Escherichia coli DnaA Protein Loads a Single DnaB Helicase at a DnaA Box Hairpin*

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The molecular engine that drives bidirectional replication fork movement from the Escherichia coli replication origin (oriC) is the replicative helicase, DnaB. At oriC, two and only two helicase molecules are loaded, one for each replication fork. DnaA participates in helicase loading; DnaC is also involved, because it must be in a complex with DnaB for delivery of the helicase. Since DnaA induces a local unwinding of oriC, one model is that the limited availability of single-stranded DNA at oriC restricts the number of DnaB molecules that can bind. In this report, we determined that one DnaB helicase or one DnaB-DnaC complex is bound to a single-stranded DNA in a biologically relevant DNA replication system. These results indicate that the availability of single-stranded DNA is not a limiting factor and support a model in which the site of entry for DnaB is altered so that it cannot be reused. We also show that 2–4 DnaA monomers are bound on the single-stranded DNA at a specific site that carries a DnaA box sequence in a hairpin structure.

The Escherichia coli chromosomal origin (oriC) has two major roles (reviewed in Refs. 1 and 2). One is to act as a site where DNA replication is controlled so that it occurs only once per cell cycle. The second is to serve as a locus where the replication fork machinery is assembled, involving a series of orchestrated steps. An important event at oriC is the binding of DnaA protein to specific sequences named DnaA boxes (3). A second essential step in the assembly process is the DnaA-dependent recruitment of DnaB (4, 5).

Studies on the native structure of DnaB have firmly established that it is a hexamer of identical subunits arranged as a toroidal structure with a central opening (6–9). Its stability requires the presence of Mg²⁺ ion; removal of the metal ion by dialysis or chelation is needed to dissociate the DnaB hexamer into its subassemblies (10). However, the form of DnaB that is required at the stage of initiation at oriC is as a complex with its partner, DnaC (11, 12). Assembly of this complex requires the binding of ATP to DnaC, with the nucleotide serving to alter the conformation of an N-terminal domain of DnaC so that it can bind to DnaB (13). Each DnaC monomer is present at a 1:1 ratio with each DnaB protomer (14–16). Whereas it is the DnaB-DnaC complex that is active at the stage of initiation, DnaB liberated from DnaC is active during DNA synthesis. The association of DnaC with DnaB inhibits the enzymatic activities of this essential helicase, and the hydrolysis of ATP bound to DnaC is required to release DnaC from DnaB (5, 12, 17).

Once DnaB is situated at the apex of the replication fork, the helicase acts to unwind the parental DNA as each DNA strand is copied by DNA polymerase III holoenzyme. These events are facilitated by a functional coupling, involving an interaction between DnaB as it moves in the 5′-to-3′ direction on the lagging strand template and the tau subunit of DNA polymerase III holoenzyme (18). When this physical interaction is maintained, DnaB moves at a 20-fold faster rate than the speed of DnaB translocation alone.

At oriC, it has been proposed that an AT-rich region unwound by DnaB protein serves as the entry site for DnaB (19). Footprinting studies map DnaB to this region, in support of the model (20). Other results indicate that only two DnaB hexamers are bound at oriC (20, 21). Because DnaA induces a limited degree of unwinding, one model is that only two DnaB hexamers can bind because the available single-stranded DNA (ssDNA) is only sufficient for one DnaB hexamer for each DNA strand. We have relied on a simple replication system to study the process of recruitment of DnaB onto DNA and to address the question of whether the availability of ssDNA influences the number of helicase molecules that can bind. With a single-stranded DNA carrying a DnaA box in a hairpin structure (M13 A-site), DnaA bound to this site forms a structure that in turn is recognized by the DnaB-DnaC complex to form an intermediate termed the prepriming complex (5, 22). Following the release of DnaC, DnaB is then free to move on the ssDNA. The transient binding of primase to DnaB results in primers that are formed at apparently random locations (23, 24). These primers are then extended by DNA polymerase holoenzyme in conversion of the ssDNA to duplex form. In this system, DNA replication is dependent on a single DnaA box-containing sequence, and only one DNA strand is synthesized on the ssDNA template. By comparison, DNA replication from oriC is more complicated because a duplex DNA is involved, and each parental DNA strand is bound by DnaB to support bidirectional replication fork movement. Priming and DNA synthesis occur on both strands of the parental duplex.

