The Escherichia coli dnaB Replication Protein Is a DNA Helicase*  

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Genetic and biochemical analyses indicate that the Escherichia coli dnaB replication protein functions in the propagation of replication forks in the bacterial chromosome. We have found that the dnaB protein is a DNA helicase that is capable of unwinding extensive stretches of double-stranded DNA. We constructed a partially duplex DNA substrate, containing two preformed forks of single-stranded DNA, which was used to characterize this helicase activity. The dnaB helicase (i) depends on the presence of a hydrolyzable ribonucleoside triphosphate, (ii) is maximally stimulated by a combination of E. coli single-stranded DNA-binding protein and E. coli primase, (iii) is inhibited by antibody directed against dnaB protein, and (iv) is inhibited by prior coating of the single-stranded regions of the helicase substrate with the E. coli single-stranded DNA-binding protein. It was determined that the dnaB protein moves 5' to 3' along single-stranded DNA, apparently in a processive fashion. To invade the duplex portion of the helicase substrate, the dnaB protein requires a 3'-terminal extension of single-stranded DNA in the strand to which it is not bound. Under optimal conditions at 30 °C, greater than 1 kilobase pair of duplex DNA can be unwound within 30 s. Based on these findings and other available data, we propose that the dnaB protein is the primary replicative helicase of E. coli and that it actively and processively migrates along the lagging strand template, serving both to unwind the DNA duplex in advance of the leading strand and to potentiate synthesis by the bacterial primase of RNA primers for the nascent (Okazaki) fragments of the lagging strand.

Genetic analyses of chromosomal DNA replication in Escherichia coli indicate that the product of the E. coli dnaB gene is an essential constituent of the bacterial replication machinery (1–5). The finding that DNA synthesis ceases abruptly when temperature-sensitive dnaB mutants of E. coli are placed at a nonpermissive temperature (1–5) suggests that the dnaB protein participates directly in the propagation of a replication fork.

Different types of multiple protein replication systems have been established in which DNA synthesis is strictly dependent on the function of the E. coli dnaB protein. This bacterial replication protein, a DNA-dependent ATPase (6, 7), is needed for the replication of the chromosomes of coliphages φX174 (8, 9) and λ (10–12) and is also required for the replication of both ColE1 plasmid DNA (13–15) and plasmid DNA containing oriC (16–18), the replication origin of the E. coli chromosome. The biochemical evidence obtained from studies of these in vitro replication systems indicates that dnaB protein enables E. coli primase (dnaG protein) to repeatedly generate the short RNA transcripts needed to prime synthesis of the nascent (Okazaki) fragments of the lagging strand at a replication fork (12, 18–20).

Biochemical analyses of the replication of φX174 virion DNA in vitro demonstrated that the dnaB protein is a constituent of the φX174 primosome, a mobile multiprotein priming apparatus that is assembled at the complementary strand replication origin on the single-stranded φX174 chromosome (19–21). It has been concluded that the φX174 primosome moves processively in an antielongation direction (5' to 3') along the template strand (22) and that this movement is fueled by nucleoside triphosphate hydrolysis by protein n', another constituent of this primosome (20).

Genetic and biochemical studies of phage λ DNA replication indicate that the λ O and P initiator proteins act to direct the bacterial dnaB protein to the λ replication origin, where it is assembled into a nucleoprotein complex (11, 12, 23–29). Model studies of this process using single-stranded DNA templates indicate that the λ O and P proteins act to guide the ATP-dependent assembly of an activated nucleoprotein complex containing dnaB protein (11, 12, 30). We have termed this complex the λ primosome, since it, like the φX174 primosome, can synthesize multiple primers on each DNA molecule when it is supplemented with primase (12). It appears to be relevant that each member of the set of three proteins that is required for the assembly of both the φX174 and λ primosomes (i.e. dnaB protein, primase, and the E. coli single-stranded DNA-binding protein (SSB')) is also known to participate in the propagation of E. coli replication forks in vivo (31). Since the λ primosomal complex does not contain protein n', it is likely that dnaB protein catalyzes the movement of primosomal complexes.

One of the tasks faced by the enzymatic machinery that replicates double-stranded DNA is the disruption of the forces that hold the two parental chains together. DNA helicases are enzymes which couple unwinding of the DNA helix to the hydrolysis of nucleoside triphosphate. The notion that dnaB protein may move processively along the lagging strand at a replication fork prompted us to determine if this DNA-dependent ATPase also contained DNA helicase activity. Two other facts also encouraged us to search for DNA helicase

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1 The abbreviations used are: SSB, single-stranded DNA-binding protein; rNTP, ribonucleoside triphosphate; dNTP, deoxyribonucleoside triphosphate; Ap0(NH)p, adenylyl-5'-imidodiphosphate; 4738-4748, 1986. Printed in U.S.A.
activity in purified preparations of dnaB protein. First, there was no compelling genetic evidence that any of the E. coli DNA helicases characterized thus far are obligatory for replication of the bacterial chromosome (32). Second, it was also known that the phage T4-coded and phage T7-coded primases were each intimately associated with a DNA helicase activity (33-35). A similar functional association of the E. coli primase with dnaB protein was indicated by findings that, with the single exception of phage G4 DNA replication, both specific and general priming by the bacterial primase usually requires prior association of the dnaB protein with the DNA template (12, 18-21, 30, 36).

We have prepared partially duplex helicace substrates that contain more than 1000 base pairs of double-stranded DNA. In this paper, we show that dnaB protein unwinds this DNA in a reaction that requires hydrolysis of a ribonucleoside triphosphate. We demonstrate that the helicase activity of dnaB protein is maximal when both primase and SSB are present. Our analysis indicates that the dnaB protein moves 5' to 3' along a strand to which it is bound and that a preformed fork is required for the protein to invade and unwind duplex DNA. These results support a model in which the dnaB protein is the primary replicative helicase of E. coli. In this model, the dnaB protein moves processively along the template for the lagging strand in the antielongation direction, both unwinding DNA to enable polymerization of the leading strand and serving as a mobile recognition locus for primase to synthesize primers for the nascent (Okazaki) fragments of the lagging strand.

