Escherichia coli strains lacking PriA are severely compromised in their ability to repair UV-damaged DNA and to perform homologous recombination. These phenotypes arise because of a lack of PriA-directed replication fork assembly at recombination intermediates such as D-loops. Naturally arising suppressor mutations in dnaC restore strains carrying the priA2::kan null allele to wild-type function. We have cloned one such gene, dnaC810, and overexpressed, purified, and characterized the DnaC810 protein. DnaC810 can support a PriA-independent synthesis of φX174 complementary strand DNA. This can be attributed to its ability, unlike wild-type DnaC, to catalyze a SSB-insensitive general priming reaction with DnaB and DnaG on any SSB-coated single-stranded DNA. Gel mobility shift analysis revealed that DnaC810 could load DnaB directly to SSB-coated single-stranded DNA as well as to D loop DNA. This explains the ability of DnaC810 to bypass the requirement for PriA, PriB, PriC, and DnaT during replication fork assembly at recombination intermediates.

PriA, a 3′ → 5′ DNA helicase (1, 2), was discovered because of its requirement, along with PriB, PriC, DnaT, DnaB, DnaC, and DnaG, for assembly of a primosome on φX174 (φX)3 viral DNA during complementary strand DNA replication in vitro (3, 4). Although the φX-type primosome could provide both the DNA unwinding (via the 5′ → 3′ DNA helicase activity of DnaB (5i) and Okazaki fragment priming (via the primase activity of DnaG (6i) functions at a replication fork (7, 8), these biochemical analyses never revealed a role for either PriA, PriB, PriC, or DnaT during chromosomal DNA replication. The advent of strains in which priA had been disrupted soon changed this (9, 10).

Based on our initial observation that the φX174 priA2::kan strain was constitutively induced for the SOS response (9) and that SOS induction could be suppressed completely by providing a mutant priA allele in trans that encoded a protein that could not act as a DNA helicase but did direct primosome assembly (11), we proposed that PriA-directed replication fork assembly became clear as detailed genetic analyses were performed with the priA null strains.

These subsequent genetic studies showed that in addition to induction of the SOS response, strains null for PriA activity had very complex phenotypes. They were poorly viable (9, 10), filamented extensively (9, 10), and were defective in homologous recombination (13, 14), the repair of both double-stranded breaks (14) and UV-damaged DNA (10, 13, 14), and in the manifestation of both inducible and constitutive stable DNA replication (15). A common theme in the processes involved in the latter four phenotypes was recombination-directed DNA replication. This led to the proposal that PriA functioned by directing the assembly of replication forks at recombination intermediates such as D loops (13, 16).

This proposal has been supported by recent studies. PriA binds D loops with high affinity, whereas it will not bind the corresponding bubble structure (17, 18) and can direct primosome assembly on D loop DNA as well (19). Significantly, PriA can also direct replication fork assembly on double-stranded templates carrying a D loop in a reaction in vitro that also requires PriB, PriC, DnaT, DnaB, DnaC, DnaG, and the DNA polymerase III holoenzyme (Pol III HE) (20).

Strains carrying priA null mutations acquire suppressor mutations rapidly (13, 14). Seventeen independent, naturally arising UVR Rec+ revertants of priA2::kan strains that carry extragenic suppressors have been isolated (13). All of these extragenic suppressors map to the C-terminal half of dnaC. Two of these extragenic suppressors alleles, dnaC809 and dnaC810, arise as a result of GAA → GGA and GAA → GGT nucleotide changes, respectively, at codon 176. This generates a E176G amino acid substitution in each case.

In order to understand the mechanism of suppression of PriA activity, we have cloned the dnaC810 allele and overexpressed, purified, and characterized the DnaC810 protein. This protein is capable of catalyzing a single-stranded DNA-binding protein (SSB)-independent general priming reaction with DnaB and DnaG. Direct examination by gel mobility shift analysis showed that, unlike wild-type DnaC, DnaC810 could transfer DnaB from a DnaB-DnaC810 complex to either SSB-coated single-stranded DNA (ssDNA) or D loop DNA. This gain of function of the mutant enzyme explains its ability to bypass the requirement for PriA, PriB, PriC, and DnaT during replication fork assembly on a template carrying a D loop (20).

MATERIALS AND METHODS

Reagents, Enzymes, and DNAs—Restriction enzymes, Pfu polymerase, and oligonucleotides were from New England BioLabs, Stratagene, and IDT, respectively. φX174 and φ1 DNAs were prepared as described (21). PriA, PriB, PriC, DnaT, DnaB, DnaG, and wild-type DnaC were purified as described by Marians (22). SSB was purified as described by Minden and Marians (7). The DNA polymerase III holoenzyme was

orC and then stalled because of encountering endogenous DNA damage (9, 12). The actual substrate for replication fork assembly became clear as detailed genetic analyses were performed with the priA null strains.

This work was supported by National Institutes of Health Grant GM34557. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

To whom correspondence should be addressed.

The abbreviations used are: φX, φX174; ss(c), single-stranded circular; oligo, oligonucleotide; PAS, primosome assembly site; PCR, polymerase chain reaction; Pol III HE, the Escherichia coli DNA polymerase III holoenzyme; RF, replicative form; ssDNA, single-stranded DNA; SSB, the E. coli single-stranded DNA-binding protein; nt, nucleotide(s).

This paper is available on line at http://www.jbc.org
reconstituted from polymerase III* and β as described by Wu et al. (8).