In this report, we sought to characterize further the molecular composition of the complex formed by the binding of DnaA, DnaB, and DnaC protein to the ssDNA carrying the DnaA box hairpin. The major objective was to test the model that the amount of ssDNA available influences the number of DnaB molecules that can bind. Several independent methods were used. In the first, a 379-nucleotide-long ssDNA bearing the DnaA box hairpin and covered by SSB was used to demonstrate

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1 The abbreviations used are: ssDNA, single-stranded DNA; SSB, single-stranded DNA-binding protein; ATPγS, adenosine 5′-O-thiotriphosphate.
the binding of DnaA in gel mobility shift assays. The second involved a primer extension assay to demonstrate the binding and positions of these proteins to the DNA box hairpin. In the third approach, we used immunoblot analysis to determine the ratio of DnaA, DnaB, and DnaC protein assembled on the ssDNA. These results show that DnaA protein bound to the DNA box hairpin recruits only a single DnaB hexamer and strongly suggest that the availability of ssDNA is not a limiting factor in the loading of DnaB onto DNA.

**EXPERIMENTAL PROCEDURES**

**DNAs and Proteins**—M13 A-site ssDNA (25), purified proteins, and antibodies have been described previously (4, 26). M13 – 40 universal primer (17-mer) and ATP/ßS were from U.S. Biochemical Corp. The large fragment of DNA polymerase I was from Roche Molecular Biochemicals. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit were from Bio-Rad. Oligonucleotides (GGCGATAA-CAATTTCCAC and CAGTGCACACGCTTGGCAAG) were synthesized by a campus facility and were used to PCR-amplify the region containing the DNA box hairpin. These primers are derived from M13mp8, the DNA vector used to construct M13 A-site. After PCR amplification, the product was digested with HindIII endonuclease that cleaves within the latter primer and then end-filled at this restriction site with [γ-32P]ATP and the large fragment of DNA polymerase I to label specifically the viral strand ssDNA fragment. In Fig. 1B, the primer corresponding to the viral strand was first radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP, and then the unincorporated label was removed by passing the sample over a Sephadex G25 spin column before PCR amplification. The PCR-amplified DNA was then combined with a 5-fold molar excess of M13 A-site ssDNA in a 1× SSC buffer, placed in a water bath at 100 °C, and allowed to cool gradually to room temperature to anneal the PCR-amplified DNA strand that is complementary to the M13 A-site viral ssDNA. The DNA of viral strand sense remains single-stranded. The sample was then electrophoresed in a 1% agarose gel in buffer containing 90 mM Tris borate, pH 8.9, and 1 mM EDTA and the isolated ssDNA fragment was determined by specific radioactivity and by agarose gel electrophoresis and staining with ethidium bromide relative to known amounts of a DNA fragment of similar size.

**Gel Mobility Shift Assay**—Reactions (10 μl) contained 5.8 fmol of [γ-32P]labeled ssDNA fragment bearing the DNA box hairpin (379 nucleotides long) in ABC Buffer (40 mM HEPES-KOH, pH 8.0, 0.38 M potassium glutamate, 4% (w/v) sucrose, 8 mM magnesium acetate, 0.1 mg/ml bovine serum albumin, and 2 mM diethiothreitol), SSB, DnaA, and ATP as indicated. Reactions were incubated at 25 °C for 10 min and then analyzed by native gel electrophoresis. Polyacrylamide gel electrophoresis (4%, 8:1 acrylamide/bisacrylamide) was in buffer containing 50 mM Tris-HCl, pH 8.0, 0.38 mM glycine, 8 mM MgCl2, and 1 mM EDTA as described (27). The gels (13 cm, 11 cm, 1.5 mm) were run in this buffer at 60–70 V for 6–7 h or at 30 V for 14 h and then were dried and autoradiographed.

