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dnaB Protein of *Escherichia coli*

PURIFICATION AND ROLE IN THE REPLICATION OF ϕX174 DNA*

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The *dnaB* gene product of *Escherichia coli* has been purified about 15,000-fold to homogeneity, in 4 to 8% yield, from wild type cells and from cells which overproduce *dnaB* protein 5-fold; the latter cells harbor a ColE1 plasmid carrying the *dnaB* gene. The protein is an oligomer of 55,000-dalton subunits; a native molecular weight of 250,000 was estimated from sedimentation in a glycerol gradient. About 20 such molecules are calculated to be present per *E. coli* cell.

Assay for *dnaB* protein is based on an absolute requirement for it, along with 12 other proteins, to reconstitute *in vitro* replication of phage ϕX174 single-stranded DNA to a duplex replicative form. The inference that *dnaB* protein is a constituent of the nucleoprotein intermediate which precedes *dnaG* protein (primase) participation in ϕX174 DNA replication (Weiner, J. H., McMacken, R., and Kornberg, A. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 752-756) was strengthened by the observation that labeled *dnaB* protein is incorporated in the complex and that anti-*dnaB* antibody destroys the replicative activity of this intermediate. This antibody inhibits primer RNA synthesis by primase by preventing formation of the replication intermediate and by interfering with its action once formed. It does not, however, affect the subsequent elongation by DNA polymerase III holoenzyme. Anti-*dnaB* antibody inhibits semiconservative *E. coli* DNA replication in cell lysates; the inhibition is reversed by *dnaB* protein.

The *dnaB* gene product of *Escherichia coli* is essential for replication of the bacterial chromosome (1-5). It participates in chromosome growth as shown by immediate cessation of DNA synthesis which occurs when temperature-sensitive mutants are shifted to a restrictive temperature (5).

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dnaB protein has also been shown to be necessary for conversion of phage ϕX single-stranded DNA[1] to the duplex replicative form (RF) (6-11) and to be required for the multiplication of RF at the stage of (−)-strand synthesis (12). Two other small coliphages with SS genomes, M13 and G4, however, do not require this gene function for their conversion to RF (6, 7, 10, 13). Hurwitz and associates purified *dnaB* protein (14) and found the native molecular weight to be about 250,000. Lark and Wechsler (15) and Kogoma (16) have analyzed DNA replication in various *dnaB* mutants *in vivo*, and pointed out the likelihood of interactions among *dnaB* protein subunits and the possibility of multiple forms of *dnaB* protein in cells.

dnaB protein has nucleoside triphosphatase activity and forms a complex with *dnaC* protein in the presence of ATP (14, 17). Recently, the ϕX SS replicative system has been successfully reconstituted (10, 11, 18) from partially purified proteins. *dnaB* protein is involved together with four other proteins (DBP, *dnaC* protein, proteins i and n) in the formation of a replication intermediate which precedes primase function (18, 19). Kinetic analysis has suggested that *dnaB* protein participates in the intermediate formation as a stoichiometric constituent of the complex rather than as a catalyst in its formation (19), but the role of this intermediate in events leading to chain elongation was not understood.

To study the role of *dnaB* protein in DNA replication, we have undertaken extensive purification of the protein and an examination of its properties. We report here the large scale purification of *dnaB* protein and some of its physical and functional properties, particularly in phage ϕX DNA replication.

MATERIALS AND METHODS

Chemicals and Enzymes

DEAE-cellulose (DE-52) and phosphocellulose (P-11) were purchased from Whatman; hydroxyapatite from Clarkson Chemical Co. (Williamsport, Pa.); Bio-Gel A-1.5m and A-5m (both 200 to 400 mesh) from Bio-Rad; [*methyl*-^{3}H]dTTP, [5,6-^{3}H]UTP, [α-^{32}P]dGTP, [^{32}P]orthophosphoric acid, NaB^3H_4 from New England Nuclear; Freund's complete adjuvant from Difco Laboratories. [γ-^{32}P]ATP

[1] The abbreviations used are: ϕX, ϕX174; SS, single-stranded; RF, replicative form; SDS, sodium dodecyl sulfate; albumin, bovine serum albumin; DBP, DNA-binding protein; holoenzyme, DNA polymerase III holoenzyme; primase, *dnaG* protein; NTP, nucleoside triphosphate.

was prepared from [^{32}P]orthophosphate. Polypeptide standards (and their molecular weights) (20) were: egg white lysozyme (14,300) from Calbiochem; chymotrypsinogen A (25,700) and ovalbumin (43,000) from Sigma; human hemoglobin (63,000), prepared as described previously (21); albumin (68,000) from Pentex; beef liver catalase (244,000) and *Escherichia coli* β-galactosidase (540,000) from Worthington. [^{32}P]φX174 *am3* phage was a gift of Dr. B. Tye of this department. Other reagents were from sources previously described (10, 11, 21).