Construction of Plasmid pET11c-dnaC810—A dnaC810 open reading frame was made by two-step overlapping polymerase chain reaction (PCR) (23). The N-terminal coding region of dnaC810 was PCR-amplified using plasmid pET11c-dnaC as a template and two flanking primers: (i) NdeI 9 primer and (ii) the BamHI primers, which is complementary to the AgeI primer and (ii) the BamHI primer (5′-TTAAGACTCTGATTTTATGACGGTTCTGGCCGACCTGTTA-3′, which carries the NdeI site at the dnaC initiation codon, and (ii) the AgeI primer, 5′-TGGTATTTCCGCCACATGAAATATCCGGTGAGCGTTCC-3′, which carries the designed point mutation (E176G, GAA → GGT). The C-terminal coding region of dnaC810 was also PCR-amplified using plasmid pET11c-dnAC as a template and two different flanking primers: (i) NdeI 9 primer and (ii) the C-terminal coding region of dnaC810 (5′-GTGAATGGATGATATTTCGATTCGGTCTG-3′), which is complementary to the AgeI primer and (ii) the BamHI primers, which carries a BamHI site just downstream of the dnaC coding region. These overlapping N- and C-terminal fragments were gel-purified after PCR and were further PCR-extended and -amplified with the two flanking NdeI and BamHI primers. The gel-purified dnaC810 open reading frame was digested with NdeI and BamHI and ligated with NdeI- and BamHI-digested pET11c plasmid DNA to give pET11c-dnaC810.

Purification of DnaC810—Because of the extreme overproduction, DnaC810 was followed during purification by SDS-polyacrylamide gel electrophoresis. BL21(DE3)pLysS carrying pET11c-dnaC810 was grown in Luria broth (24) containing 0.4% glucose and 500 μg/ml ampicillin to an A600nm of 0.4 and then induced with 1 mM isopropyl-1-thio-β-d-galactoside for 3 h. Cells were chilled, pelleted by centrifugation, and resuspended in 50 mM Tris-HCl (pH 8.4 at 4 °C) and 10% sucrose. The cell suspension (50 ml) was adjusted to 150 mM KCl, 20 mM EDTA, 5 mM dithiothreitol, 0.02% lysozyme, and 0.1% Brij 58 and incubated at 0 °C for 10 min. This suspension was centrifuged at 100,000 × g for 1 h (Sorvall SS65 rotor). The supernatant (fraction 1, 65 ml, 3510 mg of protein) was adjusted to 0.04% polymixin P by dropwise addition of 1% solution. The precipitate was removed by centrifugation at 47,000 × g in a Sorvall SS-34 rotor for 30 min. The supernatant was further subjected to (NH4)2SO4 fractionation (50% saturation) by the addition of solid. The resulting protein pellet was collected by centrifugation at 47,000 × g in a Sorvall SS-34 rotor for 30 min. The protein pellet was resuspended in 8 ml of buffer A (50 mM Tris-HCl (pH 7.5 at 4 °C), 5 mM EDTA, 10% glycerol, 0.01% Brij 58) plus 50 mM NaCl to give fraction 2 (13 ml, 1108 mg of protein). Fraction 2 was dialyzed against 2 liters of buffer B (packed in a 10-ml disposable syringe) that had been equilibrated with buffer A plus 50 mM NaCl. The column was washed with 200 ml of buffer B containing 50 mM NaCl. Fractions (15 ml) of the flow-through and wash that contained protein were pooled to give fraction 3 (81 ml, 363 mg of protein). Fraction 3 was loaded directly onto a 35-ml SP-Sepharose FF column (formed in a 60-ml disposable syringe) that had been equilibrated with buffer A plus 50 mM NaCl. The column was washed with 200 ml of buffer A plus 50 mM NaCl, and protein was then eluted with a linear gradient of 50–300 mM NaCl for 2 h using 50 mM Tris base, 40 mM boric acid, 1 mM EDTA, 2.5 mM MgOAc, and 200 mM NaCl for 12 h and then loaded onto a 100-ml DEAE-cellulose column (formed in a 60-ml disposable syringe) that had been equilibrated with buffer A plus 50 mM NaCl. The column was washed with 200 ml of buffer B containing 50 mM NaCl, and protein was then eluted with a linear gradient of 0–400 mM (NH4)2SO4 in buffer A plus 200 mM NaCl. Fractions (15 ml) of the flow-through and wash that contained protein were pooled to give fraction 4 (81 ml, 363 mg of protein). Fraction 4 was then loaded directly onto a 6-ml hydroxyapatite column (packed in a 10-ml disposable syringe) that had been equilibrated previously with buffer A plus 200 mM NaCl. The column was washed with 12 ml of equilibration buffer, and protein was eluted with a 60-ml linear gradient of 0–400 mM (NH4)2SO4 in buffer A plus 200 mM NaCl. DnaC810 eluted at 150 mM (NH4)2SO4 to give fraction 5 (23 ml, 16.5 mg of protein). Fraction 5 was loaded directly onto a 125-ml Superdex-200 fast protein liquid chromatography column that had been equilibrated with buffer A plus 50 mM NaCl. The column was washed with 200 ml of buffer A plus 50 mM NaCl, and protein was then eluted with a linear gradient of 50–300 mM NaCl for 12 h using 50 mM Tris-HCl (pH 7.8 at 25 °C), 4 mM MgOAc, 10 mM NaOAc, and 1 mM EDTA as the electrophoresis buffer. A constant voltage of 6 V/cm was applied for 4.5 h or as otherwise indicated at 4 °C with constant recirculation of the buffer. Following gel electrophoresis, the gel was either dried and autoradiographed or subjected to ECL-Western blot analysis.

The conditions for ECL-Western blot were as described by Ng and Marians (32).

