Fine Balance in the Regulation of DnaB Helicase by DnaC Protein in Replication in *Escherichia coli*  

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The DnaC protein of *Escherichia coli* chromosome from its origin (oriC) has been reconstituted in *vitro* using purified components (1). This process begins with DnaA protein binding to four 9-bp "DnaA boxes" in oriC and melting the three 13-bp iterons at one edge of the origin (2, 3). In solution, the hexameric DnaB forms a complex with six molecules of DnaC (4–6). DnaB is delivered from this complex to the melted region of oriC where it acts as the helicase at the replication forks (7–9). Upon delivery of DnaB, the DnaC protein is released from the protein-DNA complex. 2 Coating of single strands by SSB, relief of torsional stress by gyrase, and priming by primase set the stage for replication of the template by DNA polymerase III holoenzyme.  

Formation of the *E. coli* primosome also requires DnaC in addition to PriA, PriB, PriC, DnaB, DnaT, SSB, primase, and DNA polymerase III holoenzyme (10). The primosome is needed for the conversion of the viral single-stranded DNA of bacteriophage φX174 to the duplex replicative form (10) and for the replication of the lagging strand of ColE1 plasmids (11). In these replication systems, the role of DnaC is to form a tight complex with DnaB from which it delivers the helicase to its site of action on the DNA template. Unlike the other proteins required for the formation of the primosome or for chromosome initiation, even a slight excess of DnaC profoundly inhibits replication.  

The genes encoding these various proteins required for chromosomal replication have been classified as being involved in either initiation or elongation or in both. Among these genes, dnaC has some alleles defective in initiation (12–15) and others defective in elongation (12, 13, 15). Whereas DnaC is required in initiation to deliver DnaB to the replication fork, its role in elongation has remained unclear. The present study was undertaken to explore the basis for the extraordinary sensitivity of the *in vitro* initiation system to a small excess of DnaC and to determine whether such an effect is seen *in vivo*. We also explored the relationship between the *in vitro* inhibition of replication and the mutant alleles of dnaC that affect the elongation stage of DNA replication.  

**EXPERIMENTAL PROCEDURES**  

**Bacterial Strains**—*E. coli* K-12 strain MC1061 (araD139, Δ(aara, leu)7697, ΔlacX74, galU, galK, strA) was used as a host for the plasmid derivatives of pING1 (16). *E. coli* K-12 strains PC1 (F−, X−, leuB6, thyA47, rpsL353, galK2, thyA705, rpsL21, xyl-5, mtl-1, argE2, thi-1, dnaC3) were obtained from Dr. B. Bachmann (Yale University). *E. coli* K-12 strains SY562 (asm, thy, rif, strep, dnaC2, rnrT101::kan, recA56) and P7 (dnaC7, leu, thy, strep) are laboratory stocks.  

**Enzymes and Plasmid Constructions**—Restriction enzymes were obtained from either New England Biolabs or Bethesda Research Laboratories. Restriction enzyme digests and plasmid constructions were according to standard procedures (17). DNA fragments with overhanging termini were converted to blunt ends using T4 DNA polymerase (17). Purified replication proteins were prepared as previously described (1). Plasmid pBSolvC (3) contains a 678-bp HindII-PstI fragment spanning oriC (bp -189 to +499) cloned into the pBluescript vector (Stratagene, Inc.). The pLING plasmid was obtained through the courtesy of the INGENIE Corp. (16). The plasmid, pJK169, containing the dnaC gene was as described (18). The plasmid pINGK consists of the kanamycin resistance gene from pUC4K (Pharmacia LKB Biotechnology Inc.) on a 1.2-kilobase PstI fragment cloned into the pBluescript vector spanning oriC (bp -189 to +499) cloned into the pBluescript vector.  

The abbreviations used are: bp, base pair(s); Tricine, N-tris(hydroxymethyl)methylglycine; BSA, bovine serum albumin; SSB, E. coli single-stranded DNA-binding protein; NEM, N-ethylmaleimide; Fl, Form I; FlI, Form II.  

