Interaction of Escherichia coli dnaB and dnaC(D) Gene Products In Vitro

(DNA replication/DNA-stimulated ATPase)

SUE WICKNER* AND JERARD HURWITZ†

* Viral Carcinogenesis Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20014; and † Department of Developmental Biology and Cancer, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York, 10461

Contributed by Jerard Hurwitz, December 24, 1974

ABSTRACT Purified E. coli dnaB and dnaC(D) gene products interact physically and functionally in vitro. This interaction was demonstrated as follows: (a) A complex of dnaB and dnaC(D) gene products was isolated by gel filtration; ATP specifically was required for isolation of the complex. (b) The DNA-dependent ribonucleoside triphosphatase activity associated with dnaB gene product was inhibited by dnaC(D) gene product. (c) The dnaC(D) gene product was protected from inactivation by N-ethylmaleimide by the combination of dnaB gene product and ATP; this protection required ATP specifically.

Some of the proteins involved in Escherichia coli DNA replication have been defined by temperature-sensitive mutants that do not replicate their DNA at elevated temperatures. The genes that determine these proteins have been designated dnaA (1), dnaB (2), dnaC(D) (3), dnaE (4), dnaF (3), dnaII (5), dnaH (6), dnaI (7), dnaJ (8), lig (9), and polA (10). Mutants in dnaB contain thermolabile DNA polymerase II (11, 12); lig mutants contain thermolabile DNA ligase (9, 13, 14); and polA mutants are temperature-sensitive for growth when they contain thermolabile 5' to 3' exonuclease (exo- nuclease VI) (15). Of the other proteins known to be involved in E. coli DNA replication, dnaB, C(D), and G have been isolated using in vitro complementation assays in which protein preparations from wild-type cells stimulate φX174 DNA-dependent DNA synthesis in heat-inactivated crude extracts of dna temperature-sensitive cells (16–19). Each of these dna gene products has been isolated from both wild-type and temperature-sensitive strains and the thermostability of the temperature-sensitive protein has been demonstrated in the φX174 DNA-dependent complementation system. As yet, no enzymatic activity has been found associated with dnaG or dnaC(D) gene products. The dnaC and dnaD gene products have been shown to be identical in vitro (16) and in vivo (20); the protein will be referred to here as dnaC(D) gene product. Purified dnaB gene product contains ribonucleoside triphosphatase activity that is stimulated by single-stranded DNA. These triphosphatase activities and dnaB complementing activity copurify over the last 20-fold of a 40,000-fold purification procedure (21). The purpose of this report is to describe the physical and functional interaction of two of these proteins involved in replication, dnaB and dnaC(D) gene products.

MATERIALS AND METHODS

Materials, Reagents, and Methods, unless otherwise indicated, were as described (16–19).

Isolation of dnaB and dnaC(D) Gene Products. dnaB and dnaC(D) gene products were assayed by in vitro complementation in the φX174 DNA-synthesizing system. The assay conditions, definitions of units, and purification procedures were described previously for dnaB (19, 21) and dnaC(D) gene products (19, 16). The dnaB gene product contained no detectable dnaC(D) complementing activity (<5 × 10⁻⁴ U/0.4 U of dnaB), and dnaC(D) gene product contained no detectable dnaB activity [<5 × 10⁻⁴ U/0.5 U of dnaC(D)]. The addition of dnaB (0.12 U) to the dnaC(D) complementation assay system with dnaC(D) (0.015 U) did not affect the dnaC(D) complementing activity; the addition of dnaC(D) (0.15 U) to the dnaB complementation assay system with dnaB (0.012 U) did not affect the dnaB complementing activity.

Assay for ATPase. Reaction mixtures (0.03 ml) contained 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.4 mM MgCl₂, 0.05 mg/ml of bovine serum albumin, 0.2 mM [γ³²P]ATP (50 cpm/μmol), and protein fractions as indicated. After 30 min at 30°C P, was determined by the method of Conway and Lipmann (22). One unit (U) of ATPase catalyzed the production of 1 μmol of ³²P; as the above conditions.

