes 1–5; panel B, lanes 4–8*) or 1 mM ATP (*panel A, lane 6; panel B, lane 9*), 26 nM PriA, 15 nM PriB, 28 nM DnaT, 32 nM DnaB, and 32 nM DnaC, as indicated, and either DNase I (*panel A*) or S1 nuclease (*panel B*) were analyzed as described under "Materials and Methods." Maxam-Gilbert (*panel B, lanes 1–3*) sequence ladders are of the bottom strand. *pp*, this reaction mixture contained PriA, PriB, DnaT, DnaB, and DnaC. *Lane 6* of *panel A* is from the same gel as *lanes 1–5*. Intervening lanes not relevant to this figure have been removed. Curvature of the gel, which can be seen in *lanes 1–5*, prevent precise alignment of *lane 6* with *lanes 1–5*. Correspondence of the Maxam-Gilbert sequence ladder to the sequence of oligos dlp1 and 2L is indicated on the *left-hand side* of *panels A* and *B*, respectively.

strate used was not large enough to support stable binding of a complete primosome or a rearrangement of the disposition of the proteins involved was required for stable primosome assembly and this rearrangement was driven by ATP hydrolysis and thus required higher concentrations of ATP than 5 μM. Accordingly, we performed the footprinting experiments in the presence of 1 mM ATP.

Significant changes to the footprints on the D loop could be observed in the presence of PriA, PriB, DnaT, DnaB, DnaC, and 1 mM ATP. On the top strand, the region between T^{9} and T^{22} became S1 nuclease-sensitive (Figs. 6, *B, lane 9*, and 9), as if base pairing with the bottom strand had been lost. Little in the way of consistent changes in the DNase I footprint were observed. Occasionally, a generalized decrease in DNase I sensitivity was observed. However, this was very variable (*e.g.* compare Fig. 6*A, lane 9,* to 8*A, lane 5*). On the bottom strand, new DNase I-hypersensitive sites became apparent at C^{13}, G^{14}, and T^{15} as the surrounding nucleotides, T^{10}, G^{12}, and G^{17} became somewhat less sensitive to DNase I (Figs. 7, *A, lane 6,* and 9). Consistent with the increased S1 nuclease sensitivity of the left-hand end of the top strand, a similar region, between G^{64} and A^{74}, on the bottom strand also became S1 nuclease-sensitive (Figs. 7, *B, lane 9*, and 9).

We interpret the conversion of a significant portion of the left-hand flanking duplex region of the D loop from profound S1 nuclease-insensitivity to obvious S1 nuclease-sensitivity as indicating that this region of the substrate had become unwound by the action of the primosomal proteins. Both PriA and DnaB are helicases that could unwind this substrate. Thus, in order to determine whether the observed unwinding was a result of the independent action of the DNA helicases present in the reaction mixture or a result of primosome assembly, we examined the dependence of the unwinding reaction on the presence of all the primosomal proteins.

Neither PriA alone (Fig. 8, *A* and *B*, *lanes 2* and *7*, respectively), DnaB in the presence of DnaC (Fig. 8, *A* and *B, lanes 3* and *8*, respectively), or the combination of PriA with DnaB and DnaC (Fig. 8, *A* and *B, lanes 4* and *9*, respectively) showed the changes to the DNase I and S1 nuclease footprints that were indicative of unwinding of the left-hand duplex region and were obvious when PriB and DnaT were also present with PriA, DnaB, and DnaC in the reaction mixture (Figs. 6, *A, lane 9*; *B, lane 9*; 7*B, lane 9*; 8*A, lane* 5; 8*B, lane 10*). Thus, the observed unwinding was neither the result of either PriA or DnaB acting independently nor the result of the two helicases acting independently, but in a concerted manner, one after the other. Instead, we conclude that the unwinding resulted because the helicase activities intrinsic to a primosome that had been assembled on the D loop became activated in the presence of 1 mM ATP. This is consistent with the known ATP requirements for primosome helicase action (21).