Buffers

Buffer A is 50 mM Tris·Cl (pH 7.5), 20% (v/v) glycerol, 1 mM EDTA. NaCl was added as specified. Buffer B is 20 mM potassium phosphate (pH 6.5), 20% (v/v) glycerol, 1 mM EDTA. KCl was added as specified. Assay buffer contained 50 mM Tris·Cl (pH 7.5), 10% sucrose, 20 mM dithiothreitol, and 0.2 mg/ml of albumin.

Bacterial Strains and Growth

E. coli HMS83 (*polA1, polB1, thy, lys*), a strain of K12 constructed by Campbell *et al.* (22), was used for the main preparation of *dnaB* protein. H560 (F$^+$, *polA1, endA*), and BT1029 (*dnaB$_{ts}$, polA1, endA, thy*) (originally isolated by F. Bonhoeffer and co-workers) were provided by Dr. Y. Hirota (National Institute of Genetics, Japan). RLM365 (pLCll-9) is a spontaneous *polA*$^+$ derivative of HMS83 which contains a ColE1 hybrid plasmid (pLCll-9) originally obtained from clone JA200 (pLCll-9) of the Clarke and Carbon colony bank (23); the plasmid contains the chromosome of colicinogenic factor E1 and a fragment of the *E. coli* chromosome including the *dnaB* gene region and was identified (23) by screening the Clarke and Carbon colony bank for those clones which could complement thermosensitive *dnaB* mutants following conjugation.

E. coli HMS83 and RLM365 (pLCll-9) were grown in AZ broth (which contains per liter: 10 g of K_2HPO_4, 1.85 g of KH_2PO_4, 10 g of Ardamine Z yeast extract (Yeast Products Inc., Clifton, N.J.), 10 g of glucose, 50 mg of thymine, 10 mg of vitamin B_1) to A_{590} = 8 (3/4 log phase) in a Fermacell Fermentor (New Brunswick Scientific Co.) at 37° with strong aeration. The pH was maintained between 7.0 and 7.2 by addition of NaOH. Cells were harvested in a Sharples continuous flow centrifuge at 25°, suspended at A_{590} = 200 (8×10^{10} cells/ml) in 10% sucrose, 50 mM Tris·Cl (pH 7.5), and frozen in liquid N_2. Cells stored at −20° retained full *dnaB* activity for at least 6 months. H560 and BT1029 were grown similarly except that Hershey broth (7) supplemented with thymine was used and the harvest was at A_{590} = 0.5.

Preparation of Replication Proteins and DNA

Proteins necessary for φX SS to RF replication, assayed as previously described (11), and partially purified from *E. coli* HMS83 were: DBP (Fraction 3b, 1.63 mg/ml, 28,000 units/mg) (24); protein n (Fraction IV, 32,000 units/ml, 3,800 units/mg) (11); protein i (Fraction V, 80,000 units/ml, 210,000 units/mg); protein u (25) (Fraction V, 18,000 units/ml, 75,000 units/mg); *dnaC* protein (Fraction V, 10,000 units/ml, 67,000 units/mg); *dnaG* protein (Fraction V, 50,000 units/ml, 200,000 units/mg); holoenzyme (26) (Fraction IV, 50,000 units/ml, 20,000 units/mg). Procedures for purification of these proteins will be published elsewhere. DNA-cellulose-binding fraction was prepared as previously described (11).

Soluble extract of BT1029 was prepared as described for Fraction I from HMS83 (see "Results") except that heating at 37° was for 1.5 min instead of 4 min and centrifugation was for 30 min at 53,000 × *g* rather than at 32,000 × *g*.

φX SS was prepared by the method of Ray (21, 27).

Preparation of Antibodies

Antibody against protein i was prepared as previously described (19). Antibody against *dnaB* protein was prepared essentially in the same way by an initial injection of 155 μg of purified *dnaB* protein (Fraction IV, 900,000 units/mg) followed by a booster (75 μg) 3 weeks later. Blood was collected 8 days after the booster, and the γ-globulin was purified to homogeneity (28). Control γ-globulin was prepared from serum of unimmunized rabbits.

Assays of dnaB Protein

dnaB protein activity was assayed by measuring conversion of φX SS to RF in cooperation with other *E. coli* proteins. Three different systems were used.