MATERIALS AND METHODS

Reagents and Materials—Sources were the following: Hesper, Research Organics; 5'-adenylylimidodiphosphate (App[NH])p and creatine phosphate, Boehringer Mannheim; [γ-32P]ATP (3000 Ci/mmol) and [α-32P]dCTP (400-600 Ci/mmol), Amersham Corp.; unlabeled ribonucleoside triphosphates, P-L Biochemicals; hydroxylapatide, Clarkson Chemical Co.; Centicon 30 microconcentrator, Amicon; and low gelling temperature agarose, Sigma. The sources of all other materials have been described previously (10-12, 37).

Enzymes and Proteins—T4 polynucleotide kinase and terminal deoxynucleotidyltransferase were purchased from Pharmacia P-L Biochemicals. Bacterial alkaline phosphatase was purchased from Worthington (Cooper Biomedical Biochemicals). Creatine kinase was purchased from Boehringer Mannheim. BamHI and EcoRI restriction enzymes were purchased from New England Biolabs and were used according to the instructions supplied by the manufacturer. Replication proteins and their specific activities were: dnaB protein, Fraction III, 4 x 10^5 units/mg (7, 39); primase (dnaG protein), Fraction IV, 5.4 x 10^8 units/mg (57, 39); single-stranded DNA-binding protein (SSB), Fraction III, 4 x 10^4 units/mg. Each of these replication proteins was greater than 90% pure. One unit of activity is defined as the amount of protein necessary to produce 1 pmol of dNMP incorporation/min into acid-insoluble material in standard replication reactions (7, 10). Antibodies specifically directed against dnaB protein and protein I have been described (7, 10).

Phage Strains—M13oriXL and M13oriRI are derivatives of phage M13mp7 which carry the L and R strands, respectively, of a 1503-base pair bacteriophage AAl fragment that spans the oriA region. The Alu fragment was inserted in both orientations into the HincII sites of M13mp7. In each recombinant phage, the insert is flanked by adjacent BamHI sites. M13mp8oriXL is a derivative of phage M13mp8 and carries the L strand of a 1067-base pair HincII fragment of L. This fragment, which spans the oriL region, was inserted into the HincII sites of M13mp8. Construction of the recombinant phage DNAs have been described elsewhere (11).

Construction of Helicase Substrate—Isolation of a 1515-nucleotide single-stranded BamHI fragment that contained the R strand of the Alu fragment from the oriL region was accomplished as follows: M13oriRI single-stranded phage DNA (950 µg) was digested with 1000 units of BamHI in a final volume of 1.4 ml for 10 h at 37 °C (40). Approximately one-half of the Alu insert is excised under these conditions. Following the addition of 0.2 ml of 90% glycerol, the reaction products were purified by electroelution by low-temperature agarose gel (1%) using a Tris/glycine electrophoresis buffer (11) containing ethidium bromide (1 µg/ml). The section of the gel containing the 1515-nucleotide oriL fragment was removed from this gel slice by a modification previously described procedure (41). The agarose slab was melted at 70°C, and its volume (30 ml) was determined. An equal volume of 10 mM Tris/HCl, pH 8.0, 1 mM EDTA (TE buffer) was added, and the mixture was distributed equally into four 30-ml glass centrifuge tubes. An equivalent volume of neutralized phenol (equilibrated with TE buffer) was added to each tube, and the tubes were shaken vigorously for 2 min. After centrifugation at approximately 5000 x g for 2 min, the lower phenol phase was collected and placed in a separate tube, and the aqueous phase was re-extracted with phenol. The initial phenol phase was back-extracted with TE buffer, and the resulting aqueous phase was re-extracted once with phenol. All aqueous samples were subsequently pooled. The combined aqueous phase was repeatedly extracted with 1-butanol until its volume was reduced to approximately 4 ml. Finally, the concentrated sample was extracted twice more with an equal volume of ether. This material was dialyzed against 10 mM sodium phosphate, pH 7.0, and loaded onto a hydroxyapatite column. The column had 5 cm columns of 10 mM sodium phosphate, pH 7.0, and then adsorbed DNA was eluted with 2 column volumes of 0.4 M sodium phosphate, pH 7.0. The eluted DNA was dialyzed against TE buffer and concentrated to 0.65 ml using a Centricon 30 microconcentrator. The final product (48 µg) migrated as a single species during electrophoresis in an agarose gel.

The single-stranded Alu DNA (15 µg; 30 pmol of fragment) was treated with bacterial alkaline phosphatase and labeled at its 5' terminus with [32P]P using [γ-32P]ATP and T4 polynucleotide kinase (43). This [32P]-labeled DNA was copurificated with 175 µg of M13oriXL phage DNA to a final concentration of 0.5 µg of 8 M ammonium acetate and 2 volumes of cold ethanol. Following precipitation, the DNA was resuspended in 0.2 ml of 150 mM sodium phosphate, pH 7.0, 10 mM EDTA. The DNA solution was overlaid with mineral oil and incubated at 65 °C for 3 h to allow the two DNAs to hybridize. Afterwards, no unhybridized [32P]-labeled Alu fragment could be detected by agarose gel electrophoresis. The hybridized DNA was diluted 10-fold into TE buffer and used directly as a substrate in helicase assays. In order to drive the hybridization to completion, it was necessary to use an excess of M13mp8oriXL phage DNA such that a significant portion (55%) of the ss DNA circles in the hybrid substrate did not contain a hybridized AhI fragment. In these experiments, 300 units of BamHI in a final volume of 1.4 ml for 10 h at 37°C. The digestion products were separated from the DNA by gel electrophoresis (47). The reaction mixture (20 pl) contained 200 mM sodium cacodylate, pH 7.0, 1 mM CoCl₂, 1.25 µM [α-32P]dCTP (800 Ci/mmol), 2.5 µg (5 pmol) of single-stranded Alu DNA fragment, and 54 units of terminal deoxynucleotidyltransferase. The reaction was incubated for 45 min at 37°C. Approximately 3-4 nucleotides were transferred to the 5' terminus of each Alu DNA molecule. The 5' end-labeled Alu DNA (1 µg) was hybridized to M13mp8oriL phage DNA under conditions identical to those described for the 5' end-labeled DNA. Following hybridization, a 50-µl portion of each hybridization mixture was dialyzed on a Millipore microdialysis disc against TE buffer for 30 min. The 5' end-labeled and 3' end-labeled hybrids were then digested with 40 units of EcoRI (100 µl final volume) at 37°C for 3 h. Following phenol extraction, each of these EcoRI-digested hybrid was used directly as a helicase substrate.