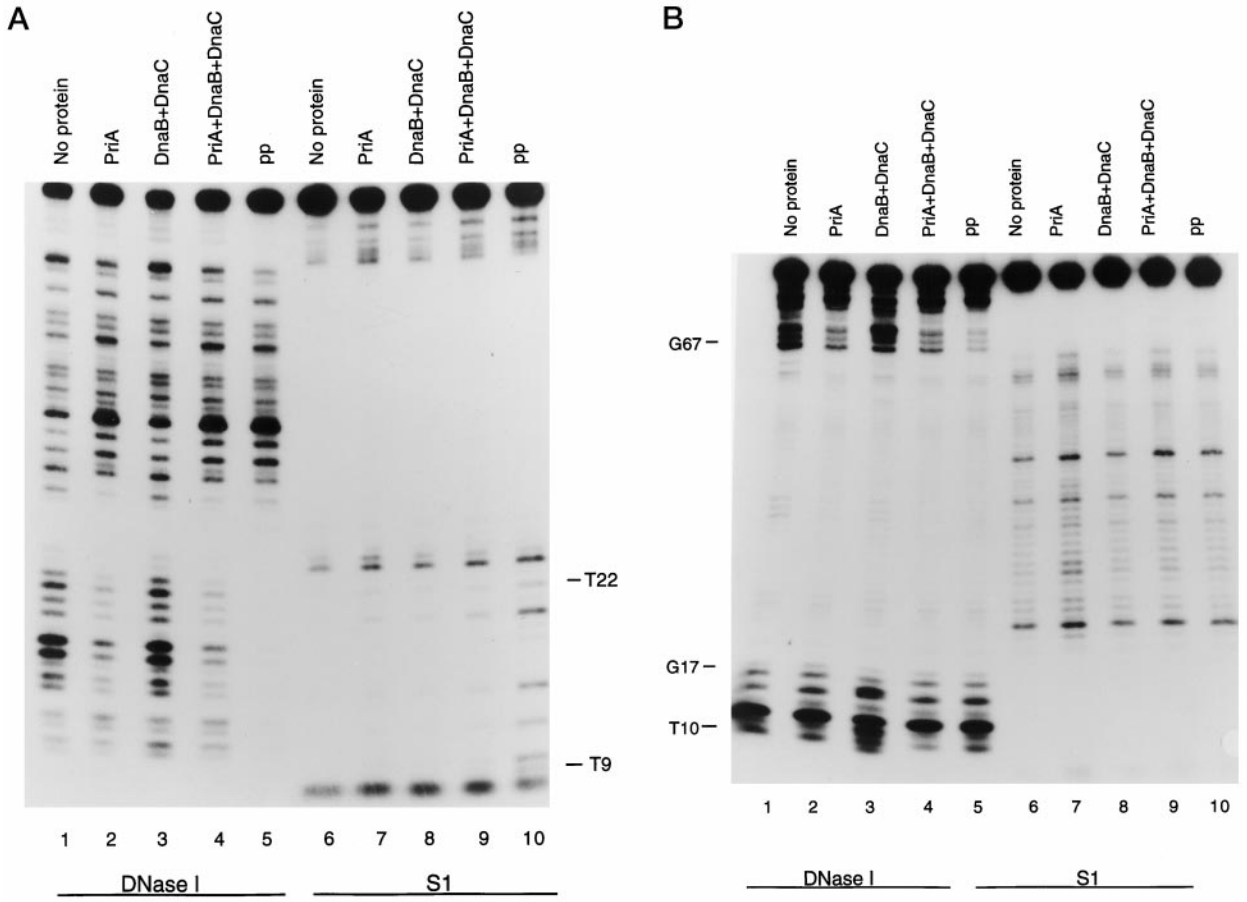

FIG. 8. **D loop unwinding requires assembly of a primosome.** Standard DNA footprinting reaction mixtures containing D loop substrate labeled on either the top (*panel A*) or bottom (*panel B*) strand, 1 mM ATP, 26 nM PriA, 15 nM PriB, 28 nM DnaT, 32 nM DnaB, and 32 nM DnaC, as indicated, and either DNase I (*lanes 1–5*) or S1 nuclease (*lanes 6–10*) were analyzed as described under "Materials and Methods." Correspondence of the cleavage patterns to the sequence of oligos dlp1 and dlp2L are indicated on the *right-hand side* of *panel A* and the *left-hand side* of *panel B*, respectively.