Assay A – The *partial reconstitution assay* identical with the "Stage III" system of Schekman *et al.* (11) consisted of 10 to 15 μl of assay buffer, DNA-cellulose-binding fraction (15 μg of protein), protein i (0.06 μg), *dnaC* protein (0.3 μg), a *dnaB* sample, and a mixture containing 100 nmol of spermidine·Cl, 20 nmol of ATP, 2.5 nmol each of GTP, CTP, and UTP, 0.3 nmol (as nucleotide) of φX DNA (SS), 0.1 μg of rifampicin, 120 nmol of $MgCl_2$, 0.45 nmol of [^{3}H]dTTP (350 Ci/mol), and 1.2 nmol each of dATP, dGTP, and dCTP (total volume, 25 μl). The components, mixed at 0° in this order, were incubated for 10 min at 30°. Incubation was terminated by chilling the mixture on ice and by adding about 40 μl of 0.2 M sodium pyrophosphate and 0.5 ml of 10% trichloroacetic acid. The resulting precipitate was collected on glass fiber filters (Whatman GF/C), washed three times with 3 ml of 1 N HCl, 0.1 M sodium pyrophosphate and once with 3 ml of ethanol, dried, and counted in a toluene-based scintillation fluid in a Nuclear Chicago scintillation spectrometer.

Assay B – The *total reconstitution assay* was identical except that (*a*) DNA-cellulose-binding fraction was replaced by five purified proteins (DBP (4.9 μg), protein n (4.2 μg), *dnaG* protein (0.5 μg), holoenzyme (1.3 μg), and protein u (2.0 μg)), (*b*) φX SS was first mixed with DBP, and (*c*) ATP concentration was lowered (6.7 nmol/25 μl).

Assay C – The *complementation assay* was performed by incubating a soluble extract (Fraction I, preheated at 37° for 10 min) of BT1029 with the *dnaB* sample, φX SS and the low molecular weight components of Assay A at 30°.

In all assays, one unit is defined as incorporation of 1 pmol of total deoxynucleotide into an acid-insoluble form in 1 min; the value for dTTP incorporation was multiplied by 4.

Two-stage DNA Replication

This was carried out as before (19). In the first stage (replication intermediate formation), the reaction mixture (incubated 20 min at 30°) contained *dnaB* protein, *dnaC* protein, DBP, protein i, protein n, protein u, φX SS, spermidine, ATP, $MgCl_2$, and assay buffer in the same amounts as in Assay B, but in a total volume of 15 μl. The second stage (primer synthesis and DNA elongation) was carried out at 30° for 3 min after addition of anti-protein i γ-globulin (8 μg), and the amounts of primase, GTP, CTP, UTP, rifampicin, [^{3}H]dTTP, dATP, dGTP, dCTP, and holoenzyme used in Assay B.

Three-stage DNA Replication

This consisted of a first stage as above (incubation time 10 min) and a second stage (primer synthesis) performed by adding primase, the three rNTPs and rifampicin, as above, and incubating for 10 min at 30°. The third stage (DNA elongation) (3 min at 30°) was performed by adding the remaining components of the two-stage replication (see above).

E. coli Chromosome Replication on Cellophane Discs

The procedure (29, 30) consisted of prelabeling H560 cells with [^{14}C]thymidine (1.8 Ci/mol) for 1 h at 37°, washing, and resuspending the cells in nonradioactive medium, and spreading and lysing the cells with lysozyme and Brij 58 on cellophane discs placed on agar. The lysate was incubated for 30 min at 30° in a DNA synthesis mixture which included [^{3}H]thymidine (315 Ci/mol). The amount of DNA synthesis was corrected for variation in the number of cells on a disc by the ^{14}C value.

Assay of ATPase

The reaction mixture (25 μl) contained 15 μl of assay buffer, 120 nmol of $MgCl_2$, 20 nmol of [γ-^{32}P]ATP (2 to 6 Ci/mol), 0.3 nmol of φX SS DNA (as nucleotide), and 0.1 μg of rifampicin; DNA was omitted in assays of the replication intermediate. Incubation was for 10 min at 30°, and the reaction was terminated by addition of 100 μl each of cold 7% perchloric acid and Norit (20% (w/v) in H_2O). The mixture was stirred on a Vortex mixer, and centrifuged for 10 min at 5,000 × *g*. ^{32}P was measured in a 25-μl aliquot of the supernatant. One unit is the release of 1 pmol of inorganic phosphate (measured as ^{32}P unadsorbed to Norit) in 1 min.

Immunodiffusion

Double diffusion (31) was performed on a microscope slide coated with 0.8% agarose containing 10 mM Tris·Cl (pH 8.0), 0.14 M NaCl, and 1% Triton X-100. Wells were punched out with a template

(Gelman Instrument Co., Ann Arbor, Mich.), and samples of 1 to 5 μl were added. Precipitates developed within 3 days at room temperature. The slide was treated with 0.5% phosphomolybdic acid for 10 min (to brighten the precipitin pattern (32)) and photographed under darkfield illumination.