Results

Purification of the DnaC810 Protein—DnaC810 was purified from BL21(DE3)pLysS cells carrying plasmid pET11c-dnaC810. A soluble lysate was prepared from iso-propyl-1-thio-β-d-galactosanox-anoside-induced cells by treatment with lysozyme followed by ultracentrifugation. DnaC810 was purified by fractionation of the lysate with (NH4)2SO4 (50% saturation), followed by chromatography on DEAE-cellulose, SP-Sepharose, hydroxyapatite, Superdex-200, and phosphocellulose. The final fraction was 99% homogeneous for DnaC810 (Fig. 1).

DnaC810 Supports SSB-insensitive General Priming—The activity of the DnaC810 suppressor protein was confirmed with that of wild-type DnaC during dX ss(c) → Replicative Form (RF) DNA Replication and General Priming—The standard reaction buffer was 50 mM HEPES-KOH (pH 8.0 at 30 °C), 10 mM MgOAc, 10 mM dithiothreitol, 0.01 mg/ml rifampicin, and 0.2 mg/ml bovine serum albumin. Both assays were stopped by the addition of 100 μl of 0.2 M NaPP. After the addition of 100 μl of 1 mg/ml heat-denatured salmon sperm DNA as carrier, trichloroacetic acid-insoluble radioactivity was determined. For the dX174 ss(c) → RF assay, reaction mixtures (25 μl) contained the standard buffer, dX174 ss(c) DNA (220 pmol as nt), 75 ng of SSB, 1 mM ATP, 100 μM CTP, GTP, and UTP, 40 μM dNTPs including [3H]dTTTP (150 cpm/μmol), 12 mM DnAβ, 100 mM DnAg, 10 mM DnAγ, 15 mM PrfA, 15 mM PrfB, 15 mM PrfC, the DNA Pol III HE (280 ng), and 1 mM MgOAc. The reaction mixtures were incubated at 30 °C for 10 min. For the general priming assay, reaction mixtures (25 μl) contained the standard buffer, dX174 ss(c) DNA (330 pmol as nt), 1 mM ATP, 200 μM CTP, GTP, and UTP, 40 μM dNTPs including [3H]dTTTP (150 cpm/μmol), 12 mM DnAβ, 50 mM DnAg, the DNA Pol III HE (280 ng), and either DnAc or DnaC810 as indicated. The reaction mixture was incubated at 30 °C for 15 min.

RESULTS

Purification of the DnaC810 Protein—DnaC810 was purified from BL21(DE3)pLysS cells carrying plasmid pET11c-dnaC810. A soluble lysate was prepared from iso-propyl-1-thio-β-d-galactosanox-anoside-induced cells by treatment with lysozyme followed by ultracentrifugation. DnaC810 was purified by fractionation of the lysate with (NH4)2SO4 (50% saturation), followed by chromatography on DEAE-cellulose, SP-Sepharose, hydroxyapatite, Superdex-200, and phosphocellulose. The final fraction was 99% homogeneous for DnaC810 (Fig. 1).

DnaC810 Supports SSB-insensitive General Priming—The activity of the DnaC810 suppressor protein was confirmed with that of wild-type DnaC during dX ss(c) → RF DNA replication. This reaction utilizes PriA, PriB, PriC, DnaB, DnaC, DnaG, and the Pol III HE to convert SSB-coated X ss(c) DNA to the double-stranded, nicked circular form (22). In this reaction, PriA directs the assembly of a primosome at a specific region on the DNA, the primosome assembly site (PAS) (25). The primosome can then synthesize a primer that is elongated by the Pol III HE. DnaC810 was slightly more active than the wild-type DnaC protein in this assay (Fig. 2A). This indicates...
that the E176G amino acid substitution present in DnaC810 does not inactivate the protein in any significant way.

Genetic studies (13) predicted that DnaC810 could direct $\phi X$-type primosome assembly in the absence of PriA. Thus, we examined the activity of the mutant protein in the $\phi X$ ss(c) → RF DNA replication reaction in the absence of PriA (Fig. 2B). The activity of the mutant protein in this reaction in the absence of PriA was 40% of what it was in the presence of PriA (Fig. 2, compare A and B), whereas wild-type DnaC was completely inactive in the absence of PriA (Fig. 2B). Thus, DnaC810 had apparently gained the ability to bypass the requirement for PriA in primosome assembly. The observed difference in DnaC810 activity in the presence and absence of PriA most likely reflects the fact that in the presence of PriA, two reactions are being measured: the bypass reaction and $\phi X$-type primosome assembly at the PAS. In the absence of PriA, only the bypass reaction is operative. Thus, we do not think the apparent reduced activity of DnaC810 in the absence of PriA actually reflects a lowered efficiency in the bypass reaction.

PriA-directed primosome assembly also requires PriB, PriC, and DnaT (3, 4). In order to determine whether DnaC810 bypassed the requirement for these proteins as well, we assessed the ability of DnaC and DnaC810 to support $\phi X$ ss(c) → RF DNA replication in the presence of only SSB, DnaB, DnaG, and the Pol III HE (Fig. 3A). DnaC810 was still active in this assay; the wild-type protein, however, could not support the reaction. With the exception of the presence of SSB and the PAS on the DNA, the replication reaction manifest in the experiment shown in Fig. 3A resembled the general priming reaction (26). During general priming, DnaB and DnaG can cooperate to synthesize a primer on any protein-free ssDNA.

DnaC stimulates this reaction significantly by forming a complex with DnaB in solution that leads to more efficient transfer of DnaB to the DNA. We therefore investigated the requirement for a PAS sequence and the effect of SSB on the reaction.