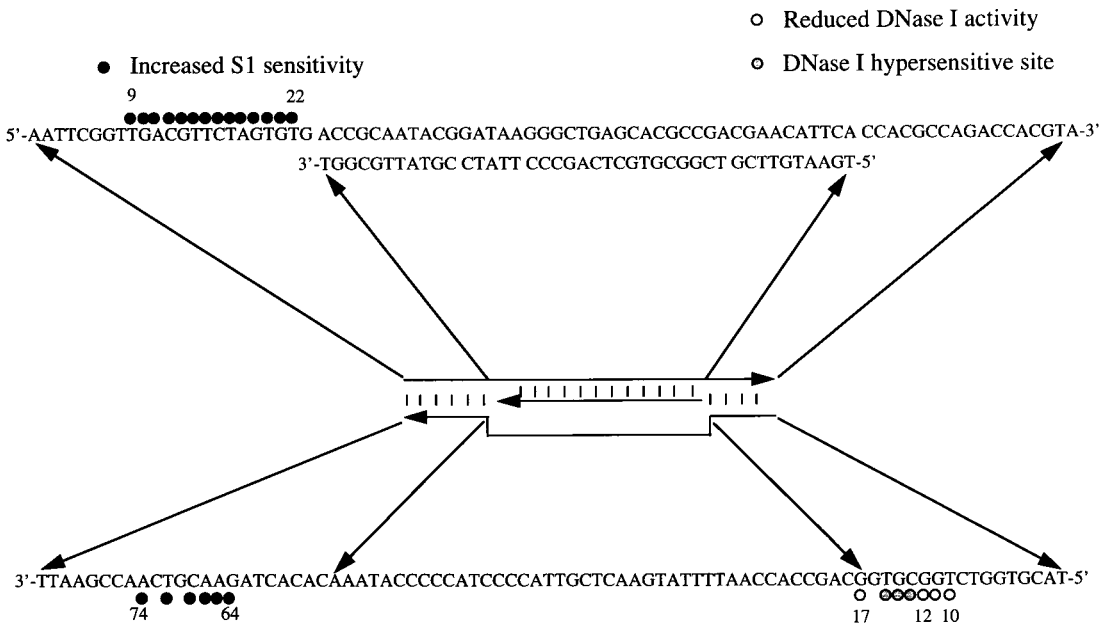

FIG. 9. **Summary of primosome-catalyzed D loop unwinding.**

DISCUSSION

PriA was discovered originally because of its requirement for conversion of viral ϕX174 DNA to a duplex replicative form *in vitro* (25, 26). Subsequent studies showed that this protein initiated the assembly of a protein-DNA complex that primed DNA synthesis of the complementary strand (6). Intensive biochemical analysis of this reaction revealed that seven proteins, now called PriA, PriB, PriC, DnaT, DnaB, DnaC, and

DnaG, were involved in assembly of this protein-DNA complex (7). On ϕX174 DNA coated with SSB, this complex is composed of PriA, PriB, PriC, DnaT, DnaB, and DnaG (13) and is capable of: (i) translocation along ssDNA in either direction (21), (ii) acting as a bidirectional DNA helicase (21), and catalyzing the synthesis of small oligoribonucleotide primers that can be used by the DNA polymerase III holoenzyme to initiate DNA synthesis (27). The ability to both unwind duplex DNA and synthesize ribonucleotide primers earned this complex the name of "primosome" (27) and illuminated an economical solution to the requirements for unwinding the duplex template DNA and providing primers for Okazaki fragment synthesis during chromosomal replication.

Currently, the term primosome now applies to any protein or multiprotein complex that is capable of providing both DNA unwinding and Okazaki fragment-priming functions at a replication fork and the one discussed specifically in this report is referred to as the ϕX-type primosome (7). The ϕX-type primosome is capable of participating in replication fork assembly *in vitro* (28) and in the replication of a number of plasmid DNAs, such as pBR322 (29). But four (PriA, PriB, PriC, and DnaT) of the seven proteins are not required for replication initiating at *oriC* (30), the bacterial chromosome origin of replication. The three ϕX-type primosomal proteins required for chromosomal DNA replication, DnaB, DnaC, and DnaG (30), all have very specific functions: DnaB is the replication fork DNA helicase (24), DnaC is required for the efficient transfer of DnaB to DNA (31), and DnaG is the primase for Okazaki fragment synthesis (32, 33). Thus, it has been unclear as to the role, if any, of PriA, PriB, PriC, and DnaT during chromosomal replication.

Genetic investigations of the phenotypes of *priA* null mutations suggested a role for this protein in the cell. These strains were constitutively induced for the SOS response (1) and were defective in homologous recombination (2, 3), both induced and constitutive stable DNA replication (4), and the repair of UV-damaged DNA (2, 3) and double-strand DNA breaks (3). The role of D loops and R loops as initiating structures for the two forms of stable DNA replication (5), and the key role of a D loop during initiation of homologous recombination (34), which is also required to initiate repair of double-strand breaks (35), led to the proposal that all these phenotypes resulted from a failure, in *priA* null strains, of replication forks to assemble at a D loop (2, 3). Recent biochemical evidence has provided significant support for this hypothesis.

PriA specifically recognizes and binds D loop DNA compared with a corresponding bubble structure (8, 9). The basis for this binding is the ability of PriA to specifically recognize bent DNA at three-strand junctions (9). In this report, we have provided evidence that PriA will also catalyze assembly of a ϕX-type primosome at a D loop. This is a necessary step in order to effect replication fork assembly on these recombination intermediates.