RESULTS

Physical Association of dnaB and dnaC(D) Gene Products. The dnaC(D) gene product was detected (as measured by stimulation in the φX174 DNA-dependent complementation assay) physically associated with the dnaB gene product (also measured by the φX174 DNA-dependent complementation system) when the two purified proteins were mixed and subjected to gel filtration through BioGel A-5m agarose in the presence of ATP (Fig. 1A). Incubation of dnaC(D) with dnaB gene product prior to gel filtration was not essential and did not result in an increased amount of dnaC(D) associated with dnaB. Fig. 1B shows that in the presence of ATP and in the absence of dnaB gene product, dnaC(D) eluted as expected for a protein with a molecular weight of about 25,000 (16). Fig. 1C shows that dnaB gene product in the presence of ATP and absence of dnaC(D) gene product eluted as expected for a protein with a molecular weight of about 250,000 (18). It eluted broadly, suggesting, as shown before (21), that dnaB gene product can exist in multiple forms that are active in the
Biochemistry: Wickner and Hurwitz

φX174 DNA-dependent complementation assay. Since the elution profile of dnaB gene product was not significantly affected by its interaction with dnaC(D) gene product, probably only one or a few dnaC(D) molecules are associated with one native dnaB molecule.

When dnaB and dnaC(D) gene products were mixed in the absence of ATP and filtered through a column in the absence of ATP, the two proteins were not associated with each other (Fig. 1D). Furthermore, most of the dnaB activity was excluded from the column; the nature of this aggregation has not been studied, nor has it been demonstrated that it was catalyzed exclusively by the dnaC(D) gene product. In the absence of ATP, dnaB and dnaC(D) gene products alone eluted as they had in the presence of ATP (results similar to those shown in Fig. 1B and C). Fig. 1E shows that dnaB and dnaC(D) activities were not associated with each other if they were incubated together for 30 min at 30°C in the presence of ATP and then filtered through the agarose column in the absence of ATP. Thus, ATP is required for detecting the physical complex of dnaB and dnaC(D) gene products. Under conditions as shown in Fig. 1A but with 0.1, 0.5, 50, and 500 μM ATP, 0.17, 33, 48, and 52% of the dnaC(D) activity, respectively, was associated with the dnaB gene product. The nucleotide requirement was specific for ATP. Under the conditions described in Fig. 1A, no dnaC(D) activity was detected associated with dnaB gene product when ATP was replaced with 10 μM dATP, dGTP, dTTP, UTP, CTP, GTP, βγ-methylene-ATP, or ADP (<5% of that associated with dnaB gene product in the presence of ATP).

The fate of ATP in this reaction is unknown. No detectable AMP was produced by dnaB, dnaC(D), or the combination of gene products. No detectable [γ-32P]ATP, [α-32P]ATP, or [3H]ATP was bound by dnaB, dnaC(D), or the combination, as measured by Millipore binding, but more sensitive means of measuring weak binding have not been used as yet. Attempts to determine if a small amount of ATP were hydrolyzed by the interaction of dnaB with dnaC(D) gene product were complicated by the ribonucleoside triphosphatase activity associated with dnaB (21), which, as shown below, dnaC(D) gene product inhibits.

The interaction of dnaB and dnaC(D) gene products depended on the ratio of these two proteins. In the experiment shown in Fig. 1A, half of the dnaC(D) activity recovered was associated with dnaB. With half the amount of dnaC(D) gene product and the same amount of dnaB gene product, all of the dnaC(D) activity was associated with the dnaB activity (Fig. 1F).

Gel filtration of dnaB, dnaC(D), and ATP was affected by the addition of φX174 DNA. Under conditions as described in Fig. 1A but with 6 nmol of φX174 DNA in the reaction, about 40% of the dnaB activity was excluded from the column and 60% eluted at its expected partially included position. About 17% of the dnaC(D) activity was excluded, 30% eluted along with dnaB gene product, and 53% was fully included. It was not clear whether dnaB was aggregated, associated with DNA directly, or associated with dnaC(D) gene product and thus associated indirectly with DNA.