A comparison of DNA synthesis supported by either $\phi X$ or $\phi 1$ ss(c) DNAs in the presence of DnaB, DnaG, DnaC810, SSB, and the Pol III HE showed that the two templates were equally active (Fig. 3B). $\phi 1$ viral DNA does not carry a PAS. Complementary strand synthesis during the phage life cycle is primed by RNA polymerase (27, 28). Thus, the DnaC810-directed replication reaction did not require a PAS, thereby distinguishing it from the PriA-directed reaction.

The DnaC810-directed replication reaction was similar in requirements to the general priming reaction. We therefore compared the activity of the wild-type and mutant proteins in that reaction directly. As noted above for the $\phi X$ ss(c) → RF replication reaction, these two proteins displayed little difference in activity (Fig. 4A). However, the differential effect of adding enough SSB to coat the ssDNA was dramatic. The reaction supported by wild-type DnaC was inhibited by SSB, whereas the reaction supported by DnaC810 was stimulated
when the ssDNA was coated with SSB (Fig. 4B). We therefore conclude that DnaC810 can bypass the requirement for PriA, PriB, PriC, and DnaT in loading DnaB to SSB-coated DNA.

In the Presence of DnaB, DnaC810 Supports Stable Complex Formation with SSB-coated ssDNA—The primary role of DnaC is to load DnaB to ssDNA (29). In the cell, it does so in cooperation with other proteins. Afterward, DnaC is not found in the complex of replication proteins on the DNA. In order to understand the properties that allow DnaC810 to bypass PriA function, we used gel mobility shift analysis to examine its ability to form stable complexes with various DNA substrates.

Formation of the DnaB-DnaC complex in solution requires ATP but not ATP hydrolysis (30). Formation of active replication complexes such as the dX-type primosome does require ATP hydrolysis (29). Although DnaB is a DNA helicase (5) and ssDNA-dependent NTPase (31), the ATP hydrolysis during loading is catalyzed by DnaC (30). Thus, binding to two oligos, 21 and 42 nt long, was assessed in both the presence and absence of ATP and SSB (Fig. 5).

Several complexes with different Rf values were formed in the presence of DnaB, DnaC, DnaC810, and SSB. In no case was any complex detected with either DnaB, DnaC, or DnaC810 individually. In the absence of SSB, the combination of DnaB and DnaC gave complex i with the 21-mer (Fig. 5, A and B, lane 3) and complex iv with the 42-mer (Fig. 5, C and D, lane 3). Formation of this complex was unaffected by the presence or absence of ATP. In the presence of SSB with the 21-mer substrate, no complex was observed in the absence of ATP (Fig. 5A, lane 9), whereas a new species, complex ii was observed in the presence of ATP (Fig. 5B, lane 9). No complexes were observed with the 42-mer substrate in the presence of SSB under any condition (Fig. 5, C and D, lane 9).

The results with DnaC810 were quite different. In the absence of SSB, the combination of DnaB and DnaC810 yielded a new complex, iii, on the 21-mer that was, like complex i, unaffected by the presence or absence of ATP but had a distinct, reduced mobility (Figs. 5, A and B, lane 4). Likewise, with the 42-mer substrate, new complexes (vi and vili), were observed that had a reduced mobility compared with complex iv (Fig. 5,
C and D, lane 4). In this case, formation of complexes vi and vii was clearly stimulated by the presence of ATP. In the presence of SSB, two complexes (ii and iii) were formed with the 21-mer substrate (Fig. 5, A and B, lane 10). The yield of these complexes seemed relatively unaffected by the presence or absence of ATP. With the 42-mer substrate, ATP was required to observe three complexes (v, vi, and vii) (Fig. 5, C and D, lane 10).

Complex i was, based on its unusual U-shaped appearance, reminiscent of the type of complex we have observed previously with DnaB and DnaC on a 304-nt-long ssDNA containing a PAS (32). ECL-Western analysis demonstrated that only DnaB was present in the complex on the PAS DNA. Thus, we believe that complex i contains only DnaB. Complexes i and iv are presumably identical in their protein content and differ only in the size of the DNA present. The same can be said for complexes iii and vii and complexes ii and v. ECL-Western analysis of the complexes formed on D loop DNA in the presence of DnaB, DnaC810, and SSB, described under “DnaC810 Can Load DnaB to D Loop DNA in the Presence of SSB” (Figs. 10 and 11), suggests that complexes iii and vii contain both DnaC810 and DnaB and that complexes ii and v contain only DnaB. The composition of complex vi is less certain, but we suspect that it does not contain DnaC810 and that either the stoichiometry of DnaB to DNA or the conformation of DnaB on the DNA is different compared with complex v.

Thus, DnaC810 appears to be able to transfer DnaB to SSB-coated ssDNA. In addition, in the presence of DnaC810, DnaB, and ATP a second larger complex can be detected on SSB-coated DNA that may contain both DnaB and DnaC810. The identical mobility of complex iii in the presence and absence of SSB argues that SSB has been displaced from the DNA. The difference between complexes i and iii suggests that DnaC810 is either slower than DnaC to release DnaB to the DNA or requires an interaction with another protein to do so.

Wild-type DnaC could load DnaB to SSB-coated 21-mer but not to the 42-mer. This is most likely a result of the stability of the SSB on the DNA. The observed difference in complex formation with DnaB and DnaC is a function of the size of the oligomer. The most likely aspect of the binding reaction that can be affected by oligomer size is the number of SSB molecules bound to the DNA. The simplest way to think of this is that one SSB tetramer is bound to the 21-mer, whereas two SSB tetramers are bound to the 42-mer. This allows the SSB tetramers bound to the 42-mer to interact. The energy gain from this.