**Primer Extension Assay**—Reactions (25 μl) contained M13 A-site ssDNA annealed to the ~ 40 universal primer (50 ng), SSB (1 μg), DnaA (45 ng), DnaB (50 ng), and DnaC (25 ng) as indicated and ATP-ßS (0.1 mM) as indicated in ABC Buffer. After incubation for 10 min at 30 °C, deoxynucleotides (50 μM each including [γ-32P]ATP, 400 cpn/pmol of total nucleotide) and the large fragment of DNA polymerase I (2 units) were added followed by incubation at 37 °C for 5 min. Samples were denatured in a boiling water bath and then electrophoretically separated on a sequencing gel followed by autoradiography to visualize the extension products. DNA sequencing reactions by the dideoxy chain terminating method with the singly primed M13 A-site template described above provided molecular weight markers to map the 3′-ends of the primer extension products.

**Isolation of Prepriming Complexes**—Reactions of prepriming complex formation (100 μl), a 50-fold scale up of a standard replication reaction in terms of DNA and replication protein components, contained M13 A-site ssDNA (5 μg), SSB (30 μg), DnaA (2.8 μg), DnaB (10 μg), and DnaC (5 μg) as indicated in ABC Buffer supplemented with 0.1 mM ATP or 0.1 mM ATP-ßS. After incubation at 25 °C for 10 min, samples were applied onto gel filtration columns (Sepharose 4B, 0.7 × 13 cm; Amersham Biosciences) equilibrated in ABC Buffer supplemented with 0.1 mM ATP or 0.1 mM ATP-ßS, as indicated. Fractions of 200 μl were collected, and the isolated prepriming complexes were analyzed by quantitative immunoblotting.

**Quantitative Analysis of Complexes**—Reactions from gel filtration chromatography were analyzed by agarose gel electrophoresis to identify void volume fractions containing M13 A-site ssDNA and, where indicated, to quantitate the amount of DNA by comparison with known amounts of M13 A-site ssDNA that was co-electrophoresed and used to prepare a standard curve. The ethidium bromide-stained gels were photographed and analyzed with an Eastman Kodak Co. EDAS 120 gel documentation system. To quantitate the amounts of the respective proteins bound to the ssDNA, samples were electrophoretically separated by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (BA-85; Schleicher and Schuell) and visualized with known amounts of the respective purified protein to prepare a standard curve. The blots were probed with either M43 monoclonal antibody for DnaA (28) or a combination of affinity-purified polyclonal antibodies for DnaB and DnaC. Chemiluminescence (SuperSignal; Pierce) of immune complexes of horseradish peroxidase conjugated to the secondary antibody was analyzed with a Bio-Rad model GS505 molecular imager and associated software.

**RESULTS**

**DnaA Bound to the DNA Box Hairpin Forms a Single Discrete Complex by Gel Mobility Shift Assay**—Previous studies indicate that DnaA protein recognizes and binds to a DNA box sequence in a putative hairpin structure, as indicated by footprinting studies with DNase I and dimethyl sulfate and by mutational analysis of the DNA box hairpin (22). To confirm these observations, we developed assay conditions to measure the binding of DnaA protein to a 379-nucleotide-long ssDNA fragment carrying the DNA box hairpin. Because prepriming complex formation occurs on an ssDNA covered by SSB, the amount of SSB needed to saturate the DNA was first explored. The binding site size for SSB is 65 ± 3 nucleotides at the moderate ionic strength used in this experiment (29–31), and recent studies confirm that the ssDNA wraps around the SSB tetramer, with each dimer capable of binding an oligonucleotide of 35 residues (32). The hairpin of 62 bases is stable in the presence of SSB because it remains sensitive to DNase I cleavage (22). The left arm of 110 nucleotides can accommodate two tetramers, with one tetramer bound to 65 nucleotides and the second tetramer bound to 35 nucleotides via one of its dimers. The right arm of 207 nucleotides is long enough to be bound by three SSB tetramers. Our observation of five complexes with increasing SSB is consistent with these expectations.