SDS-Polyacrylamide Gel Electrophoresis

Analytical electrophoresis was carried out in a slab gel of 10% polyacrylamide and 0.1% SDS. The buffer system of Marco *et al.* (33) and the Studier apparatus (34) were used. Samples were precipitated with 10% trichloroacetic acid, dissolved in 50 μl of sample buffer (2 M Tris, 30% glycerol, 10% SDS, 1 M 2-mercaptoethanol, 0.1% bromphenol blue), neutralized with 5 μl of 1 N HCl, and heated for 2 min at 100° before application to the slab. A constant current of 12 mA per slab (15 × 11 × 0.1 cm) was applied until the dye marker entered the gel; 36 mA was used thereafter.

Glycerol Gradient Sedimentation

A 0.1-ml mixture of *dnaB* protein and standard proteins was applied to a 5-ml glycerol gradient (15 to 35%) containing 20 mM potassium phosphate (pH 6.8), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM $MgCl_2$. Gradients were centrifuged for 15 h at 0° at 50,000 rpm in a Beckman SW 50.1 rotor. Two-drop fractions were collected from the tube bottom.

Gel Filtration

Gel filtration of *dnaB* protein was performed by applying a 0.14-ml sample to a column of Bio-Gel A-1.5m (200 to 400 mesh, 0.7 × 21 cm) and eluting with Buffer B containing 0.1 M KCl and 50 μg/ml of albumin at a flow rate of 2 ml/h. Three-drop fractions were collected.

Gel filtration of the replication intermediate containing ^{3}H-labeled *dnaB* protein (see below) was performed by filtering 78 μl of a first stage reaction mixture (from a two-stage DNA replication assay; see above) through Bio-Gel A-5m (200 to 400 mesh, 0.7 × 8 cm) (19). Four-drop fractions were collected, chilled, and assayed for both replication activity (by incubating with the second stage mixture) and radioactivity.

^{3}H-labeling of dnaB Protein

Radioactive labeling of *dnaB* protein was performed at 0–2° by a modification of the reductive alkylation method of Rice and Means (35). The *dnaB* protein (Fraction V; 12 μg in 100 μl of 0.1 M potassium phosphate (pH 6.5), containing glycerol, KCl, and EDTA) was brought to pH 8 with 0.2 M NaOH, and treated with 160 nmol of formaldehyde. After 30 s, NaB^3H_4 (160 nmol, 6 Ci/mmol, freshly dissolved in 0.01 M NaOH to 20 mM) was added. After 2 min, 6 μl of 2 M Tris·Cl (pH 6.5) was added and the total mixture was dialyzed against 300 ml of Buffer A. The outer liquid was changed after 2 h and again after 4 h. Specific radioactivity of the preparation was 6 × 10^4 cpm/μg (0.15 μCi/μg); only about 10% of the original *dnaB* activity was recovered. For preparation of replication intermediate, ^{3}H-labeled *dnaB* protein was further purified on a Bio-Gel A-5m column; only the fractions containing active *dnaB* protein were used.

Other Methods

β-Galactosidase activity was determined by the method of Craven *et al.* (36), catalase by the method of Beers and Sizer (37), hemoglobin by absorbance at 405 nm, and protein by the method of Lowry *et al.* (38) with albumin as the standard.

RESULTS

Purification

dnaB protein was purified approximately 15,000-fold from a lysate of HMS83 cells (Table I). (Unless noted, all operations were performed at 0–4°).

Preparation of Soluble Extract—Frozen *Escherichia coli* HMS83 cell paste (2900 g) was thawed at 0–4° in 300- to 500-g portions, and adjusted to pH 7.5 with solid Tris base. To the suspension were added lysozyme, NaCl, and spermidine·Cl, to achieve final concentrations, respectively, of 0.2 mg/ml, 0.1 M, and 0.01 M. After gentle mixing, the suspension was distributed in 250-ml centrifuge bottles and, after 45 min at 0°, the bottles were placed in a 37° bath for 4 min, mixed by inversion each min, and then centrifuged for 60 min at 27,000 × *g* in a Sorvall GSA rotor. The supernatant fluid was decanted (Fraction I; 10 liters).