Helicase Assay—Helicase reactions were assembled at 0°C. Unless otherwise indicated, the standard reaction mixture (15 µl) contained 40 mM Hepes/KOH, pH 7.6, 50 µg of bovine serum albumin/ml, 11 mM magnesium acetate, 3.4 mM ATP, 0.01% of 5' end-labeled Alu fragment (approximately half were hybridized) and 5'-end-labeled single-stranded Alu fragment from the oriL re-
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RESULTS

E. coli dnaB Protein Is a Helicase—We have assayed purified E. coli dnaB protein for helicase activity by determining its capacity to unwind partially duplex DNA substrates. Since dnaB protein binds preferentially to single-stranded DNA (45, 46), the substrate was designed to contain a long region of single-stranded DNA to promote binding by the dnaB protein. The helicase substrate (Fig. 1) was constructed by hybridizing a single-stranded DNA fragment, labeled with \(^{32}\)P at its 5' terminus, to the circular single-stranded chromosome of an M13mp8 phage recombinant that contains a complementary insert (see “Materials and Methods” for details). Because the insert contained in M13mp8 is shorter than the labeled DNA fragment, 356 nucleotides at the 5' end and 99 nucleotides at the 3' terminus of the linear single-stranded chain remain unannealed. This substrate, therefore, contains two forks, each with adjacent single-stranded regions. The assay, depicted in Fig. 2, utilizes gel electrophoresis to separate unwound radioactive product from unreacted helicase substrate. The helicase substrate contains two species (see legend to Fig. 2), each of which is a satisfactory substrate for the dnaB protein helicase.

When dnaB protein was incubated with the helicase substrate in the presence of ATP, approximately 10% of the hybridized \(^{32}\)P-labeled DNA fragments were released from the...
partially duplex substrate molecules (Fig. 3, lane 3; Fig. 5). The dnaB protein helicase activity was stimulated about 2-fold by the presence of SSB (Fig. 3, lane 4; Fig. 5) and about 6-fold when both primase and SSB were present (Fig. 3, lane 5; Fig. 5). The addition of primase did not stimulate the helicase activity of dnaB protein when SSB was absent from the reaction mixture (Fig. 3, lane 6; Fig. 5). Control experiments (Fig. 3, lanes 7–9) demonstrated that neither SSB nor primase possessed detectable helicase activity in the absence of dnaB protein.

The helicase activity was inhibited by an antibody directed specifically against dnaB protein and could be restored by adding back excess dnaB protein to an antibody-inhibited reaction (Fig. 4, lanes 1–10). A control IgG preparation did not inhibit the helicase reaction (Fig. 4, lanes 11 and 12). The ratio of anti-dnaB protein IgG to dnaB protein that was necessary to inhibit helicase activity was similar to that required to inhibit the ATPase activity of dnaB protein (data not shown). These findings suggest that the helicase activity resides in the dnaB polypeptide and not in a minor contaminant.

We prepared a potential helicase substrate in which the entire 1515-nucleotide AluI single-stranded fragment from the R strand of phage λ was base paired to circular M13mp80oriXL (11). DNA. However, no detectable unwinding was observed with this substrate (data not shown). Thus, the dnaB protein, in order to initiate helicase action, apparently requires single-stranded DNA on both strands at the border.

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![Fig. 4. Inhibition of helicase activity by antibody directed against dnaB protein. Helicase reactions were carried out as described under “Materials and Methods” except that dnaB protein (263 ng) was preincubated with either IgG or with buffer (40 mM Hepes/KOH, pH 7.6, 11 mM magnesium acetate, 50 μg of bovine serum albumin/ml) for 10 min at 0°C before being added to reaction mixtures. All reaction mixtures were supplemented with SSB, primase, and rNTPs. The reaction products were subjected to agarose gel electrophoresis and detected by autoradiography. Lanes 1 and 2, dnaB protein was preincubated with buffer; lanes 3 and 4, dnaB protein was preincubated with 12.3 μg of anti-dnaB protein IgG; lanes 5 and 6, dnaB protein was preincubated with 12.3 μg of anti-dnaB protein IgG and then supplemented with excess dnaB protein (350 ng) before being added to the helicase reaction mixture; lanes 7 and 8, dnaB protein was preincubated with 14.7 μg of anti-dnaB protein IgG; lanes 9 and 10, dnaB protein was preincubated with 14.7 μg of anti-dnaB protein IgG and then supplemented with excess dnaB protein (350 ng) before being added to the helicase reaction mixture; lanes 11 and 12, dnaB protein was preincubated with 12.3 μg of anti-protein 1 IgG. The average percentages of conversion of substrate into product were: lanes 1 and 2, 48%; lanes 3 and 4, 7%; lanes 5 and 6, 35%; lanes 11 and 12, 53%; lanes 7–10, not determined.](image-url)

of the duplex region, i.e. unwinding by the dnaB protein requires a preformed fork.

The amount of fragment displaced in the helicase assay depends on the amount of dnaB protein added to the reaction (Fig. 5). At high dnaB protein levels 70–90% of the hybridized fragment is released from the M13 template. The inability of the dnaB helicase to displace all of the fragment from the template under optimal assay conditions may mean that a portion of the released fragment renatemplates to the template. The addition to the helicase reaction mixture of SSB or primase, or both, had no significant effect on the amount of dnaB protein required for maximal activity. At saturation in the helicase assay, dnaB protein is present at approximately a 60-fold molar excess over phage DNA circles and at a 120-fold molar excess over helicase substrate.