§ Under conditions described in footnote †, at pH 7.5, neither dnaB nor dnaC(D) activity was in the excluded volume when the two proteins were mixed with 6 nmol of φX174 DNA and subjected to gel filtration. Part of the dnaC(D) activity was associated with the dnaB gene product and part was free.

Fig. 1. BioGel A-5m agarose gel filtration of dnaB, dnaC(D), and the combination of gene products. (A) A reaction mixture (0.075 ml) containing dnaB (1.2 U), dnaC(D) (1.5 U), 0.5 mM ATP, 1.0 mM MgCl₂, 0.05 mg/ml of bovine serum albumin, 30 mM potassium phosphate (pH 6.0), and 10 mM dithiothreitol was applied to a 0.5 × 20-cm column of BioGel A-5m agarose equilibrated with 0.5 mM ATP, 1.0 mM MgCl₂, 0.05 mg/ml of bovine serum albumin, 30 mM potassium phosphate (pH 6.0), and 10 mM dithiothreitol at room temperature. The column was developed with the same buffer at room temperature, and 0.1 ml fractions were collected in tubes on ice containing 1 nmol of ATP. Fractions were assayed as described in Materials and Methods for dnaB (●) and dnaC(D) (○) complementing activities. (B) A reaction mixture minus dnaB gene product was filtered through an A-5m agarose column as described in part A. (C) A reaction mixture minus dnaC(D) gene product was filtered as described in part A. (D) A reaction mixture minus ATP was filtered as described in part A but with ATP omitted from the column buffer. (E) A reaction mixture containing dnaB (1.2 U), dnaC(D) (1.5 U), 0.5 mM ATP, 1.0 mM MgCl₂, 30 mM potassium phosphate (pH 6.0), 10 mM dithiothreitol, and 0.05 mg/ml of bovine serum albumin was incubated 30 min at 30°C. The reaction was then filtered as described in part A but with ATP omitted from the column buffer. (F) A reaction mixture as described in part A containing dnaB (1.2 U) and dnaC(D) (0.75 U) was filtered as described in part A.
Fig. 2. Association of ATPase with dnaB and inhibition of ATPase activity with dnaC(D) gene product. (A) dnaB gene product was sedimented through a 5.2-ml, 15-35% glycerol gradient containing 0.2 M KCl, 20 mM potassium phosphate (pH 7.5), 1 mM EDTA, and 1 mM dithiotreitol for 9 hr at 50,000 rpm in a Spinco SW 50.1 rotor. Twenty-five fractions were collected and assayed for dnaB complementing activity (C), ATPase activity in the absence of DNA (O), ATPase in the presence of 2 nmol of φX174 DNA (●), ATPase activity by 10 μl of each fraction in the absence of DNA in the presence of 0.2 U of dnaC(D) gene product (△), and ATPase activity by 10 μl of each fraction in the presence of 2 nmol of φX174 DNA and 0.2 U of dnaC(D) (▲). (B) dnaC(D) gene product was sedimented through a 5.2-ml, 15-35% glycerol gradient as described above for 27 hr at 60,000 rpm in a Spinco SW 65 rotor. 26 fractions were collected and assayed for dnaC(D) complementing activity (●) and inhibition of dnaB ATPase activity (0.1 U of dnaB) by 2 μl of each fraction (O).