**Fig. 5.** DnaC810- and DnaB-dependent complex formation on SSB-coated ssDNA. Standard gel mobility shift reaction mixtures containing either no ATP (A and C) or 8 μM ATP (B and D), DnaB (16 nM), and either DnaC or DnaC810 (32 nM; given here and in all other figure legends as hexamer equivalent to facilitate comparisons of stoichiometry with DnaB) as indicated were incubated and analyzed as described under “Materials and Methods.” Autoradiograms of the dried gels are shown. The reaction mixtures contained either the 32P-labeled 21-mer oligo (1 nM; A and B) or 32P-labeled 42-mer oligo (1 nM; C and D) as the DNA substrate. SSB (4 nM) was present in the reaction mixtures analyzed in lanes 7–12 of A–D.
interaction would make it more difficult to displace SSB from the 42-mer than from the 21-mer. Alternatively, only one protomer in the SSB tetramer may be bound to the 21-mer, whereas two protomers of the tetramer may be bound to the 42-mer. This scenario would also make it more difficult for SSB to be displaced from the 42-mer than from the 21-mer.

Lehrn et al. (33) demonstrated that it is actually DnaC that first binds to ssDNA while transferring DnaB via a cryptic DNA-binding activity that becomes activated when bound to DnaB. The relative affinities of DnaC and SSB for the DNA thus become the crucial factor in determining whether DnaC can transfer DnaB to the DNA. Because of the potential for greater cooperative interactions on the larger DNA, less energy would be required for DnaC to displace SSB from the 21-mer than from the 42-mer. Because DnaC810- and DnaB-catalyzed general priming is SSB-insensitive (Fig. 4B), the E176D mutation in DnaC810 apparently increases its affinity for ssDNA in the presence of DnaB so that it maintains the ability to displace SSB no matter the size of the DNA. The observation that DnaB- and DnaC810-dependent complex formation on the 42-mer, but not on the 21-mer, requires ATP is also consistent with this explanation.

DnaC810 Can Load DnaB to D Loop DNA in the Presence of SSB—We have shown that PriA can direct the assembly of a replication fork on a template DNA carrying a D loop in vitro. As with the Δx ss(c) → RF DNA replication reaction described above, DnaC810 could bypass the requirement for PriA, PriB, PriC, and DnaT and direct the assembly of a replication fork at D loop DNA (20). This implies that DnaC810 can load DnaB directly to SSB-coated D loop DNA. We therefore investigated this directly by gel mobility shift analysis. We first assessed the ability of DnaC810 to bind a bubble formed from two 82-nt-long oligos. This DNA substrate has, going 5′ → 3′ on the top strand, 23 nt of duplex, a 42-nt long nonhomologous bubble, and then 17 nt of duplex (18). In the absence of SSB, two complexes could be observed in the presence of DnaC810 and DnaB that were unaffected by either the presence or absence of ATP (Fig. 6A, lanes 1–3). These were similar in relative mobility to the complexes (iii and iv) observed on the oligo substrates and were expected based on the predicted ssDNA present in the nonhomologous region of the bubble substrate. Interestingly, wild-type DnaC was unable to form any similar complexes with DnaB, although it could on the oligo substrates.

Under standard reaction conditions in the presence of SSB, no binding to the bubble DNA could be detected in the presence of DnaB and either DnaC or DnaC810 either in the presence or absence of ATP (Figs. 6). This was surprising, because, as demonstrated above, DnaC810 could form a complex with DnaB on, and transfer DnaB to, SSB-coated ssDNA (Fig. 5). We investigated whether this was simply a result of a reduced affinity of the DnaC proteins for the ssDNA in the bubble. However, no binding was observed in either the presence or absence of ATP even when the concentration of DnaC and DnaC810 was increased to very high levels (data not shown).

Our previous studies have shown that PriA binds D loop DNA with high affinity but does not bind to bubble DNA at all, although it does not require a free end to bind DNA (18). Through the use of various forms of bubble DNA substrates, we concluded that this was because, although the nonhomologous region was clearly in single-stranded form (based on nuclease sensitivity), the single strands were still twisted about each other as in a tangle. This is also probably why we did not find complex formation on either the bubble substrate in the presence of DnaB and DnaC or on the SSB-coated bubble substrate in the presence of DnaB and DnaC810. Complex formation in the presence of DnaB and DnaC810 on the bubble in the absence of SSB reinforces the suggestion that the E176D mutation has increased the affinity of DnaC810 for DNA.

Binding of DnaB in the presence of either DnaC or DnaC810 to D loop DNA was investigated next. The D loop DNA substrate was formed by annealing a 42-nt-long invading strand that was homologous to the top strand of the bubble (18). In the presence of ATP, neither DnaB, DnaC, nor DnaC810 could form a stable complex on D loop DNA either in the presence or absence of SSB (data not shown).

In the absence of SSB, the combination of DnaB and DnaC gave little evidence of complex formation in the absence of ATP (Fig. 7A, lanes 2–7) and formed primarily two complexes, b and c, in the presence of ATP (Fig. 7B, lanes 3–7). Complex c exhibited the distinctive U-shaped appearance also shown by complexes i and iv that we have attributed to the presence of only DnaB on the DNA (32). Increasing the DnaC concentration significantly did little to change the distribution between complexes b and c. The combination of DnaB and DnaC810 also yielded complexes b and c. In addition, there were trace amounts of a slowly moving complex (a) (Fig. 7A, lanes 8–12). In the presence of ATP, complexes b and c diminished in abundance as the concentration of DnaC810 was increased, and complex a became very prominent (Fig. 7B, lanes 9–13).

Both complexes b and a always appeared as doublets. The resolution of the dimer pair varied from gel to gel. In general, the slower moving complex in the pair was generally the most populated, but we could not associate a difference in distribution with a particular set of conditions in the reaction mixture. We assume that these dimer pairs relate either to a difference in stoichiometry of the proteins present or to a difference in conformation of one or both of the proteins present.