The requirement for a large molar excess of dnaB protein in the helicase assay might reflect a relatively low affinity of dnaB protein for single-stranded DNA. At the concentration of DNA used in the helicase assay, only a small proportion of the dnaB protein molecules may be bound to the helicase substrate. Alternatively, the need for excess dnaB protein could indicate that stoichiometric binding of a large number of dnaB protein molecules is required for unwinding of each
point except that dnaB protein levels were varied as indicated. Each data point in the figure represents the average of duplicate determinations. Primase and SSB were added to reaction mixtures as indicated.

![Figure 5. Titration of the amount of dnaB protein required for maximal helicase activity.](image)

Figure 5. Titration of the amount of dnaB protein required for maximal helicase activity. Helicase reaction mixtures were assembled and treated as described under “Materials and Methods” except that dnaB protein levels were varied as indicated. Each data point in the figure represents the average of duplicate determinations. Primase and SSB were added to reaction mixtures as indicated.

![Figure 6. Titration of dnaB protein binding to the DNA helicase substrate as determined with a filter-binding assay.](image)

Figure 6. Titration of dnaB protein binding to the DNA helicase substrate as determined with a filter-binding assay. Binding of dnaB protein to 32P-labeled DNA helicase substrate was measured with the nitrocellulose filter-binding assay described under “Materials and Methods.” Each point represents the average determined from duplicate samples.

It has been well documented that SSB inhibits the binding of dnaB protein to single-stranded DNA (11, 12, 19, 21, 36). Accordingly, when saturating levels of SSB are added to the helicase substrate prior to the addition of dnaB protein, no unwinding is detected (Fig. 8). In contrast, if dnaB protein is permitted to bind to uncoated single-stranded DNA prior to the addition of SSB, as in the standard helicase reaction, then the presence of SSB stimulates the dnaB protein helicase activity (Figs. 3, 5, and 8). The time course of dnaB protein activation on the helicase substrate at 30°C prior to the addition of SSB is depicted in Fig. 9A. Maximal stimulation of the dnaB protein helicase activity requires 1–3 min under standard helicase reaction conditions. This presumably reflects, at least in part, the time required for dnaB protein to...
bind to every helicase substrate. Once activated, the dnaB protein mediates unwinding of the hybridized fragment within 1 or 2 min at 30 °C in the presence of SSB (Fig. 9B).

**Nucleotide Requirements for dnaB Protein Helicase Activity**—The dnaB protein requires a hydrolyzable ribonucleoside triphosphate cofactor in order to unwind DNA (Fig. 10, Tables I and II). Either in the presence (Table II) or absence (Table I) of SSB, the nucleotide requirement for helicase function was best satisfied by ATP; GTP and CTP were somewhat less active as cofactors, and UTP was significantly less active. Of the four dNTPs, only dATP and dCTP supported slight helicase activity. Maximal unwinding required an ATP concentration of approximately 2 mM (data not shown), a concentration well above the reported Kₐ of the DNA-dependent ATPase activity of dnaB protein for ATP (45, 46). However, in the presence of an ATP regeneration system, 1 mM rNTP sustained optimal unwinding. No detectable helicase activity was found in the absence of Mg²⁺ (data not shown).

The nonhydrolyzable ATP analogue App(NH)p was not capable of serving as a nucleotide cofactor for DNA unwinding catalyzed by the dnaB protein (Fig. 10 and Table II), even though this analogue enables dnaB protein to bind to single-stranded DNA (46, 48). However, for maximal dnaB protein helicase activity only App(NH)p need be present with dnaB protein and the helicase substrate prior to the addition of SSB, provided that ATP or another hydrolyzable ribonucleoside triphosphate is added after SSB (Fig. 10 and Table II). Control experiments indicated that either a hydrolyzable or nonhydrolyzable ribonucleoside triphosphate must be present in the reaction mixture together with dnaB protein and the helicase substrate prior to the addition of SSB in order to obtain detectable helicase activity (data not shown). These results suggest that the initial step in the dnaB protein helicase reaction is ribonucleotide-dependent binding of dnaB protein to single-stranded regions of the helicase substrate, a characteristic shared by several other known helicases (49). We infer that this initial binding step is followed by migration of one or more dnaB protein molecules from the single-stranded region to the duplex portion of the substrate and subsequent unwinding of the duplex DNA. One or both of these last two steps is apparently fueled by ATP hydrolysis.

**Direction of Unwinding by dnaB Protein**—Each helicase examined in this paper appears to unwind DNA with a specific polarity (49). With partially duplex substrates, each DNA helicase is thought to bind first to single-stranded DNA and then to approach and unwind duplex DNA in a particular direction. To determine if dnaB protein also unwinds duplex...
TABLE II
Nucleotide effectors for dnaB protein helicase activity in the presence of SSB

| Nucleotide       | Fragment displaced |
|------------------|-------------------|
| None             | 0                 |
| ATP              | 36                |
| GTP              | 31                |
| CTP              | 28                |
| UTP              | 10                |
| App(NH)p         | 0                 |
| App(NH)p + ATP   | 38                |

*The initial 3-min incubation at 30°C was carried out in the presence of App(NH)p (100 μM). After the addition of SSB, the reaction mixture was supplemented with ATP (6.4 mM) and incubated for an additional 5 min at 30°C.

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The experimental results indicate that in the presence of dnaB protein alone only minimal helicase activity is detected (Fig. 12, lanes 3 and 4), since the concentration of dnaB protein is very low (e.g. see Fig. 5). In the presence of SSB or SSB and primase, however, the dnaB protein helicase displaced approximately 10 times more of the 3' end-labeled fragment (Fig. 11, fragment b) than of the 5' end-labeled fragment (Fig. 12, lanes 5-8). This result strongly suggests that the dnaB protein moves in a 5' to 3' direction along the DNA strand to which it is bound. As anticipated, at higher dnaB concentrations (i.e. the dnaB concentration used in the standard helicase assay) the bias in favor of displacing the 3' end-labeled fragment decreases (to approximately 2-3-fold) (Fig. 13, lanes 5-8). Remarkably, at this higher dnaB protein concentration, when SSB is omitted from the reaction mixture, there actually is a reversal of the observed bias in fragment displacement such that 5-fold more 5' end-labeled fragment (Fig. 11, fragment a) than 3' end-labeled fragment was released (Fig. 13, lanes 3 and 4).