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A. Kornberg, unpublished observations from many studies in this laboratory.
into the EcoRI site of pINC<sub>so</sub> placing it downstream of dnaC. The pINC<sub>so</sub> plasmid contains the translation initiation sequences of bacteriophage T7 gene 10 linked to the pINC<sub>so</sub> plasmid contains the translation initiation sequences of then cloned into the SmuI site of pINGK. The resulting dnaC coding region was

to the sulfhydryl-specific reagent, NEM (Table I). Treatment of DnaC with 

TABLE I

NEM inactivates the DnaC replication and inhibitory activities

| DNA synthesis | pmol |
|---------------|------|
| A. DnaC sample | 1    |
| None          |      |
| Untreated     | 141  |
| Mock-treated  | 167  |
| NEM-treated   | 7    |
| B. Additional DnaC | |
| None          | 126  |
| Mock-treated  | 108  |
| 1 pmol        | 30   |
| 2 pmol        | 11   |
| NEM-treated   | 96   |

Given that the functional form of DnaC is in a (DnaB<sub>so</sub>-DnaC<sub>so</sub>) complex, inhibition of replication by DnaC may depend on the amount of DnaB present, that is on the ratio of DnaC to DnaB. In the previous experiments where 1 pmol of DnaC was optimal, DnaB was present at 1.25 pmol per reaction, a ratio of 0.8 pmol of DnaC to 1.0 pmol of DnaB. To address the importance of the ratio of DnaC to DnaB, replication was assayed in reactions containing 3.0 pmol of DnaC and increasing amounts of DnaB (Fig. 2). As expected with the normal 1.25 pmol of DnaB, 3.0 pmol of DnaC completely inhibited replication (2.4 pmol of DnaC: 1.0 pmol of DnaB). Additional DnaB “stimulated” replication (relieved inhibition) to saturation at 2.75 pmol of DnaB which point the ratio was nearly 1:1 (0.8 pmol of DnaC to 1.0 pmol of DnaB). This relief of inhibition by additional DnaB also implies that the inhibition is a property of free DnaC protein with which it is known to interact (4–6).

Excess DnaC Inhibits the Function of the Prepriming Complex, Not Its Formation—DnaC protein might inhibit replication in one of several ways: by preventing the formation of prepriming complexes, by destabilizing formed complexes,
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The standard prepriming complex reaction was carried out with excess DnaC present (3.0 pmol/20-μl reaction). After the incubation at 37 °C for 30 min, the reactions were placed on ice and more DnaB was added, as indicated. Replication was assayed by addition of the elongation components and incubation at 18 °C for 15 min.

**Fig. 2. DnaB overcomes inhibition of the prepriming complex by DnaC.** The standard prepriming complex reaction was carried out with excess DnaC present (3.0 pmol/20-μl reaction). After the incubation at 37 °C for 30 min, the reactions were placed on ice and more DnaB was added, as indicated. Unwinding was performed at 28 °C for 6 min and topoisomers resolved by agarose gel electrophoresis as described under “Experimental Procedures.” The migration of nicked circular (FI), supercoiled (FI*), and highly underwound (FI***) DNA and the unwinding intermediate is indicated at the right.

**Table II**

**Removal of DnaC by gel filtration relieves inhibition**

The prepriming complex reaction was scaled-up to 5-fold and carried out in the presence of the normal amount of DnaC (1 pmol/20 μl) or with a high, inhibitory level of DnaC (4.5 pmol/20 μl). Protein-DNA complexes were isolated by gel filtration, and 20-μl portions were assayed for replication activity at 18 °C for 15 min after addition of the elongation components. An additional 4.25 pmol of DnaB/20 μl was added to the reaction before the elongation components, where indicated (+ DnaB). The isolated prepriming complexes were supplemented with 2 pmol of DnaC per 20 μl prior to the addition of the elongation components (+ DnaC).

| DnaC level during prepriming | Fraction (± supplement) | DNA synthesis (pmol) |
|-----------------------------|-------------------------|----------------------|
| Normal                      | Load                    | 107                  |
|                             | Void                    | 127                  |
|                             | Void + DnaC             | 8                    |
|                             | Load                    | 0                    |
|                             | Load + DnaB             | 107                  |
|                             | Void                    | 108                  |
|                             | Void + DnaC             | 2                    |

or by inhibiting the function of formed complexes. To distinguish among these possibilities, a prepriming reaction containing excess, inhibitory DnaC protein was performed. Protein-DNA complexes were isolated by gel filtration chromatography and assayed for replication activity (Table II). As before, excess DnaC completely inhibited replication. Supplementing this fraction with additional DnaB revealed that active prepriming complexes were present. Moreover, simply removing the excess DnaC by gel filtration allowed recovery of all of the prepriming complex replication activity.