In the presence of SSB, no complexes were evident when DnaB and DnaC were included in the reaction mixture either in the absence (Fig. 8A, lanes 2–7) or presence of ATP (Fig. 8B, lanes 2–7). This was consistent with the inability of this combination of proteins to form a complex on SSB-coated ssDNA that was larger than a 21-mer. In contrast, the combination of DnaB and DnaC810 formed primarily complex b at low concentrations of DnaC810 and complex a at high concentrations of...
DnaC810. Small amounts of another complex (c9) were also evident as a smeared thickening of the amount of labeled material on the sides of the lane just above the position of the SSB-coated D loop DNA (Fig. 8B, lanes 9–12). Complex c9 is presumably related to complex c, but we label it differently because of the presence of SSB in the reaction mixture.

The identical nature of complexes a and b formed in the absence (Fig. 7) and presence (Fig. 8) of SSB was confirmed when these reactions were analyzed side-by-side on the same gel (Fig. 9) as described below.

Thus, the combination of DnaB and DnaC810 was clearly able to form at least two complexes on SSB-coated D loop DNA, whereas the combination of DnaB and DnaC could not. This was similar to the results obtained when SSB-coated ssDNA was used as the substrate. In that case, it appeared as if DnaC810 was capable of displacing SSB from the DNA. To assess if this were the case with the D loop DNA as well, we compared on the same gel the mobility of complexes formed in the presence and absence of SSB (Fig. 9).

In the presence of DnaB and DnaC810 and in the absence of ATP, complex b was the major product (Fig. 9A). Much higher concentrations of DnaC810 were required to form substantial amounts of complex b in the presence of SSB than in its absence. Nevertheless, it is clear that the mobility of complex b formed in either the presence or absence of SSB was identical. This strongly suggests that SSB has been displaced. Note the large shift in mobility of the D loop when SSB is bound (compare lane 7 with lane 1 in Fig. 9A). As shown below, complex b contains DnaB. If only one 300-kDa hexamer of this protein were bound to the D loop in addition to SSB, there should be a significant change in mobility of the complex compared with when SSB is absent.

Similar results were obtained in the presence of ATP where complex a is the major species formed (Fig. 9B). Here as well, the mobility of complex a in the presence and absence of SSB was identical. This complex contains both DnaB and DnaC810 (see below). Thus, one would expect the difference in mobility if SSB were also bound to the D loop compared with its absence to be even greater than that for complex b. Thus, we conclude that during formation of both complexes a and b, SSB is displaced from the DNA by the action of the DnaC810 protein.

ECL-Western blotting was used to determine the distribution of DnaB and DnaC in complexes a and b. These analyses were confounded somewhat by the fact that under the conditions of electrophoresis used for gel mobility shift analysis, the free DnaB and DnaC migrated into the gel. However, the data were clear enough to allow conclusions to be made.

The mobility of free DnaB was very close to that of complex...
b (Fig. 10), so that under standard conditions, when DnaB was in vast excess over the DNA substrate, the smearing of the anti-DnaB antibody-reactive material obscured the position of complex b (Fig. 10, lanes 10–12). To circumvent this problem, D loop binding assays were performed with concentrations of the D loop substrate that were in excess of the concentration of DnaB (Fig. 10). This is why little complex formation is evident on the autoradiogram (Fig. 10, lanes 4–6). However, under these conditions, the presence of DnaB in complex b could be clearly observed in the Western blot (Fig. 10, lanes 10–12). On the other hand, the presence of DnaB in complex a could be clearly observed under standard conditions as the concentration of DnaC810 in the reaction mixture was increased (Fig. 10, lanes 4–6 and 10–12).

The situation was reversed in assessing the presence of DnaC810 in these two complexes. Free DnaC810 barely moved into the gel, obscuring the position of complex a (Fig. 11). However, as the concentration of DnaC810 was increased in the reaction mixture, a band could be observed at the trailing edge of the smear of free DnaC810 in the Western blot coincident with the position of complex a that was not present in each case when DnaB was omitted from the reaction mixture so that no complex would form (Fig. 11, lanes 2–7 and 9–14). No DnaC810 signal was observed in the position of either complex b or c. We therefore conclude that complex a contains both DnaB and DnaC810, whereas complex b contains only DnaB.

We have no direct evidence for the composition of complex c. Thus, any conclusion made as to its identity must be considered speculative. Based on the relative mobilities of complexes a, b, and c, we suspect that it contains only DnaB. This is consistent with our previous conclusion with respect to the nature of these U-shaped bands (32).

We make no claim that the Western analyses can be used to determine relative stoichiometries of DnaB and DnaC810 in the complexes. Although polyclonal antibodies were used, major antigenic determinants can be buried and inaccessible in one complex but not another. Rather, these data can only help to suggest which proteins are present in which complex.

These data indicate that whereas both DnaC and DnaC810 can load DnaB to a D loop in the absence of ATP to give complex b, only DnaC810 can do so in the presence of SSB. In addition, in both the absence and presence of SSB, DnaC810 can form a...
complex with DnaB on the D loop DNA (complex a). Both DnaC810-directed loading of DnaB and formation of the DnaB-
DnaC810 complex result in the displacement of SSB. This
ability of DnaC810 to load DnaB onto SSB-coated DNA, which
represents a gain of function over the properties of the wild
type, accounts for its ability to bypass the function of PriA,
PriB, PriC, and DnaT during replication fork formation at a D
loop (20).