TABLE I

Purification of dnaB protein

Fraction	Total activity	Total protein	Specific activity	Yield	Purification
	units × 10^{-3}	*mg*	*units/mg*	%	*-fold*
I. Lysate	22,300	343,000	65	100	1
II. Ammonium sulfate	7,100	6,440	1,100	32	17
III. DEAE-cellulose	2,150	27	80,000	9.6	1,230
IV. Phosphocellulose	830	1.91	435,000	3.7	6,690
V. Hydroxyapatite	830	0.83	1,000,000	3.7	15,400

Ammonium Sulfate Fractionation—Solid ammonium sulfate (0.226 g/ml) was added to Fraction I. The mixture was stirred for 30 min; the precipitate was collected by centrifugation (17,000 × *g* for 25 min), resuspended in a solution (1/8 volume of Fraction I) of ammonium sulfate in Buffer A (0.2 g of salt to each ml of Buffer A), and collected again by centrifugation (17,000 × *g*, 25 min). The precipitate was washed with Buffer A containing ammonium sulfate (0.16 g of salt added to each milliliter of buffer) (1/50 volume of Fraction I), collected by centrifugation (31,000 × *g* for 15 min) and dissolved in a minimal volume of Buffer B containing 1 mM dithiothreitol[2] (Fraction II, 132 ml).

DEAE-cellulose—Fraction II was dialyzed against 2.5 liters of Buffer B containing 0.08 M KCl and 1 mM dithiothreitol for 9 h, and clarified by centrifugation (27,000 × *g* for 20 min). Forty-milliliter portions were diluted with 2 volumes of Buffer B containing 1 mM dithiothreitol to achieve the same conductivity as the buffer used to equilibrate the column (see below). The diluted sample was applied (over a 6-h period) to a DEAE-cellulose column (7.5 × 12.5 cm) equilibrated with Buffer B containing 0.08 M KCl and 1 mM dithiothreitol. The column was washed with 100 ml of the equilibrating buffer and then with 820 ml of Buffer B containing 0.27 M KCl and 1 mM dithiothreitol. Elution of adsorbed *dnaB* protein was with 750 ml of Buffer B containing 0.37 M KCl at 150 ml/h; 12.5-ml fractions were collected. *dnaB* protein activity appeared in the first fractions in which conductivity was increasing. Peak fractions were combined, diluted 3.5-fold with Buffer B, and applied (over a 6-h period) to a second DEAE-cellulose column (10.4 cm^3, 1.6 × 5.2 cm) equilibrated with Buffer B containing 0.1 M KCl. The column was washed with 75 ml of Buffer B containing 0.27 M KCl and *dnaB* protein was eluted with a 60-ml gradient of KCl from 0.27 to 0.40 M in Buffer B at 50 ml/h; 1.7-ml fractions were collected. The *dnaB* protein peak fractions, eluted between 0.29 and 0.39 M KCl, were combined (Fraction III, 60 ml).

Phosphocellulose—Solid ammonium sulfate (0.3 g/ml) was added to Fraction III. The mixture was stirred for 30 min; the precipitate was centrifuged (30 min at 44,000 × *g*), suspended in 3.5 ml of Buffer B, dialyzed for 4 h against 1 liter of Buffer B, and clarified by centrifugation for 15 min at 15,000 × *g*. The supernatant was diluted 3-fold with Buffer B (final conductivity corresponding to 0.03 M KCl in Buffer B), applied

[2] *dnaB* protein does not require sulfhydryl reducing agents for activity and stabilization. Dithiothreitol was added in early stages of purification because other replication proteins were prepared from side fractions of these steps.

to a phosphocellulose column (1 × 11 cm) equilibrated with Buffer B, and eluted at 3.5 ml/h with 5-ml portions of Buffer B containing in succession 0.05 M, 0.1 M, 0.14 M, and 0.2 M KCl; 0.8-ml fractions were collected. Under these conditions, *dnaB* protein (>95%) eluted slightly behind the bulk of unadsorbed protein. Ammonium sulfate (0.3 g/ml) was added, the mixture stirred for 30 min, and centrifuged at 35,000 × *g* for 25 min. Precipitate was dissolved in 1.9 ml of Buffer B containing 0.1 M KCl and clarified by centrifugation for 15 min at 30,000 × *g*. The supernatant was dialyzed against 1 liter of Buffer B for 2 h and again for 6 h. The dialyzed material was applied to a second phosphocellulose column (1 × 10 cm) equilibrated with Buffer B. The column was washed with 8 ml of the same buffer and eluted at 2 ml/h with a linear gradient (40 ml) of KCl from 0.03 to 0.4 M in Buffer B; 0.8-ml fractions were collected. Totally adsorbed *dnaB* protein activity was eluted between 0.2 and 0.32 M KCl (Fraction IV, 14 ml) (Fig. 1*A*).

Hydroxyapatite—A portion of Fraction IV (0.5 mg of protein) was applied to a hydroxyapatite column (0.7 × 3 cm) equilibrated with Buffer B containing 0.25 M KCl. The column was washed with 4.5 ml of the same buffer solution and then 2.5 ml of the buffer solution containing 35 mM phosphate. Elution was with a linear gradient (13 ml) of potassium phosphate from 35 to 200 mM at 40 ml/h; 1-ml fractions were collected. The activity peak appeared at 60 mM phosphate (Fig. 1*B*). Peak fractions from identical columns were pooled (Fraction V) and stored in liquid nitrogen.