The full recovery of replication activity after gel filtration implies that the excess, inhibitory DnaC was not stably associated with the prepriming complex. If prepriming complexes and free DnaB-DnaC complexes were in dynamic equilibrium, addition of excess DnaC might destabilize the prepriming complexes by mass action. Yet there were as many active complexes isolated when DnaC was in excess as when it was present at low levels. This equivalence suggests that excess DnaC does not act by destabilizing prepriming complexes already formed. Prepriming complexes isolated from either the high-level DnaC and the normal-level DnaC reactions were equally susceptible to inhibition by additional DnaC (Table II). These experiments imply that excess DnaC affects replication by inhibiting the function but not the formation of the prepriming complex.

**DnaC Inhibits the DnaB Helicase Present in Prepriming Complexes**—Prepriming complexes contain several activities. Among them is the ability of DnaB protein at the forks to unwind the template (in the presence of gyrase and SSB) to generate a highly unwound structure called Form I* (FI*) (8, 9). This FI* can be resolved from supercoiled template (FI), nicked circular template (FII), and other topoisomers by agarose gel electrophoresis in the presence of ethidium bromide. By uncoupling unwinding from priming and DNA synthesis (as in the FI* assay), the susceptibility of unwinding to additional DnaC can be independently assessed. The addition of more DnaC to a reaction mixture already containing 1 pmol inhibited FI* formation (Fig. 3). At a total of 3 pmol of DnaC, FI* formation was greatly inhibited and completely so at 4 pmol of DnaC.

The topoisomer that migrates more slowly than FI is an unwinding intermediate with a superhelicity between that of FI and FI* (data not shown). At levels of DnaC that completely inhibit FI* formation and replication, this unwinding intermediate was still present. Eventually at higher levels of DnaC, its formation was inhibited. These findings suggest that small excesses of DnaC act by slowing the DnaB helicase, thereby preventing FI* formation but still allowing formation of the early intermediate. Higher amounts of DnaC inhibited the helicase sufficiently that the intermediates were not formed either.

**Overproduction of DnaC Inhibits Cell Growth**—Strains bearing plasmids which allow the controlled expression of dnapC exhibited a slowed growth rate when DnaC was overproduced. A plasmid derivative of pING1 (16) was constructed which contained the kanamycin resistance gene, pINGK. Open reading frames were cloned into pINGK under control of the ara promoter including the dnuC gene with its natural translation initiation signals (pINC_{SSD}) and the dnapC gene with the bacteriophage T7 gene 10 translation initiation signals (pINC_{SSD}). Strains harboring pINC_{SSD} induced with 1% arabinose for 2 h overexpressed DnaC, producing a visible band on a Coomassie-stained polyacrylamide gel and ~1000-fold more DnaC replication activity compared with control strains.
harvesting plNGK (data not shown). Strains bearing plINCnat overexpressed less DnaC; no protein was visibly overproduced on a Comassie-stained gel although induction of DnaC was seen on Western blots (data not shown). In the absence of induction, strains with pINGK, pINCnat, or pINCsd all grew equally well with the same doubling time (39 min, data not shown). After induction, the pINGK-containing strains continued to grow at the same rate (Fig. 4). Strains overexpressing DnaC continued growing rapidly for a period after which cell growth slowed considerably. The length of lag before the downshift of growth rate correlated with the level of DnaC produced. The pINCsd strain shifted after 60 min, whereas the pINCnat strain shifted after 120 min.