**DISCUSSION**

Current models suggest that the replication forks formed at
oriC often stall and collapse either at DNA damage such as
nicks and noncoding lesions or by colliding with protein road-
blocks on the DNA (12, 13, 16, 34, 35). Correction of the lesion
requires both repair and recombination proteins and continued
viability of the cell requires replication fork restart. Restart is
likely to occur in the majority of instances through recombin-
ation-directed DNA replication, where a recombination inter-
mediate such as a D loop serves to provide both the primer for
leading-strand synthesis (the 3'-OH of the invading strand)
and the site at which a replisome assembles.

Because strains carrying a disruption in priA were constitu-
tively induced for the SOS response (9) and shown to be defec-
tive in homologous recombination (13, 14), the repair of both
double-strand breaks (14) and UV-damaged DNA (10, 13, 14)
and inducible stable DNA replication (15) (which requires both
recA and recBCD (36)), it was proposed that PriA was involved in
directing replication fork assembly at recombination inter-
mediates (13, 16). This has been supported by the demonstra-
tion that PriA binds specifically to D loop DNA (17, 18) and can
direct the assembly of both a dX-type primosome (19), and a
replication fork (20) at a D loop in vitro.

In this report, we have investigated the properties of the
DnaC810 protein, encoded by dnaC810, a naturally arising,
eXogenous suppressor of all of the phenotypes of the priA2::kan
mutation (13). Genetic analysis has indicated that dnaC810
bypasses PriA function. This suggested that DnaC810 could
also direct replication fork assembly at a D loop. This proved to
be the case (20). Here we show that the underlying gain of
function that allows DnaC810 to bypass PriA activity is an
ability to load DnaB, the replication fork DNA helicase (7, 37,
38), to SSB-coated DNA.

Although DnaB can bind ssDNA to act in vitro as a ssDNA-
dependent NTPase (31), a DNA helicase (5), and a landing pad
for DnaG during general priming (26), the protein is, in gen-
eral, prevented from binding indiscriminately to ssDNA in vivo
by its association with DnaC in a tight stoichiometric complex
(36). While in this complex, DnaC can transfer DnaB to exposed
ssDNA but not to SSB-coated ssDNA. This transfer is mediated
by a cryptic DNA-binding site in DnaC that is activated by
binding of DnaB (33). While in the form of a DnaB-DnaC
complex, DnaB cannot bind ssDNA (33). Under normal circum-
stances, any exposed ssDNA will be coated with SSB. Access of
DnaB to the DNA is therefore effectively limited to targeting by
other mechanisms that involve additional DNA replication pro-
teins to generate a SSB-free region of ssDNA for DnaB binding.
The two mechanisms that operate in vivo to do this are DnaA-
directed initiation of DNA replication at oriC (37) and PriA-
directed assembly of a primosome at recombination intermedi-
ates such as a D loop (20).

DnaC810 was able to load DnaB to both SSB-coated ssDNA
and D loop DNA. We have previously shown by DNA footprint-
ing studies that during assembly of the dX-type primosome,
DnaB is loaded to the displaced strand in the D loop (19). This
is consistent with the ability of DnaC810 to form a replication
fork at a D loop in the presence of SSB, DnaB, and the Pol III
HE, where the invading strand is used as the leading strand
primer (20). This also places DnaB on the displaced strand that
becomes the lagging strand template. Thus, we believe that
DnaC810 is also loading DnaB to the displaced strand of the D
loop. Because this is the only region of the D loop that can bind
SSB, this would also explain the inability of the wild-type
DnaC to load DnaB to the D loop in the presence of SSB.

The most likely explanation for the gain of function exhibited
by DnaC810 is that the affinity for ssDNA of its cryptic DNA
binding activity has been increased. This would account for its
ability to compete with SSB for binding to the ssDNA. In
addition to having gained the ability to transfer DnaB to SSB-
coated DNA, DnaC810 can also be found on the same DNA as
DnaB. Although there is no direct evidence that DnaC810 and
DnaB are together in a complex on the DNA, we believe that
this is the case.

DnaC810 alone will not bind to ssDNA, either in the pres-
ence or absence of SSB. Thus, in order for both DnaB and
DnaC810 to be on the same DNA, a DnaB-DnaC810 complex
would have to have interacted with the DNA and then rear-
arranged in such a manner that both proteins were left on
the DNA. This seems unlikely, because free DnaB can be found on
the DNA, transferred from the DnaB-DnaC810 complex, but
free DnaC810 (or DnaC, for that matter) is never found bound
to the DNA. In addition, DnaC is not present on the DNA after
formation of the dX-type primosome (32, 39).

The nature of this DnaB-DnaC810 complex on the DNA,
however, is unclear. The argument advanced above would sug-
gest that it is bound in a stable fashion as a result of the DnaC

---

**FIG. 11.** DnaC810 is present in complex a formed on SSB-coated D loop DNA by the action of DnaC810 and
DnaB. Standard gel mobility shift reaction mixtures containing 1 nm 32P-labeled D-loop DNA, 8 μM ATP, 4 nM SSB, 16 nM
DnaB, and the indicated concentrations of DnaC810 (in hexamer equivalents) were incubated and analyzed as described un-
der “Materials and Methods.” An autoradiogram of the wet gel is shown (lanes 1–6). ECL-Western blot analysis using
anti-DnaC antisem was performed on nitrocellulose paper after transfer from the wet gel (lanes 7–12).
DNA-binding site and not that of DnaB. Presumably, transfer of DnaB to the DNA ejects DnaC from the complex. This could be seen to be consistent with the requirement for higher concentrations of DnaC810 to form this complex compared with the concentration required to transfer DnaB directly to the DNA.

Based on our biochemical analyses described here, can we account for the properties of the dnaC810 mutation in vivo? The answer is mixed. The ability to bypass PriA function and rescue all of the phenotypes of the priA2::kan mutation can clearly be attributed to the gain of function that allows DnaB directly to the DNA ejects DnaC from the complex. This could easily change the apparent biochemical requirements for replication fork restart.