Purification of *dnaB* protein on a smaller scale (100 g of cells) was successfully completed even when the first DEAE-cellulose and phosphocellulose chromatography steps were omitted.

Purification of dnaB protein from E. coli H560 and RLM365 (pLC11-9) Cells—*dnB* protein was also purified from *E. coli* strains H560 and RLM365 (pLCll-9). From 350 g of H560 cells a preparation with 1,100,000 units/mg of protein (16,000-fold purification) was obtained in 8% yield. Similar yield and specific activity were obtained from RLM365 (pLCll-9), the strain with a 5-fold enhanced level of *dnaB* protein activity due to the presence of the *dnaB* gene on a hybrid ColE1 plasmid.

Criteria of Purity and Stability

Purified *dnaB* protein migrated as a single band on electrophoresis in a 10% polyacrylamide gel slab with 0.1% SDS in a Tris/glycine system (Fig. 2). An impurity could have been detected if present at a level of 2%.

A single precipitin line was produced between anti-*dnaB* antibody and purified *dnaB* protein in an Ouchterlony double immunodiffusion analysis (Fig. 3). Antibody had been developed against the purest peak fractions of *dnaB* protein (900,000 units/mg) derived from the second phosphocellulose step (Fraction IV). A single precipitin line with no *spur* in response to the antibody given by all *dnaB* protein samples from the various stages of purification (Fractions III, IV, and V) indicates that the preparation used for immunization was relatively pure and that there is a single and common antigen at various stages of purity.

dnaB protein at the DEAE-cellulose step or beyond was stable for at least 6 months when stored in liquid nitrogen. At 0°, the protein (40 μg/ml) lost 50% of its activity after a month; at 30°, 50% was lost after 10 min.

Physical Properties

Molecular Weight of Subunit—The single protein band in SDS-polyacrylamide gel electrophoresis indicates that *dnaB* protein is made up of one or more polypeptides of molecular weight about 55,000 (Fig. 4); the same molecular weight was obtained for the protein purified from H560 cells.

Glycerol Gradient Centrifugation—The native molecular weight of *dnaB* protein was estimated by glycerol gradient centrifugation from a single peak which sedimented slightly faster than a catalase marker (Fig. 5*A*) indicating a sedimentation coefficient of about 11.5 S and corresponding to a molecular weight, for a globular protein, near 250,000. This agrees with values reported by Wright *et al.* (9) and Schekman *et al.* (11). Under these conditions, *dnaB* protein appears to behave as a tetramer. When sedimentation analysis was performed without $MgCl_2$, essentially no *dnaB* activity was recovered from the gradient (Fig. 5*B*), suggesting that Mg^{2+} is needed to stabilize the active tetrameric structure under these conditions.

Bio-Gel A-1.5m Filtration—The behavior of *dnaB* protein on gel filtration suggests multiple forms depending on $MgCl_2$. In its presence, *dnaB* protein emerged with hemoglobin, suggesting a monomeric or an asymmetric form penetrating the gel. In the absence of $MgCl_2$, *dnaB* activity appeared in two peaks corresponding to molecular weights of 220,000 and 110,000 (Fig. 6*B*), suggestive of tetrameric and dimeric forms.

Functional Properties

Requirement of dnaB Protein for Conversion of ϕX SS to RF—The requirement for *dnaB* protein (6, 9, 11, 18) was

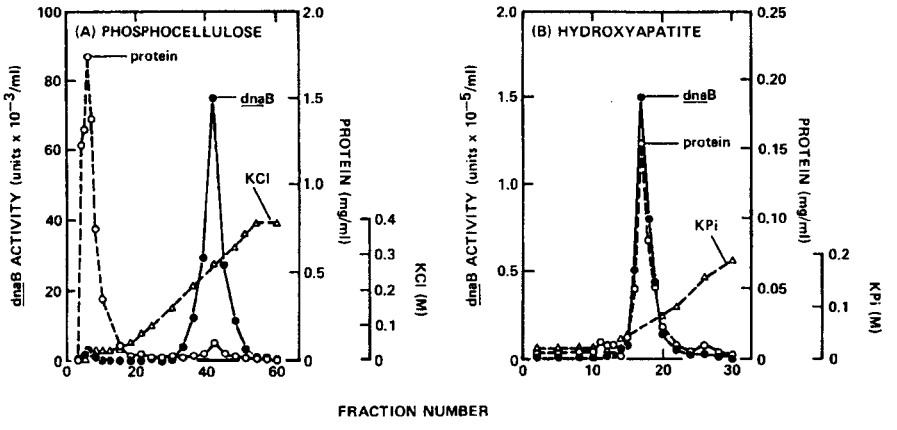


FIG. 1. Chromatography of *dnaB* protein on phosphocellulose and hydroxyapatite.