Once conditions established the level of SSB that was saturating for this ssDNA fragment, the effect of DnaA on the mobility of the SSB-ssDNA complex was examined. At the highest levels of DnaA, a single, more slowly migrating complex was detected (Fig. 1B). Complexes of intermediate mobility were not observed. As described below, 2–4 DnaA monomers are bound to the DNA box hairpin. Thus, binding of DnaA to this sequence appears to be concerted because only one single shifted complex was observed. It is noteworthy that in this replication system, the level of SSB is not critical as long as it is at or above the level needed to cover the ssDNA (data not shown). The conclusion from this set of experiments is that DnaA is able to bind to the hairpin structure (as will be shown below also) to form a single discrete complex despite the abundant presence of SSB.

Experiments were also performed to measure the binding of DnaA to the ssDNA fragment without SSB or at subsaturating SSB (data not shown). Under either condition, DnaA did not form discrete complexes but bound nonspecifically to the ssDNA fragment based on the formation of complexes that migrated as a smear. This was observed only at the highest
levels tested (under reaction conditions as described in Fig. 1B; range of DnaA from 0.09 to 2.8 pmol in increments that varied 2-fold). These results suggest that the binding of SSB to the ssDNA masks sites of nonspecific binding of DnaA.

Because of the appearance of discrete SSB-ssDNA complexes at subsaturating SSB, the possibility arises that SSB is not binding randomly and that the stem-loop structure imposes an order in the binding of SSB to the ssDNA. However, Sun and Godson observed the formation of discrete complexes using ssDNA from the lacZ coding region (33). They concluded that SSB did not bind to this DNA in a phased manner from results of 

**Discussion.** Since DnaA physically interacts with DnaB in the DnaB-DnaC complex in the recruitment of the helicase into the prepriming complex (4), the primer extension results suggest that the DnaB-DnaC complex is bound directly adjacent to DnaA at the DnaA box.

Interestingly, the abundance of the termination product attributed to the binding of DnaA was elevated upon supplementation of the reaction containing DnaA with ATPγS, DnaB, and DnaC (Fig. 2, lane 9 compared with lane 6). As shown below, the presence or absence of DnaB and DnaC did not alter sta-
statistically the ratio of DnaA monomers bound to the DnaA box hairpin, so DnaB and DnaC do not stabilize DnaA at this site. One possible explanation for the elevated termination due to the binding of DnaA is that DnaA in the prepriming complex assumes a conformation that impedes movement of the DNA polymerase during primer extension.

Assembly of the Prepriming Complex Requires the DnaA Box Hairpin—The results of Fig. 2 support the model of a specific nucleoprotein structure. To determine its molecular composition, quantitative immunoblot analysis of the proteins that form this nucleoprotein complex was performed. As a control, we demonstrated that the assembly of DnaA, DnaB, and DnaC on the ssDNA was specific for the DnaA box hairpin (Fig. 3). The M13 derivative carrying the DnaA box hairpin or wild type M13 was incubated with DnaA, DnaB, DnaC, and SSB. As in Fig. 2, ATP$_{S}$ was included to support formation of the DnaB-DnaC complex but not the release of DnaC from the prepriming complex. In B, the 3' terminus of the primer extension product that reflects the binding of DnaA protein (open arrow) maps to the DnaA box sequence. The filled arrows represent the binding of the DnaB-DnaC complex. The calculated thermodynamic stability ($\Delta G$) of the DnaA box hairpin is $-17.6$ using “DNA mfold” (48).

Regardless of whether DnaB and DnaC were also included, 2–4 DnaA monomers were bound to the DnaA box hairpin. The stoichiometry of DnaA monomers per ssDNA circle is statistically indistinguishable whether the complexes were assembled and isolated with ATP or ATP$_{S}$. When DnaB and DnaC were included under conditions that support formation of the DnaB-DnaC complex, the stoichiometry of $4 \pm 0.9$ and $4.2 \pm 0.8$ DnaB monomers bound per ssDNA is consistent with a single hexamer bound in the prepriming complex. The nucleotide analogue ATP$_{S}$ maintains the association of DnaC with DnaB, because ATP hydrolysis is required for the release of DnaC after DnaB has become stably bound to the ssDNA. The comparable stoichiometries of DnaC and DnaB in the prepriming complex under this condition support the conclusion that a single DnaB$_6$-DnaC$_6$ complex is bound per ssDNA. When the prepriming complex was assembled with ATP instead, the inability to detect DnaC in the isolated complex indicates that DnaC has been released.