Inhibition of dnaB Ribonucleoside Triphosphatase Activity by dnaC(D) Gene Product. The dnaB gene product has associated with it a ribonucleoside triphosphatase activity that is stimulated by single-stranded DNA but not by double-stranded DNA or RNA (21). The products of the reaction are P$_1$ and NDP. Table 1 shows that in the absence of DNA, dnaB ATPase activity was inhibited by dnaC(D) gene product. GTPase activity was also inhibited, but 10 times higher concentrations of dnaC(D) were required; similarly, UTP hydrolysis by dnaB was inhibited by high concentrations of dnaC(D) gene product. In the presence of φX174 single-stranded DNA, dnaC(D) gene product only slightly inhibited dnaB ATPase or GTPase; similar results were obtained with fd DNA.

The following experiment demonstrated that the inhibition of the ATPase associated with dnaB was due to the action of dnaC(D) gene product on dnaB gene product. Fig. 2A shows that ATPase that cosedimented through a glycerol gradient with dnaB activity was inhibited by dnaC(D) gene product. The dnaB complementing activity and ATPase activity were previously shown to copurify over the last 20-fold of an extensive purification to apparent homogeneity (21).

Fig. 2B shows that dnaC(D) complementing activity cosedimented through a glycerol gradient with the inhibition of dnaB ATPase. In addition, inhibition of ATPase activity and dnaC(D) complementing activity were inactivated by heat at the same rate (50% in 3 min at 55°C). Also, treatment of the dnaC(D) gene product preparation with N-ethylmaleimide resulted in inactivation of both dnaC(D) complementing activity [as previously shown (16)] and inhibition of dnaB ATPase activity.

The inhibition by dnaC(D) of dnaB ATPase activity occurred rapidly. Fig. 3 shows that addition of dnaC(D) gene product to reactions in which dnaB gene product was catalyzing the hydrolysis of ATP immediately prevented further hydrolysis. This is consistent with the data shown above, that the physical interaction of dnaB and dnaC(D) gene products occurred rapidly.

Protection of dnaC(D) Gene Product from N-Ethylmaleimide Inactivation by dnaB Gene Product. As mentioned above, the complementing activity of dnaC(D) gene product is inhibitory.

![Fig. 3. Rapid inhibition of dnaB ATPase by dnaC(D) gene product. ATPase reaction mixtures were as described in Materials and Methods. After the indicated times of incubation at 25°C, [γ-$^32$P]ATP production was measured catalyzed by dnaB gene product (0.05 U) alone (●); by dnaB (0.05 U) with dnaC(D) (0.2 U) added at 0 time (△); or by dnaB (0.05 U) with dnaC(D) (0.2 U) added 8 min after the start of the incubation (○).](image)

<table>
<thead>
<tr>
<th>Additions</th>
<th>ATP hydrolysis (nmol of $^32$P/30 min)</th>
<th>GTP hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>− dnaB</td>
<td>1.13</td>
<td>0.96</td>
</tr>
<tr>
<td>dnaC(D) (0.20 U)</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>dnaB + dnaC(D) (0.03 U)</td>
<td>0.74</td>
<td>0.94</td>
</tr>
<tr>
<td>(0.10 U)</td>
<td>0.24</td>
<td>0.90</td>
</tr>
<tr>
<td>(0.30 U)</td>
<td>0.04</td>
<td>0.48</td>
</tr>
<tr>
<td>+ φX174 DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dnaB</td>
<td>2.03</td>
<td>2.11</td>
</tr>
<tr>
<td>dnaC(D) (0.45 U)</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>dnaB + dnaC(D) (0.15 U)</td>
<td>1.61</td>
<td>2.20</td>
</tr>
<tr>
<td>(0.45 U)</td>
<td>1.45</td>
<td>1.40</td>
</tr>
</tbody>
</table>

Reaction mixtures were as described in Materials and Methods with 0.06 U of dnaB gene product, 0.5 nmol of φX174 viral DNA, [γ-$^32$P]ATP (130 cpm/pmol), or [γ-$^32$P]GTP (250 cpm/pmol), where indicated, and dnaC(D) gene product as indicated.
Table 2. Protection of dnaC(D) gene product from 
N-ethylmaleimide inactivation by dnaB gene product