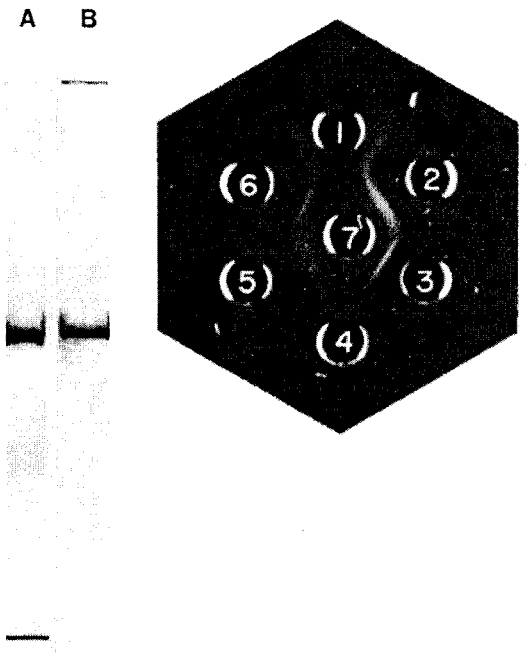


FIG. 2 (*left*). SDS-polyacrylamide gel electrophoresis of *dnaB* protein. *A*, Fraction V from HMS83 (6 μg); *B*, Fraction V from H560 (4 μg).

FIG. 3 (*right*). Double immunodiffusion analysis of *dnaB* protein. *Wells 1*, *7*, and *4* contained γ-globulins; *1*, anti-*dnaB* protein 14 μg (1 μl); *7*, anti-*dnaB* protein 70 μg (5 μl); *4*, control 40 μg (5 μl). Other wells contained *dnaB* protein preparations: *2*, Fraction III 30 units (5 μl); *3* and *6*, Fraction V 30 units (5 μl); *5*, Fraction IV 35 units (4 μl).

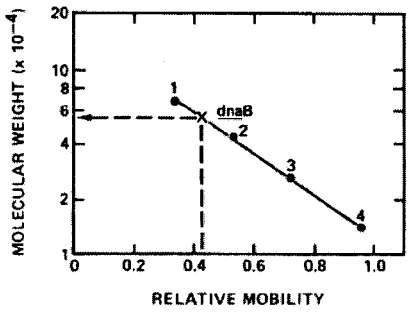


FIG. 4. Determination of *dnaB* polypeptide molecular weight by SDS-polyacrylamide gel electrophoresis. Protein standards were: *1*, albumin (68,000); *2*, ovalbumin (43,000); *3*, chymotrypsinogen A (25,700), and *4*, lysozyme (14,300). Mobilities are expressed relative to the marker dye, bromphenol blue.

observed in the reconstituted system (Fig. 7). Similar results were also obtained with the partially reconstituted system (data not shown). Rate of DNA synthesis was a linear function of *dnaB* protein concentration up to 8 units (Fig. 7*A*). A decrease in DNA synthesis at higher *dnaB* protein concentrations may be due to direct inhibition by excess *dnaB* protein. DNA synthesis proceeded almost linearly for about 10 min at 30° and leveled off when about 70% of the added template circles were replicated (Fig. 7*B*).

Complementation of dnaB Extract – Purified *dnaB* protein was capable of complementing extracts from the *dnaB* mutant

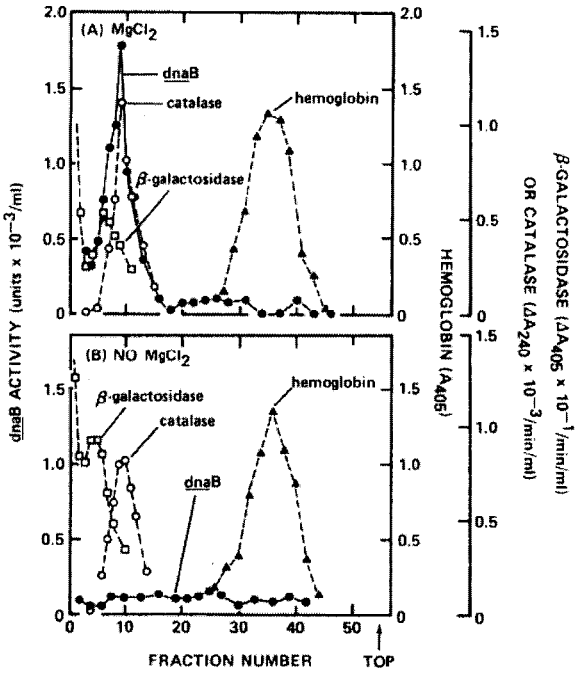


FIG. 5. Glycerol gradient centrifugation of *dnaB* protein. The *dnaB* protein applied was Fraction V (1600 units). Recovery of *dnaB* activity in *A* was about 60%.