Conditions for the assembly of the prepriming complex include an incubation step at 20 °C for 10 min followed by isola-
entirely single-stranded, with the exception of the DnaA box

The M13 derivative carrying the DnaA box sequence is bound to the ssDNA were detected by immunoblot analysis as described under “Experimental Procedures.” For the indicated fractions from 5 to 14, 50 µl of sample was trichloroacetic acid-precipitated and resuspended in Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Those fractions from 16 to 31 (6 µl/lane) were electrophoresed without trichloroacetic acid precipitation. Markers (40 ng) of the indicated proteins are in the leftmost lane of each blot. With M13 ssDNA lacking a binding site for DnaA, the levels of DnaA, DnaB, and DnaC detected in the void volume fractions are 0.01, 0.07, and 0.03, respectively, the level (normalized to 1) of the corresponding protein in the isolated prepriming complex formed on the M13 A-site carrying the DnaA box hairpin. Their relative levels were determined by scanning densitometry of respective void volume fractions in B.

FIG. 3. Prepriming complex formation requires the DnaA box hairpin. Prepriming complexes with M13 A-site ssDNA (left panels) or wild type M13 (right panels) were assembled and isolated as described under “Experimental Procedures” but with 0.1 mM ATPγS instead of ATP. In A, samples (10 µl) from the indicated fractions were subjected to agarose gel electrophoresis, and DNA was visualized by ethidium bromide staining to identify void volume fractions (fractions 10–12). M13 A-site or wild type M13 ssDNA (100 ng each) served as markers. In B, prepriming proteins bound to the ssDNA were detected by immunoblot analysis as described under “Experimental Procedures.” For the indicated fractions from 5 to 14, 50 µl of sample was trichloroacetic acid-precipitated and resuspended in Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Those fractions from 16 to 31 (6 µl/lane) were electrophoresed without trichloroacetic acid precipitation. Markers (40 ng) of the indicated proteins are in the leftmost lane of each blot. With M13 ssDNA lacking a binding site for DnaA, the levels of DnaA, DnaB, and DnaC detected in the void volume fractions are 0.01, 0.07, and 0.03, respectively, the level (normalized to 1) of the corresponding protein in the isolated prepriming complex formed on the M13 A-site carrying the DnaA box hairpin. Their relative levels were determined by scanning densitometry of respective void volume fractions in B.

tion of the protein-DNA complex that takes another 10 min. Because the rate of DnaB translocation is estimated at 35 nucleotides/s at 30 °C (18), the incubation period and time required to isolate the complex is more than sufficient for DnaB to move away from its entry site and to allow another DnaB hexamer to bind. The finding that only a single DnaB hexamer is bound to the ssDNA suggests that a mechanism acts to limit the number of DnaB hexamers that can bind to a single molecule.

DISCUSSION

At oriC, formation of an intermediate termed the prepriming complex involves the recruitment of the DnaB-DnaC complex by a specific interaction between DnaB and DnaA bound to the respective DnaA boxes in oriC (4, 5). We recently determined the stoichiometry of proteins in the oriC prepriming complex (21). One important conclusion was that only two DnaB hexamers were bound at oriC, one for each replication fork that then moves in opposing directions. The finding of two replicative helicases per oriC raises the question of whether a mechanism controls the number of helicase molecules that can enter at oriC. Motivated by the need to understand this mechanism, we wanted to test the model that the amount of ssDNA available at oriC restricts the number of DnaB hexamers that can bind. Studies based on potassium permanganate sensitivity yield an estimate that 26 nucleotides become single-stranded when the ATP-bound form of DnaA induces strand opening at oriC (34). In contrast, the amount of ssDNA character contained in a supercoiled oriC plasmid of 6.6 kb is about 400 nucleotides as judged by the extent of unwound DNA induced by DnaB helicase activity in the absence of DNA gyrase (35, 36). The M13 derivative carrying the DnaA box sequence is entirely single-stranded, with the exception of the DnaA box hairpin and additional secondary structures in the M13 origin region. This ssDNA is the ideal substrate to test whether the availability of ssDNA is the controlling factor that limits the number of DnaB molecules at oriC to only two.