DISCUSSION

Prior to this report three different E. coli gene products had been shown to possess DNA helicase activity, namely rep protein, DNA helicase II (the product of the uurD gene), and DNA helicase III. Although each of these enzymes is capable of unwinding long stretches of double-stranded DNA, none of them had been implicated by genetic analyses of bacterial DNA replication mutants as the sole replicative DNA helicase (49). The recent discovery that uurD-rep double mutants are inviable (32), combined with biochemical studies that demonstrated that helicase II and rep proteins have opposite polarities of movement (49), led to a proposal that these two helicases cooperate to unwind the two strands at a replication fork (32). On the other hand, numerous genetic and biochemical studies have confirmed that the E. coli dnaB protein is essential for the propagation of chromosomal replication forks (31, 50). Thus, the demonstration that highly purified dnaB protein has an associated DNA helicase activity strongly suggests that this protein functions as the major replicative DNA helicase in vivo. This report confirms the hypothesis of Staufenbauer et al. (14) that the dnaB protein might be a
DNA helicase that unwinds DNA ahead of the leading strand, a proposal based on the effects of anti-dnaB protein antibodies on the properties of ColEl plasmid DNA replication in vitro.

In general, the capacity of the E. coli primase (the dnaG gene product) to synthesize primers for DNA replication is functionally linked to interactions with the dnaB replication protein (19-22, 36, 50). During the replication of the φX174 viral strand this interaction is believed to take place in the context of the primosome, a large mobile multiprotein priming complex that is believed to contain dnaC protein and proteins i, n, n', and n" in addition to dnaB protein and primase (20-22, 50). In the presence of SSB, both the dnaB protein (Fig. 12) and the primosome (22) move in a direction opposite to DNA chain elongation, 5' to 3' along the template strand to which they are bound. This polarity of dnaB protein translocation is consistent with a model of dnaB protein function in which the dnaB protein processively migrates along the lagging strand template at a replication fork, acting both as a DNA helicase (to unwind the DNA duplex in advance of the DNA polymerase that synthesizes the leading strand) and as a coparticipant with primase in the synthesis of the multiple primers needed for production of the lagging strand nascent DNA, which they are bound. This polarity of dnaB protein translocation is particularly notable since this protein is believed to be responsible for the movement of the φX174 primosome (20).

These findings, coupled with the biochemical properties of the dnaB protein, suggest that the dnaB protein itself is primarily responsible for the movement of replication forks in the E. coli and λ chromosomes. The involvement of protein n' rather than dnaB protein in the processive movement of the φX174 primosome was largely based on the assumption that only protein n' is capable of hydrolyzing dATP, a nucleotide which could, under certain conditions, enable the synthesis of multiple primers on φX174 DNA (20). It may be significant, however, that our preparation of dnaB protein contains a dATPase activity that (i) is sensitive to antibody directed against dnaB protein and (ii) coelutes with highly purified native dnaB protein during gel permeation chromatography. Furthermore, this dATPase activity is not due to the presence of contaminating n' protein in our dnaB protein preparation, since the dATPase activity of protein n' is unaffected by our anti-dnaB protein IgG preparation. Nevertheless, the capacity of dATP to serve as an effector of dnaB protein movement is uncertain, since dnaB protein hydrolyzes ATP considerably faster than dATP and since high levels of containing either the E. coli replication origin (oriC) (18) or the bacteriophage λ replication origin (oriλ) (5). Both replication systems can be efficiently reconstituted with sets of highly purified replication proteins. Although both primase and the dnaB protein helicase are absolutely required for propagation of replication forks from oriC or oriλ, other known helicases as well as E. coli proteins i, n, n', or n" can be omitted from these in vitro replication systems without effect (18). The lack of a requirement for protein n' is particularly notable since this protein is believed to be responsible for the movement of the φX174 primosome (20).

These findings, coupled with the biochemical properties of the dnaB protein, suggest that the dnaB protein itself is primarily responsible for the movement of replication forks in the E. coli and λ chromosomes. The involvement of protein n' rather than dnaB protein in the processive movement of the φX174 primosome was largely based on the assumption that only protein n' is capable of hydrolyzing dATP, a nucleotide which could, under certain conditions, enable the synthesis of multiple primers on φX174 DNA (20). It may be significant, however, that our preparation of dnaB protein contains a dATPase activity that (i) is sensitive to antibody directed against dnaB protein and (ii) coelutes with highly purified native dnaB protein during gel permeation chromatography. Furthermore, this dATPase activity is not due to the presence of contaminating n' protein in our dnaB protein preparation, since the dATPase activity of protein n' is unaffected by our anti-dnaB protein IgG preparation. Nevertheless, the capacity of dATP to serve as an effector of dnaB protein movement is uncertain, since dnaB protein hydrolyzes ATP considerably faster than dATP and since high levels of

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**Fig. 12.** Determination of the polarity of movement of the dnaB protein helicase. Helicase assay mixtures were assembled as described under "Materials and Methods." Lanes 1 and 2, no proteins were added; lanes 3 and 4, dnaB protein (25 ng) alone was added; lanes 5 and 6, dnaB protein (23 ng) and SSB (380 ng) were added; lanes 7 and 8, dnaB protein (23 ng), dnaG primase (150 ng), and SSB (380 ng) were added. Reaction mixtures contained either 50 ng of the 5' end-labeled linearized substrate (odd-numbered lanes) or 50 ng of the 3' end-labeled linearized substrate (even-numbered lanes). See "Materials and Methods" and the legend to Fig. 11 for additional description of the substrates. Helicase reactions were performed, analyzed, and quantitated as described under "Materials and Methods." The reaction products are displayed on a 1% neutral agarose gel. The percentages of fragment displaced from the substrate were 0, 0, <2, <2, 24, 3, and 48 for lanes 1-8, respectively.