<table>
<thead>
<tr>
<th>Additions</th>
<th>Treatment</th>
<th>dnaC(D) complementing activity (pmol/20 min at 30°)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaC(D)</td>
<td>Dithiothreitol + N-ethylmaleimide</td>
<td>39.2</td>
<td>100</td>
</tr>
<tr>
<td>dnaC(D) + ATP</td>
<td>N-ethylmaleimide</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>dnaC(D) + dnaB</td>
<td>“</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>dnaC(D) + dnaB + ATP</td>
<td>“</td>
<td>2.5</td>
<td>6.4</td>
</tr>
<tr>
<td>dnaC(D) + dnaB + ATP + dATP, dUTP, dGTP, dCTP, αβ-methylene-ATP, or βγ-methylene-ATP</td>
<td>“</td>
<td>33.5</td>
<td>86</td>
</tr>
</tbody>
</table>

Mixtures (0.02 ml) contained dnaC(D) (0.2 U), 30 mM potassium phosphate (pH 6.5), 0.5 mM dithiothreitol, 1 mM MgCl2, 0.05 mg/ml of bovine serum albumin, and 0.5 mM ATP or other nucleotides and dnaB (0.3 U) where indicated. They were incubated with 10 mM N-ethylmaleimide or the combination of 10 mM N-ethylmaleimide and 50 mM dithiothreitol as indicated. After 20 min at 25°, mixtures containing N-ethylmaleimide were adjusted to 50 mM dithiothreitol. Samples (5 μl) were then assayed for dnaC(D) complementing activity as described in Materials and Methods.

Activated by N-ethylmaleimide. This inactivation was prevented by dnaB gene product and ATP (Table 2). Neither of these components alone protected dnaC(D) complementing activity nor could other nucleoside triphosphates be substituted for ATP. The extent of protection of dnaC(D) from inactivation by N-ethylmaleimide depended on the ratio of dnaB to dnaC(D) gene product and also on the concentration of ATP. At higher ratios of dnaB to dnaC(D) gene product, higher concentrations of ATP were required to protect dnaC(D) from inactivation, possibly because of hydrolysis of ATP by the excess dnaB; at low ratios of dnaB to dnaC(D) gene product there was only partial protection of dnaC(D) from N-ethylmaleimide inactivation, and the protection required lower concentrations of ATP. The protection of dnaC(D) against N-ethylmaleimide inactivation by the dnaB gene product was influenced by the presence of DNA. The addition of φX174 or fd DNA increased the inactivation of dnaC(D) by N-ethylmaleimide in the presence of dnaB and ATP; DNA alone or with ATP did not protect dnaC(D) from inactivation.

The addition of N-ethylmaleimide to reactions used for the measurement of ATPase activity (containing dnaB and dnaC(D) gene products and ATP as described in Materials and Methods) did not reverse the inhibition of dnaB ATPase by dnaC(D). This is consistent with the observation that the combination of dnaB and ATP protect dnaC(D) from N-ethylmaleimide inactivation. In these experiments the rate of ATP hydrolysis by dnaB (0.06 U) was 1.6 nmol in 30 min at 30° in the presence or absence of 10 mM N-ethylmaleimide (under conditions as described in Materials and Methods with 1 mM dithiothreitol). The addition of dnaC(D) (0.07 U) in the absence of N-ethylmaleimide inhibited hydrolysis 83%; the addition of dnaC(D) (0.07 U) treated with N-ethylmaleimide prior to its addition to the ATPase assay mixture inhibited hydrolysis 6%. The addition of N-ethylmaleimide (10 mM final concentration) after the start of ATPase reactions containing dnaB and dnaC(D) gene products had no effect on the inhibition of ATP hydrolysis catalyzed by dnaC(D). The dnaB ATPase activity after a 30 min incubation was inhibited 91 and 79% when N-ethylmaleimide was added at 1 and 10 min, respectively, after the start of the reaction.