One DnaB-DnaC Complex or a Single DnaB Hexamer Is Loaded in the Vicinity of the DnaA Box—When the prepriming complex was assembled with ATPγS, we concluded that a single DnaB-DnaC complex was bound per ssDNA circle. Under conditions that permit the release of DnaC from DnaB (in which the prepriming complex was assembled and isolated in buffer containing ATPγS), the results point to a single DnaB hexamer bound to the DNA. ATP should support the movement of DnaB from its original site of entry to allow other helicase molecules to bind, yet the number of bound DnaBs is limited to one. These findings show that the availability of ssDNA is not the factor controlling the number of DnaB hexamers that can bind at the DnaA box hairpin or at oriC.

Models on the Loading of DnaB—Various models come to mind that may explain these observations. One that can be excluded is the possibility that hydrolysis of ATP bound to DnaA is coordinated with the loading of DnaB at the DnaA box hairpin and that DnaA complexed to ADP is no longer active in loading. First, Masai and Arai described that DnaA bound to either ATP or ADP supported DNA replication with the ssDNA carrying the DnaA box hairpin (37). Our unpublished observations with DnaA46 protein that is defective in ATP binding show that this protein is also active in this DNA replication system.2 The model that the nucleotide-bound form of DnaA controls the loading of DnaB is unlikely.

Several observations suggest that the DnaB-DnaC complex

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might be bound on the 5’ side of the DnaA box hairpin. First, DnaB has a 6-fold preference for the 5’ ssDNA tail over the 3’ ssDNA tail of a synthetic replication fork (38). Since the ssDNA flanking the hairpin and the hairpin itself resemble a synthetic replication fork, this could place the DnaB-DnaC complex to the left of the hairpin stem. The underlying assumption here is that DnaB and not DnaC is primarily responsible for binding to ssDNA in the prepriming complex. Second, a stop site on the 5’ side of the hairpin is seen when the DnaB-DnaC complex is assembled into the prepriming complex with ATP (Fig. 2, lane 8). This primer extension product may reflect the binding of the DnaB-DnaC complex to the left of the hairpin. Because an interaction between DnaA and DnaB is involved in the loading of DnaB from the DnaB-DnaC complex (4, 5), these observations suggest that the ssDNA upstream of the hairpin is the site of initial binding. This model, if correct, predicts that the translocation of DnaB should displace DnaA bound to the hairpin. However, the amount of DnaA bound to the ssDNA when the prepriming complex is formed and isolated with ATP is statistically indistinguishable (Table I). A second prediction is that once the entry site for the DnaB-DnaC complex is no longer occupied by virtue of DnaB movement, additional DnaB-DnaC complexes can bind. This expectation was not met; the stoichiometry of DnaA bound to the ssDNA under conditions that permit DnaB movement is still limited to a single molecule. Third, if the DnaB-DnaC complex is bound on the 5’ side of the DnaA box hairpin, the DNA that maps to the 3’ side of the hairpin base is not explained. The model that the entry site of the DnaB-DnaC complex is at the 5’ side of the DnaA box hairpin is not attractive, because the predictions are not supported by the results. A third possibility is that DnaB remains at its initial site of binding to occlude the binding of additional DnaB molecules while simultaneously tracking on the DNA. This possibility is not favored based on what is known about DnaB. DnaB functions as a helicase by translocation in the 5’-to-3’ direction (39). Fluorescent energy transfer experiments indicate that 20 ± 3 nucleotides of ssDNA bound to a single DnaB protomer pass

![Figure 4](image-url)
through the central cavity of DnaB (40). This evidence does not support two DNA binding sites, the minimum needed for DnaB to remain bound at the site of its initial binding while also tracking on the DNA. These observations strongly suggest that DnaB does not remain bound to one site while threading the ssDNA through the central cavity during DNA unwinding.