**Fig. 13.** Polarity of dnaB protein helicase action at high concentrations of dnaB protein. Helicase reactions were performed and analyzed exactly as described in the legend to Fig. 12, except that, when present, 350 ng of dnaB protein was added to each reaction mixture. Lanes 1 and 2, no proteins were added; lanes 3 and 4, dnaB protein (350 ng) alone was added; lanes 5 and 6, dnaB protein (350 ng) and SSB (380 ng) were added; lanes 7 and 8, dnaB protein (350 ng), dnaG primase (150 ng), and SSB (380 ng) were added. Reaction mixtures contained either 50 ng of the 5' end-labeled linearized substrate (odd-numbered lanes) or 50 ng of the 3' end-labeled linearized substrate (Fig. 11). The percentages of fragment displaced from the substrate were 0, 0, 51, 11, 34, 72, 41, and 92 for lanes 1-8, respectively.

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5 K. Mensa-Wilmot, unpublished results.
dATP do not support significant levels of helicase activity by dnaB protein (Table I), even in the presence of SSB and primase.

The notion that the replication apparatus involved in the initiation of the nascent Okazaki fragments of the lagging strand in the E. coli chromosome is relatively simple (Fig. 14) and consists solely of a helicase (dnaB protein), a primase (dnaG protein), a single-stranded DNA-binding protein (SSB), and a multisubunit DNA polymerase (DNA polymerase III holoenzyme) is consistent with replication fork propagation mechanisms elucidated for the chromosomes of coliphages T4 and T7. In the case of phage T4, synthesis of the lagging strand requires the coupled action of the T4 gene 41 protein helicase and the gene 61 primase, as well as the gene 32 single-stranded DNA-binding protein and the T4 DNA polymerase (51). Likewise, synthesis of phage T7 lagging strand fragments by the T7 gene 5 DNA polymerase involves the coupling of helicase and primase activities, both of which reside in the T7 gene 4 product (35, 52). The available evidence from in vitro T4 and T7 phage replication systems suggests that the viral primase remains stably associated with a DNA helicase that moves along the template for the lagging strand of a replication fork (51, 52). The precise nature and structure of the protein or protein complex that acts processively at an E. coli replication fork is uncertain. It remains to be determined if the E. coli primase is tightly bound to the bacterial dnaB protein helicase on the lagging strand template, or whether the primase repeatedly associates with and dissociates from the moving dnaB protein before and after priming the synthesis of each Okazaki fragment (as depicted in Fig. 14). In this regard, we have been unable to detect, in the absence of DNA, the formation of stable complexes between the dnaB protein helicase and the dnaG primase. Primase does, however, enhance the stability of a ternary complex of dnaB protein, single-stranded DNA, and ATP (48).

The discovery that the dnaB protein is a DNA helicase clarifies its functional roles in the various stages of the replication of duplex DNA chromosomes. We suggest the following mechanisms for the action of dnaB protein in the establishment and propagation of replication forks in the chromosomes of E. coli and bacteriophage λ. During the initiation phase of the reaction, the dnaB protein is positioned between the DNA strands at or near the unique chromosomal origin. Because dnaB protein binds weakly and nonspecifically to single and double-stranded DNA (46, 48), transfer of dnaB protein onto duplex DNA at a chromosomal replication origin requires specific protein-protein and protein-DNA interactions, such as interaction of a dnaB-dnaC protein complex with dnaA protein bound at oriC (18) or interaction of a complex of dnaB protein and λ P protein with λ O protein bound at oriλ (11, 12, 28, 29). Once positioned between the strands, the dnaB protein migrates processively with 5' to 3' polarity along the strand to which it is bound, unwinding the duplex DNA as it moves. The single-stranded DNA generated in the origin region is stabilized and prevented from reannealing by the binding of SSB. Primase interacts with the dnaB protein helicase and periodically synthesizes primers that are extended by the DNA polymerase III holoenzyme into the nascent (Okazaki) fragments of the lagging strand. The first primer synthesized may, during bidirectional DNA replication, be extended into the continuous leading strand of a second replication fork that is established by another dnaB helicase molecule that moves along the other strand in the opposite overall direction to the first (19).

E. coli SSB stimulates the helicase activity of dnaB protein if the dnaB protein is permitted to bind to the helicase substrate prior to the addition of SSB to the reaction mixture (Figs. 3 and 5). If the order of addition is reversed, no helicase activity is detected (Fig. 8), presumably because the dnaB protein cannot bind to the helicase substrate once regions of single-stranded DNA are coated with SSB. The presence of SSB during strand unwinding by the dnaB protein stimulates both the rate (Figs. 7 and 9) and extent of helicase activity (Fig. 5). Although it remains to be proven, we believe that it is likely that SSB stimulates helicase activity simply by binding to the unwound DNA product and preventing reannealing of the separated DNA strands. If so, the lower rate of helicase activity observed in the absence of SSB does not imply that the dnaB protein helicase is unwinding DNA more

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*Fig. 14. Proposed mechanism for function of E. coli replication proteins at a replication fork. See text for details.*

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C. Alfano, M. Dodson, and H. Echols, unpublished results.
slowly than when SSB is present. Instead, multiple passages of the dnaB protein through the 1060-base pair duplex portion of the substrate may be required to successfully displace the hybridized single-stranded fragment from the circular M13 chromosome. Alternatively, SSB may function as a positive effector of the DNA helicase activity of dnaB protein, mediated, perhaps, by direct protein-protein interactions that appear to stabilize the binding of dnaB protein to single-stranded DNA (21).