Primosome assembly on PAS sequences in ssDNA occurs in five discrete steps resulting in, sequentially, the following protein-DNA complexes (13): (i) PriA-PAS DNA, (ii) PriA-PriB-PAS DNA, (iii) PriA-PriB-DnaT-PAS DNA, (iv) PriA-PriB-DnaT-DnaB-PAS DNA (the preprimosome), and (v) PriA-PriB-DnaT-DnaB-DnaG-DNA (the primosome). PriC is present in the latter two complexes if they are formed on large DNAs. We were able to demonstrate, using gel mobility shift analysis, the formation of a PriA-PriB-DnaT-D loop DNA complex, but could not, using this methodology, observe any of the higher order complexes.

DNA footprinting revealed that PriA bound preferentially to the left-hand side of the D loop. This is consistent with our observation, reported in the accompanying manuscript (9), that PriA preferred to bind three-strand junctions with 5′-single-stranded tails. PriA binding resulted in the modification of the DNase I digestion pattern over a large portion of the D loop. At the three-strand junction formed by the top, invading, and bottom strands, PriA binding reduced DNase I activity on all three strands. This region of protection extended from the three-strand junction, 10 base pairs into the duplex formed by the top and invading strands and 18 base pairs in the opposite direction into the duplex formed by the top and bottom strands. Given that steric interference in a DNase I footprint generally leads to an overestimate of binding site size, this is a region that could be covered by a protein that has a Stokes radius of 36 Å (22).

Interestingly, however, the footprint was more complex. PriA binding also generated an alternating pattern of protection from and enhancement of cleavage by DNase I essentially throughout the length of the duplex formed by the top and invading strands. The entire region effected by PriA binding covered almost 200 Å of DNA, far too much to be covered by one PriA molecule. Although the obvious explanation, that PriA wraps a portion of the D loop about itself, is attractive, we do not have any corroborating evidence to support it. The alternative explanation, that multiple molecules of PriA are bound to the D loop, cannot be ruled out at this time.

In the presence of 5 μM ATP, a concentration sufficient to support primosome assembly (13), the addition of the other primosomal proteins did not result in any significant changes in the footprint. This was surprising because we expected, if a primosome was being assembled, to observe a footprint on the displaced strand corresponding to the loading of DnaB. However, we were uncertain as to whether a substrate this small could support stable binding of a complete primosome. Our previous studies utilized a 304-nt long ssDNA that contained a PAS sequence roughly in the center of the fragment (13). When a smaller substrate was used composed solely of the sequence of the PAS, we could only observe PriA binding.[2] Thus we searched for functional evidence for primosome assembly.

Primosome DNA helicase activity was detected in the presence of 1 mM ATP because of the appearance of S1 nuclease-sensitive sites in both the top and bottom strands of the left-hand flanking duplex region. It was clear that this was a primosomal function because: (i) all the preprimosomal proteins were required, and (ii) neither PriA or DnaB alone nor a combination of the two helicases was sufficient for unwinding to occur. It seems likely that the components of the primosome assembled on a D loop are binding to both strands of DNA. PriA binding alone spanned each of the three strands at the left-hand junction. The 5′ $\rightarrow$ 3′ directionality of the DnaB helicase activity argues that this protein is loaded onto the displaced strand. If it were bound to either the top or invading strand, we would have expected to observe either displacement of the invading strand or unwinding of the right-hand flanking duplex.

The disposition of the other proteins in a primosome assembled on a D loop is not clear. In the presence of 1 mM ATP, we observed both protection from and enhancement of DNase I cleavage in an 8-nt region on the bottom strand in the right-hand flanking duplex region. However, no corresponding effects were observed on the top strand. Thus, we think it unlikely that unwinding of the right-hand flanking duplex was occurring. Instead, we take this to indicate a rearrangement on the DNA of some of the other primosomal proteins to accommodate loading of DnaB.

Although to simplify the analysis, these experiments were

[2] K. H. Zavitz and K. J. Marians, unpublished data.

conducted in the absence of the single-stranded DNA-binding protein (SSB), it is clear, because we can observe replication fork assembly at a D loop (16), that primosome occurs on D loops in the presence of SSB as well.

It seems likely, then, that the normal substrates for PriA-directed primosome assembly in the cell are D loops that are formed by the recombination proteins. This is supported by our demonstration of primosomal protein- and DNA polymerase III holoenzyme-dependent replication fork assembly on form II DNA templates carrying a D loop (16). Thus, one can conclude, much as bacteriophage λ evolved the λP protein to steal DnaB from DnaC and suborn it to replication of its own genome, bacteriophage ϕX174 evolved to steal an existing cellular replication system and direct it to its own survival by mimicking the substrate for primosome assembly.

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PriA-directed Assembly of a Primosome on D Loop DNA

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