Growth Inhibition by DnaC Is Suppressed by DnaB—Inasmuch as additional DnaB protein alleviated the inhibition of replication by excess DnaC in vitro, we explored the possibility that DnaB might also relieve the growth inhibition. Two additional plNGK derivatives were constructed: one that contained the dnaB gene with its natural Shine-Dalgarno sequence (pINB) and one derived from pINCnat with the dnaB reading frame downstream of that for dnaC (pINC). Western blots confirmed that the strain harboring pINB expressed DnaB and that with pINCB expressed both DnaB and DnaC when induced (data not shown). The ability of these strains to grow after induction was determined by measuring their plating efficiencies with and without arabinose (Table III). In agreement with the growth rate studies, induction of pINGK-bearing strains had no effect on its ability to grow. In addition, both pINCnat and pINCsd strains failed to plate when induced with 1% arabinose. Strains bearing pINGK grew equally well with or without induction implying that the overproduction of a replication protein is not, in general, inhibitory to cell growth. When the pINCsd strains were induced with 1% arabinose, they plated 260-fold better than without DnaB DnaC (pINCnat) though still not as well as strains overexpressing only DnaB.

At lower levels of induction, similar results were obtained. Consistent with the different times of downshift in growth rate between pINCnat and pINCsd strains, pINCnat strains plated more efficiently than did pINCsd ones. Also, co-overproduction of DnaB rescued the low plating efficiency of DnaC-overproducing strains. In all instances of measurable plating, the strains were viable and continued to grow as observed by increased colony size after a second overnight incubation.

**TABLE III**

| Plasmid          | Overexpressed protein | Plating efficiency (+Ara/-Ara) |
|------------------|-----------------------|--------------------------------|
| pINC           | DnaC: high            | 0.31 0.001                     |
| pINCsd         | DnaC: low             | <0.002 <0.002                   |
| pINCnat        | DnaC: high            | 0.90 0.98                      |
| pINCssD        | DnaC and DnaB: both   | 0.61 0.26                      |

**TABLE IV**

The dnaC* gene fails to complement elongation-defective dnaCts alleles

The strains were transformed with the indicated plasmid, and transformants were selected by plating with ampicillin (50 μg/ml) at the permissive temperature. A single colony was grown overnight in L broth with ampicillin at the permissive temperature, diluted, plated with selection, then incubated overnight at 42 °C or at the permissive temperature, as indicated. The same plating procedure was employed with the strains lacking plasmids except that ampicillin was omitted. The strains are all temperature-sensitive (ts) mutants defective in initiation or elongation, as indicated; the wild-type gene is designated dnaC*. All plasmids are derivatives of pBR322; pBR322 was used in some instances as the parental, control plasmid.

| Strain          | dnaCts allele | Plasmid | Plating efficiency (42 °C/30 °C) |
|-----------------|---------------|---------|----------------------------------|
| SY562           | Initiation    | None    | <10^-4                           |
| SY562(pK169)    | Initiation    | dnaC*   | 0.95                             |
| CT28-3b(pBR322) | Initiation    | Control | <10^-4                           |
| CT28-3b(pK169)  | Initiation    | dnaC*   | 1.0*                             |
| PC1(pBR322)     | Elongation    | Control | <10^-5                           |
| PC1(pK169)      | Elongation    | dnaC*   | <10^-4                           |
| PC7             | Elongation    | None    | <10^-5                           |
| PC7(pK169)      | Elongation    | dnaC*   | <10^-6                           |

*The permissive temperature for CT28-3b is 25 °C rather than 30 °C.

**DISCUSSION**

The initiation of chromosomal replication requires the DnaC protein both in vivo (12-15) and in vitro (1). Prior to
its action at oriC, DnaC forms a complex in solution with DnaB (4-6). From this complex DnaC performs its role of delivering DnaB to a template. This action of DnaC is manifest in a general priming system, containing only DnaB, primase, and single-stranded DNA, where DnaC increases the affinity of DnaB for the single-stranded DNA (24) but is not essential (25). In contrast, for the oriC-specific initiation system, DnaC is absolutely essential and larger amounts of DnaB do not overcome the requirement for DnaC. These findings argue that DnaC has some other function in this specific reaction, such as a direct interaction with DnaA in loading DnaB (26). Upon delivering DnaB to form a prepriming complex, DnaC is released and is not needed for the elongation stage in vitro. Genetic experiments, however, indicate that DnaC can affect elongation (12, 13, 15). Moreover, slight excesses of DnaC added during elongation inhibited replication in vitro (Fig. 1).