This suggests that PriB and PriC have redundant functions in a pathway that involves PriA. Although the biochemical properties of DnaC810 would lead one to expect that the dnaC810 mutation should be able to completely reversion of the phenotype of DnaC810 to load DnaB to SSB-coated D loop DNA. This allows replication fork restart downstream of recombination intermediates required for continued cell viability. Biochemically, neither PriA, PriB, PriC, nor DnaT are required for this bypass step. Thus, it is interesting that genetically, priA2::kan, dnaC810 triple mutant strain is still as viable as wild-type. PriA2::kan, dnaC810 mutation should be able to completely suppress the multiple phenotypes associated with the priA2::kan mutation and was even less viable and grew more slowly (40).

In contrast to what was predicted based on the pathway of assembly of the φX-type primosome (32), neither priB nor priC disruptions exhibit any of the phenotypes associated with the priA2::kan mutation (40). Remarkably, however, the priB, priC double mutant did display the multiple phenotypes associated with the priA2::kan mutation and was even less viable and grew more slowly (40).

REFERENCES
1. Lee, M. S., and Marinos, K. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8345–8349
2. Lasken, R. S., and Kornberg, A. (1986) J. Biol. Chem. 261, 5512–5518
3. Wickner, S., and Hurwitz, J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4120–4124
4. Schekman, R., Weiner, J., Weiner, A., and Kornberg, A. (1975) J. Biol. Chem. 250, 5859–5865
5. LeBowitz, J. H., and McMacken, R. (1986) J. Biol. Chem. 261, 4738–4748
6. Bouche, J.-P., Zechel, K., and Kornberg A. (1975) J. Biol. Chem. 250, 5995–6001
7. Minden, J. S., and Marinos K. J. (1985) J. Biol. Chem. 260, 9316–9325
8. Wu, C. A., Zechner, E. L., and Marinos, K. J. (1992) J. Biol. Chem. 267, 4030–4044
9. Nurse, P., Zavitz, K. H., and Marinos, K. J. (1991) J. Bacteriol. 173, 6686–6693
10. Lee E. H., and Kornberg, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3029–3032
11. Zavitz, K. H., and Marinos, K. J. (1992) J. Biol. Chem. 267, 6933–6940
12. Zavitz, K. H., and Marinos, K. J. (1991) Mol. Microbiol. 5, 2869–2873
13. Sandler, S. J., Sawra, H. S., and Clark, A. J. (1996) Genetics 143, 5–13
14. Kogoma, T., Cadwell, G. W., Barnard, K. G., and Asai, T. (1996) J. Bacteriol. 178, 1258–1264
15. Masai, H., Asai, T., Kubota, Y., Arai, K.-I., and Kogoma, T. (1994) EMBO J. 13, 5328–5345
16. Kogoma, T. (1996) Cell 85, 3523–3545
17. McGlynn P., Al-Deib, A. A., Liu, J., Marinos, K. J., and Lloyd, R. G. (1997) J. Mol. Biol. 270, 212–221
18. Nurse, P., Liu, J., and Marinos K. J. (1999) J. Biol. Chem. 274, 25026–25032
19. Liu J., and Marinos, K. J. (1999) J. Biol. Chem. 274, 25033–25041
20. Liu, J., Xu, L., Sandler, S. J., and Marinos, K. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3552–3555
21. Model, F., and Zinder, N. (1974) J. Mol. Biol. 83, 231–251
22. Marinos, K. J. (1995) Methods Enzymol. 262, 507–521
23. Morton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 61–68
24. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Marinos, K. J. (1984) CRC Crit. Rev. Biochem. 17, 153–215
26. Arai, K.-I., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 76, 4308–4312
27. Wickner, W., Brutlag, D., Schekman, R., and Kornberg, A. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 965–969
28. Wickner, R., Wright, M., Wickner, S., and Hurwitz, J. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3223–3227
29. Marinos, K. J. (1992) Annu. Rev. Biochem. 61, 673–719
30. Wahl, E., Lasken, R. S., and Kornberg, A. (1989) J. Biol. Chem. 264, 2463–2468
31. Wickner, S., Wright, M., and Hurwitz, J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 783–787
32. Ng, J. Y., and Marinos, K. J. (1996) J. Biol. Chem. 271, 15642–15648
33. Cox, M. M. (1998) EMBO J. 17, 5386–5391
34. Kuzminov, A. (1995) Mol. Microbiol. 16, 373–384
35. LeBowitz, J. H., and McMacken, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1154–1159
36. Kogoma, T. (1997) Microbiol. Mol. Biol. Rev. 61, 212–238
37. Kaguni, J. M., and Kornberg A. (1984) Cell 38, 183–190
38. Mok, M., and Marinos, K. J. (1997) J. Biol. Chem. 272, 15649–15655
39. Ng, J. Y., and Marinos, K. J. (1996) J. Biol. Chem. 271, 15649–15655
40. Sandler, S. J., Marinos, K. J., Zavitz K. H., Coutu, J., Parent, M. A., and Clark, A. J. (1999) Mol. Microbiol. 34, 91–101
Purification and Characterization of DnaC810, a Primosomal Protein Capable of Bypassing PriA Function
Liewei Xu and Kenneth J. Marians

J. Biol. Chem. 2000, 275:8196-8205.
doi: 10.1074/jbc.275.11.8196

Access the most updated version of this article at http://www.jbc.org/content/275/11/8196

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 39 references, 25 of which can be accessed free at http://www.jbc.org/content/275/11/8196.full.html#ref-list-1