Formation of the DnaB-DnaC complex in which DnaB and DnaC are present at a 1:1 molar ratio occurs with ATP or ATPγS (11, 12). As ATPγS is not hydrolyzed, DnaC remains bound to DnaB when the DnaB-DnaC complex assembles into the prepriming complex (5). However, this intermediate is not active because of the inability to release DnaC. If assembly of the prepriming complex is with ATP, DnaB ends up encircling the ssDNA upon release of DnaC. At some point in the loading process, the helicase must assume a conformation in which one of the interfaces between DnaB protomers is open in this ring-shaped protein in order for the helicase to encircle the ssDNA.

A fourth admittedly speculative model is presented here (Fig. 5). In this model based on results from primer extension assays (Fig. 2), the entry site is altered on binding of the first DnaB-DnaC complex so that additional molecules do not bind. The primer extension method measures terminations that reflect the binding of particular proteins in the prepriming complex assembled at the DnaA box hairpin. A prominent extension product due to bound DnaA mapped to the DnaA box (Fig. 2), confirming the results from methylation protection assays (22). Terminations dependent on the DnaB-DnaC complex mapped on either side at the base of the hairpin stem. Because the hairpin structure is stable in vitro as indicated by its sensitivity to DNase I and is destabilized by mutations that disrupt base pairing within the hairpin stem (see Fig. 3 of Ref. 22), the DnaB-DnaC complex apparently first assembles into the prepriming complex with the hairpin structure in predominantly duplex form. Inasmuch as neither DnaB nor DnaC alone or in the DnaB-DnaC complex binds directly to duplex DNA but interacts with ssDNA, the possibility arises that this portion of the hairpin becomes single-stranded upon binding of the DnaB-DnaC complex. Indeed, the stop site on the 3′ side of the hairpin to that in the DnaA box sequence where DnaA is bound is separated by 10 nucleotides. The termination site on the 5′ side (left) of the hairpin stem is 10 nucleotides from the site opposite to where DnaA-dependent termination is mapped.

Cryoelectron microscopy of the DnaB-DnaC complex in comparison to DnaB reveals that DnaC is bound to one end of the toroidal shaped DnaB (43). Each DnaB protomer is in a side-by-side arrangement in the hexamer with each protomer aligned in the same direction. From limited proteolysis, the DnaB protomer is composed of a smaller N-terminal domain linked to a larger C-terminal domain via a hinge region (44). DnaB has been shown to have a unique orientation on a forked DNA molecule with a 5′ ssDNA tail (42). Based on this orientation, the smaller N-terminal domain is aligned upstream to the C-terminal domain when the helicase is translocating on a ssDNA template.

Because the N-terminal domain of DnaB protects DnaC from inactivation by N-ethylmaleimide, one interpretation is that this domain interacts with DnaC (44). Thus, DnaC in the DnaB-DnaC complex is expected nearer the smaller domain of each DnaB protomer of hexameric DnaB. Due to the orientation of DnaB bound to ssDNA, this places DnaC on the 5′ side of DnaB. The cryptic ssDNA binding activity of DnaC may assist in stabilizing the DnaB-DnaC complex at the DnaA box hairpin (45). We speculate that this latent activity of DnaC accounts for the termination at the 5′ side near the base of the hairpin.

Primer Extension—Masai et al. (22) performed primer extension experiments similar to those of Fig. 2 but with modified T7 DNA polymerase (Sequenase). The large fragment of DNA polymerase I was used in Fig. 2. In the cited article, multiple terminations were observed within the DnaA box sequence. These terminations were solely dependent on DnaA bound to the DnaA box hairpin. Our results show a single termination event within the DnaA box sequence. These differences may be due to the different DNA polymerases used for primer extension. Sequenase lacks the proofreading exonuclease of T7 DNA polymerase and consequently has “terminal transferase” activity (46, 47). The multiple terminations observed by Masai et al. (22) may be due to the addition of unpaired nucleotides by this
DNA polymerase. Primer extension products made by the large fragment of DNA polymerase I do not have additional unpaired nucleotides at the 3' end, because this DNA polymerase also possesses its proofreading exonuclease. Another difference was that Masai et al. observed multiple terminations dependent on DnaA on the 3' side at the base of the hairpin, whereas we did not unless the DnaB-DnaA complex was also present. The reason for these differences is not clear but may relate to the propensity of DnaA to self-aggregate.

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