In studies of the nature of this inhibition, we observed that stable, isolable prepriming complexes are formed in the presence of inhibitory levels of DnaC (Table II), implying that DnaC neither destroys prepriming complexes nor prevents their formation. Excess DnaC was found to slow the DnaB helicase at the replication fork. Because the rate of replication is limited in vitro by the rate of unwinding by DnaB (9), slowing or stalling of this unwinding is sufficient to account for the inhibition of replication. Additional effects of DnaC on the activity of primase to synthesize primers cannot be ruled out and might further inhibit replication.

When free in solution, DnaB exhibits an ATPase activity which DnaC completely inhibits (6, 24, 25) when they form a complex. On model helicase substrates, increasing amounts of DnaC first stimulate then inhibit the DnaB helicase (24). After delivering DnaB to the prepriming complex, DnaC leaves, allowing DnaB to display its ATPase and helicase activities. Since DnaC is not found in isolated prepriming complexes, it must have a lower affinity for DnaB in this context than in solution. The extreme sensitivity of the system to DnaC is unusual and is not well understood. Presumably, some conformation of DnaB at the replication fork is especially susceptible to binding and inhibition by additional DnaC.

Initiation of replication at the bacteriophage λ origin proceeds in a manner similar to that at oriC, except that the λ and AP proteins act as the respective analogues of DnaA and DnaC. DnaB forms a complex in solution with λ protein even in the presence of DnaC (27). Like DnaC, λ protein completely inhibits the ATPase of DnaB. In directing DnaB to the replication fork, the AP protein has been shown to interact with λ protein (28) as proposed for DnaC interacting with DnaA in delivering DnaB (26). Furthermore, an excess of the AP protein can inhibit DNA synthesis at a rolling-circle replication fork.5

Although similarities exist between the action of the DnaC and AP proteins, they are not alike in all respects. For λ, several heat shock proteins including DnaK are required to activate the DnaB protein after its delivery. The DnaK protein removes the AP protein from the complex at ori (29) to form stable complexes with the AP protein (30), and may thereby prevent its reassociation with DnaB. In the oriC system, the addition of DnaK had no effect on the inhibition by DnaC.4

The existence of elongation mutants of dnaC implies that the mutant gene product is present at the replication fork. If these mutant proteins reflect the in vitro action of DnaC at the fork, they may have a higher affinity for DnaB at the nonpermissive temperature and thereby bind and stall the helicase. The dominance of these elongation alleles over the wild-type gene lends support to this proposal. If the temperature sensitivity were a consequence of temperature-induced overexpression of wild-type DnaC, then protein synthesis would be necessary to see this elongation defect. The elongation mutants used in this report display their temperature-sensitive phenotype in the presence of chloramphenicol (12, 13, 15) implying that new protein synthesis is not required for this effect. These alleles might also be expected to affect initiation by preventing early unwinding, but initiation defects cannot be seen in vivo when elongation defects are also present. Although DnaC can clearly have negative effects on elongation, there is no evidence that it has a positive role other than for initiation.

The dnaC and dnaT genes are in the same operon. Had these elongation alleles been classified as dnaC-defective when they were actually in dnaT, the wild-type dnaC gene would then not be able to complement them. This simple possibility was ruled out by demonstrating that a plasmid which complements a dnaTts strain fails to complement the elongation alleles of dnaC.4 It remains possible, however, that these elongation-defective strains bear an additional mutation which would not be complemented by the dnaC+ gene.

It seems paradoxical that DnaC inhibits the function of a complex whose formation it promotes. This dual action may reflect a feedback control mechanism. When conditions are unfavorable for rapid DNA synthesis, the cell may utilize this control to slow the replication fork and lengthen the replication (C) period. In addition, the precise ratio of DnaC and DnaB necessary for optimal replication suggests that the cell has a mechanism to regulate the coordinate expression of these two genes located in different operons. Little is known about the expression of dnaC and dnaB as a function of cell cycle or growth conditions. There may also be factors in the cell that attenuate the inhibitory actions of DnaC.

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