Maximal activity of the dnaB protein helicase requires that it be present in a large molar excess over that of the helicase substrate. This is not unusual for a replicative DNA helicase, since both the phage T4 gene 41 protein helicase and the phage T7 gene 4 protein helicase each must be present at greater than 50-fold molar excess, relative to the helicase substrate, to obtain maximal unwinding (33, 35). Because of this requirement for excess dnaB protein, we have not been able to determine if the dnaB protein helicase acts in a processive or distributive fashion. The behavior of the dnaB protein in two different single-stranded DNA replication systems in vitro indicates that the protein is acting in a catalytic and processive fashion during the synthesis of multiple primers on SSB-coated DNA (11, 12, 19, 36, 48). The low intracellular concentration of dnaB protein, approximately 15–20 molecules/E. coli (7, 53, 54), suggests that this protein functions processively even in vivo as well. Filter-binding analysis of the binding of dnaB protein to the helicase substrate under optimal conditions for DNA unwinding (Fig. 6) indicated that about 3–4 molecules of dnaB protein hexamer were bound to the substrate at the time of addition of SSB. In the presence of the nonhydrolyzable ATP analogue App(NH)p or in the presence of ATP at 0°C, we presume that the bound dnaB protein molecules are randomly distributed on the single-stranded regions of the helicase substrate at the time of addition of SSB. When ATP hydrolysis is subsequently permitted, the bound dnaB protein molecules apparently migrate in a 5′ to 3′ direction along the SSB-coated single strand. The fraction of bound dnaB molecules that successfully migrates to one of the forks in the helicase substrate is uncertain. Nevertheless, even if each bound dnaB protein molecule moved to a fork in the helicase substrate, it appears unlikely that 4 dnaB protein molecules could unwind 1060 base pairs of duplex DNA in a single-stoichiometricaly helicase activity owing to the duplex DNA. Processive movement of one or more dnaB protein molecules during DNA unwinding is suggested.

It is not possible to obtain an accurate measurement of the rate of movement of the dnaB helicase through duplex DNA with the helicase substrate used here. Since detectable amounts of the short 1060-nucleotide-long fragment can be displaced within 30 s at 30°C (Fig. 9), the dnaB protein helicase must move at a rate of >35 base pairs/s. Substrates with long duplex regions will need to be used to determine if the optimal rate of strand unwinding in vitro approaches the high rate of fork movement that obtains during replication of the E. coli chromosome in vivo (800–900 base pairs/s at 37°C). The rate of unwinding observed in vitro is a minimal estimate and may primarily be a reflection of a slow step that occurs prior to the initiation of strand displacement, e.g., migration of dnaB protein along SSB-coated single-stranded DNA before it reaches the preformed fork. It might be expected that dnaB protein traverses SSB-coated single-stranded DNA slowly, since it presumably rarely encounters bound SSB in its path during its action as a replicative helicase.

In the absence of specific protein transfer reactions at a duplex replication origin, the dnaB protein helicase, like the phage T7 gene 4 helicase/primase (35), requires a preformed fork adjacent to the duplex region to initiate unwinding of model helicase substrates. We have not determined the minimum length of single-stranded DNA in the template strand that will enable dnaB protein to bind and initiate unwinding of a duplex DNA. Nuclease protection experiments, however, suggest that bound dnaB protein interacts with approximately 80 nucleotide residues of single-stranded DNA (48). The helicase substrate used in this report contains a 99-nucleotide-long single-stranded region at the 3′ terminus of the annealed fragment. dnaB protein, preferentially bound to the long single-stranded region of the circular M13 chromosome and moving in a 5′ to 3′ direction, apparently requires that the complementary strand contain a 3′-terminal extension of single-stranded DNA in order to initiate helicase action. The minimal length of this 3′-terminal extension that is required for dnaB protein helicase activity has not been precisely determined but is apparently >40 nucleotides. We base this conclusion on the finding that complementary strand fragments of 24 and 1503 base pairs are not unwound by dnaB protein, even if the fragments contain extensions of approximately 40 nucleotide residues of poly(dC) at their 3′ termini. 4

We found, in the experiments to determine helicase directionality (Figs. 12 and 13), that the bias toward preferential displacement of the 3′ end-labeled fragment (Fig. 11) is reduced at high dnaB protein concentrations (Fig. 13). In fact, at these elevated levels of dnaB protein, this bias is actually reversed when SSB is omitted from the reaction mixture, with the result that 5-fold more 5′ end-labeled fragment than 3′ end-labeled fragment is displaced from the linearized helicase substrate (Fig. 13, lanes 3 and 4). It is highly unlikely that the polarity of dnaB protein movement depends on the concentration of dnaB protein. Instead, we suggest that the binding of multiple dnaB protein molecules to the long central stretch of single-stranded DNA in the helicase substrate (Fig. 11) somehow interferes with movement of the dnaB protein toward the 3′ end-labeled fragment. Due to the much smaller size of the ss DNA tail (356 nucleotides) present in the 5′ end-labeled fragment (Fig. 11), it is much less probable that multiple dnaB protein molecules bind to this region of the helicase substrate. Of course, as the dnaB concentration increases, the probability of a dnaB molecule binding to the ss tail of the 5′ end-labeled fragment also increases. This fragment could then be displaced by normal 5′ to 3′ helicase action of the bound dnaB protein. How could attachment of multiple dnaB protein molecules to a long stretch of ss DNA interfere with helicase action? Conceivably, this could occur if (i) each dnaB protein hexamer binds to single-stranded DNA at multiple and widely separated sites (such that loops of single-stranded DNA are formed by independent binding of the individual dnaB protein subunits) and if (ii) additional dnaB protein molecules can bind to the domains of single-stranded DNA created by the binding of other dnaB molecules. Such multipoint attachment of a single dnaB protein molecule to widely separated sites on ss DNA has not been reported and is presumably precluded in vitro by the binding of SSB, but it is known that there are at least 4 binding sites for single-stranded DNA/dnaB protein hexamer (34). Multiple ss DNA-binding sites are presumably involved in the interaction of dnaB protein with the two strands of duplex DNA during its physiological function as a helicase.