Thus, dnaB and dnaC(D) gene products mutually affect one another in reactions involving ATP; they bind to one another, the ATPase of dnaB is inhibited by dnaC(D), and the N-ethylmaleimide sensitivity of dnaC(D) is prevented by dnaB gene product.

**DISCUSSION**

The dnaB and dnaC(D) gene products and ATP interact physically and functionally in vitro. It is not known if this interaction occurs in vivo. However, if the two proteins interact physiologically, then (a) dnaB gene product would be implicated in initiation of chromosome replication since dnaA mutant is required for this process (3), (b) dnaC(D) gene product would be implicated in chromosome elongation since dnaB is required for elongation (2), or (c) both proteins would be implicated in both chromosome initiation and elongation. Consistent with the possibility that these two proteins function jointly, some dnaC(D) mutants stop DNA synthesis immediately at the elevated temperature (J. Wechsler, personal communication). This observation suggests that dnaC(D), like dnaB, is involved in chromosome elongation.

In addition to being required for E. coli DNA replication, both dnaB and dnaC(D) gene products are required for conversion in vitro of φX174 single-stranded DNA to duplex DNA in combination with eight other purified proteins: dnaG gene product, DNA polymerase III, DNA elongation factors I and II, DNA binding protein, and DNA replication factors X, Y, and Z (23). DNA synthesis by this reconstituted system of proteins requires ATP specifically (23). Three partial reactions have been described that may contribute to this ATP requirement. One is the interaction of dnaB and dnaC(D) gene products demonstrated here. A second is the ribonucleoside triphosphatase activity of dnaB gene product that utilizes NTPs but not dNTPs (21). A third is the elongation of RNA- or DNA-primed single-stranded DNA that requires ATP or dATP. It is possible that other partial reactions of the φX174 DNA-synthesizing system will be found to involve ATP.

With the reconstituted φX174 DNA replicating system it is now possible to further dissect the system into its partial

---

* The factor previously referred to as DNA replication factor Y has been resolved into two protein fractions; they will now be referred to as replication factors Y and Z. DNA binding protein, elongation factors I and II, and replication factors X, Y, and Z are as yet undefined by genetic loci.

† We have previously reported that the elongation reaction catalyzed by DNA polymerase III and DNA elongation factors I and II requires ATP or dATP (24). Wickner and Kornberg (25) have reported a similar reaction in the presence of polymerase III* and DNA polymerase III* that requires ATP.
reactions. So far the reaction has been resolved into two steps. Step one requires dnaB and dnaC(D) gene products in addition to DNA binding protein and replication factors X, Y, and Z, ATP, and φX174 DNA, and involves reactions prior to dNMP incorporation; dnaA gene product, DNA polymerase III, and DNA elongation factors I and II are not required during step 1 but are required for dNMP incorporation during the second step after the addition of dNTPs (23). Recently we have physically separated these two steps (results to be published elsewhere). Incubation of the components required for step 1 followed by BioGel A-5m gel filtration resulted in the isolation of a complex associated with φX174 DNA in the excluded volume. The addition of the components required for step 2 to this complex resulted in dNMP incorporation. Analysis of the complex isolated after gel filtration indicated that 25–50% of the dnaB gene product recovered was associated with the φX174 DNA in the excluded volume; the rest was partially included in the gel. In contrast, <5% of the dnaC(D) gene product recovered was associated with the DNA; all detectable activity was associated with dnaB in the partially included volume or free in the fully included volume. Thus, it is possible that the complex of dnaB and dnaC(D) gene products participates in a reaction resulting in the transfer of dnaB to φX174 DNA and the concomitant release of dnaC(D) gene product.

This research was carried out while S.W. was a guest scientist in Dr. W. Parks' laboratory. The authors are indebted to Dr. Parks for his hospitality. The work was supported in part by grants from the National Institutes of Health, National Science Foundation and the American Cancer Society. S.W. is a National Institutes of Health Postdoctoral Fellow.


Interaction of dnaB and dnaC(D) Gene Products