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REFERENCES

1. Bonhoeffer, F. (1969) Z. VererbungsL. 98, 141-149
2. Horrie, Y., Ryter, A., and Jacob, F. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 677-693
3. Fangman, W. L., and Novick, A. (1968) Genetics 60, 1-17
4. Carl, P. L. (1970) Mol. Gen. Genet. 109, 107-122
5. Wechsler, J. A., and Gross, J. D. (1971) Mol. Gen. Genet. 113, 273-295
6. Wickner, S., Wright, M., and Hurwitz, J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 783-787
7. Ueda, K., McMacken, R., and Kornberg, A. (1978) J. Biol. Chem. 253, 261-269
8. Wickner, S., and Hurwitz, J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4120-4124
9. Schekman, R., Weis, J. W., Weiner, A., and Kornberg, A. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 5859-5865
10. Wold, M. S., Mallory, J. B., Roberts, J. D., LeBowitz, J. H., and McMacken, R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6176-6180
11. LeBowitz, J. H., and McMacken, R. (1984) Nucleic Acids Res. 12, 3069-3088
12. LeBowitz, J. H., Zylicz, M., Georgopoulos, C., and McMacken, R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3988-3992
13. Staudenbauer, W. L., Lanka, E., and Schuster, H. (1978) Mol. Gen. Genet. 158, 243-249
14. Staudenbauer, W. L., Scherzinger, E., and Lanka, E. (1979) Mol. Gen. Genet. 177, 113-120
15. Minden, J. S., and Marians, K. J. (1985) J. Biol. Chem. 260, 9316-9325
16. Fuller, R. S., Kagami, J. M., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7370-7374
17. Kagami, J. M., and Kornberg, A. (1984) Cell 38, 183-190
18. van der Ende, A., Baker, T. A., Ogawa, T., and Kornberg, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3954-3958
19. McMacken, R., Ueda, K., and Kornberg, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4190-4194
20. Arai, K., Low, R. L., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 707-711
21. Arai, K., Low, R., Kobori, J., Shlomai, J., and Kornberg, A. (1981) J. Biol. Chem. 256, 5275-5280
22. Arai, K., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 69-73
23. Georgopoulos, C. P., and Herskowitz, I. (1971) in The Bacteriophage Lambda (Hershey, A. D., ed) pp. 553-564, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Furth, M. E., McLeester, C., and Dove, W. F. (1978) J. Mol. Biol. 126, 185-255
25. Wickner, S. H. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 303-310
26. Klein, A., Lanka, E., and Schuster, H. (1980) Eur. J. Biochem. 105, 1-6
27. Tsurimoto, T., and Matsubara, K. (1981) Nucleic Acids Res. 9, 1789-1799
28. McMacken, R., Wold, M. S., LeBowitz, J. H., Roberts, J. D., Mallory, J. B., Wilkinson, J. A. K., and Loehrlein, C. (1983) in Mechanisms of DNA Replication and Recombination (Cozzarelli, N. R., ed) pp. 819-848, Alan R. Liss, Inc., New York
29. Dodson, M., Roberts, J. D., McMacken, R., and Echols, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4678-4682
30. LeBowitz, J. H., and McMacken, R. (1984) in Advances in Experimental Medicine and Biology (Hilbersch, U., and Spadari, S., eds) Vol. 179, pp. 77-89, Plenum Press, New York
31. Kornberg, A. (1980) DNA Replication, Freeman Publications, San Francisco
32. Taeuber-Scholz, G., Abdel-Monem, M., and Hoffmann-Berling, H. (1983) in Mechanisms of DNA Replication and Recombination (Cozzarelli, N. R., ed) pp. 65-76, Alan R. Liss, Inc., New York
33. Venkatesan, M., Silver, L. L., and Nossal, N. G. (1982) J. Biol. Chem. 257, 12426-12434
34. Liu, C.-C., and Alberts, B. M. (1981) J. Biol. Chem. 256, 2813-2820
35. Matson, S. W., Taihor, S., and Richardson, C. C. (1983) J. Biol. Chem. 258, 14017-14024
36. Arai, K., and Kornberg, A. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4308-4312
37. Wold, M. S., and McMacken, R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4907-4911
38. Arai, K., Yasuda, S., and Kornberg, A. (1981) J. Biol. Chem. 256, 5247-5252
39. Rowen, L., and Kornberg, A. (1978) J. Biol. Chem. 253, 758-764
40. Bee, M. D., and Champoux, J. J. (1983) Methods Enzymol. 101, 90-98
41. Burns, D. M., and Bechem, I. R. (1983) Anal. Biochem. 135, 48-54
42. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) in Molecular Cloning, pp. 122-123, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
43. Nelson, T., and Brugler, D. (1979) Methods Enzymol. 68, 41-50
44. Woodbury, C. P., Jr., and von Hippel, P. H. (1983) Biochemistry 22, 4730-4737
45. Reha-Krantz, L. J., and Hurwitz, J. (1978) J. Biol. Chem. 253, 4051-4057
46. Arai, K., and Kornberg, A. (1981) J. Biol. Chem. 256, 5253-5259
47. Sanger, F., Coulson, A. R., Hong, G. F., Hill, D. F., and Petersen, G. B. (1982) J. Mol. Biol. 162, 729-773
48. Arai, K., and Kornberg, A. (1981) J. Biol. Chem. 256, 5960-5966
49. Geider, K., and Hoffmann-Berling, H. (1981) Annu. Rev. Biochem. 50, 233-260
50. Kornberg, A. (1982) 1982 Supplement to DNA Replication, Freeman Publications, San Francisco
51. Nossal, N. G., and Alberts, B. A. (1983) in Bacteriophage T4 (Mathews, C. K., Kutter, E. M., Mosig, G., and Berget, P. B., eds) pp. 71-81, American Society of Microbiology, Washington, D.C.
52. Richardson, C. C. (1983) in Replication of Cellular and Viral Genomes (Becker, Y., ed) pp. 153-204, Martinus Nijoff, Boston
53. Reha-Krantz, L. J., and Hurwitz, J. (1978) J. Biol. Chem. 253, 4045-4050
54. Lanka, E., Edelbluth, C., Schlicht, M., and Schuster, H. (1978) J. Biol. Chem. 253, 5847-5851