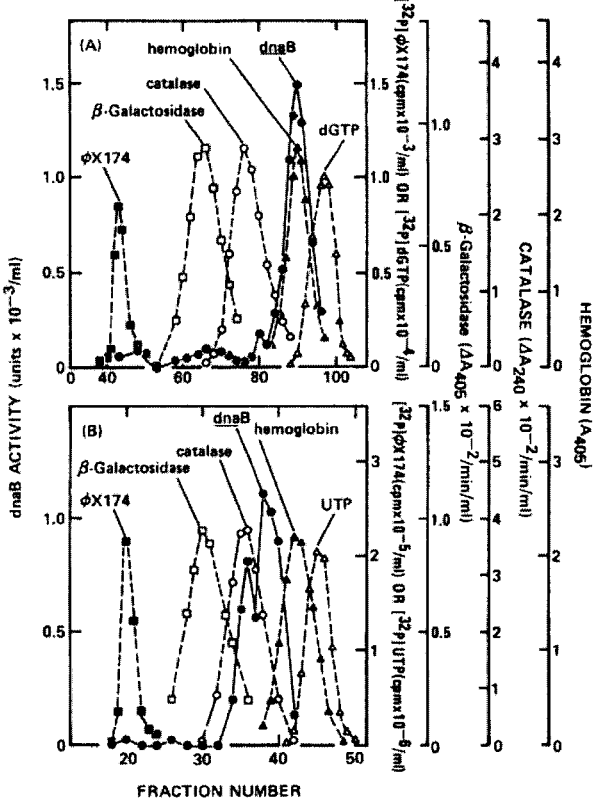
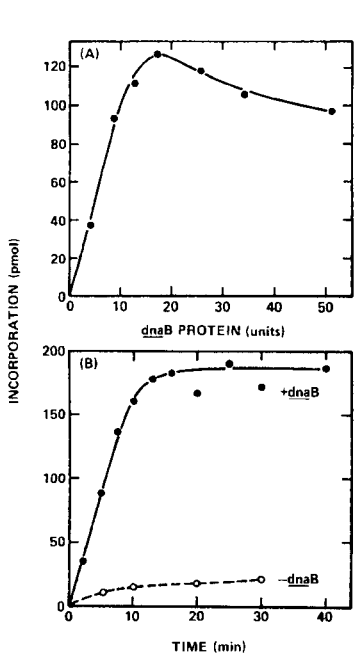
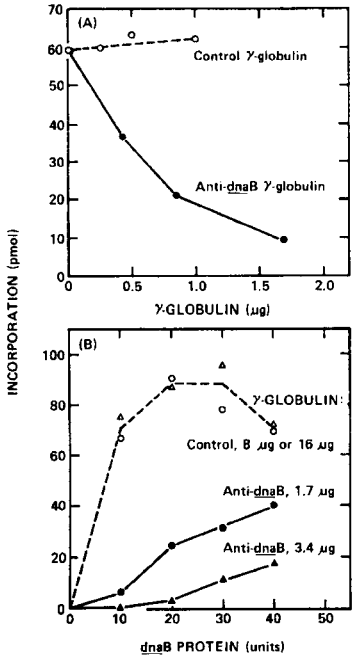


FIG. 6. Bio-Gel A-1.5m filtration of *dnaB* protein. *dnaB* protein (Fraction V, 3000 units) was filtered in the presence (*A*) or absence (*B*) of 10 mM $MgCl_2$. In *A*, a larger column (0.7 × 30 cm) was used and 2-drop fractions were collected. Recovery of *dnaB* activity was about 40% in both cases.





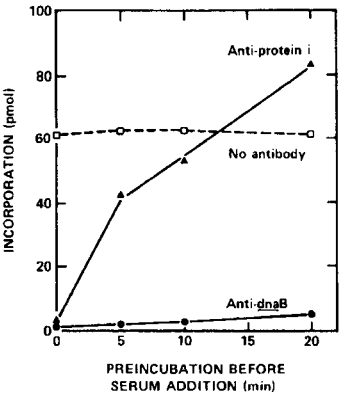


FIG. 7 (*left*). Dependence of φX DNA replication on *dnaB* protein in the total reconstitution system. *A*, varying amounts of *dnaB* protein (Fraction V) were added to the standard assay mixture; *B*, a 12-fold standard mixture containing 120 units of *dnaB* protein (Fraction V) was incubated at 30°; 25-μl aliquots were analyzed at intervals.

FIG. 8 (*center*). Effects of anti-*dnaB* antibody on φX DNA replication. *A*, the reaction (standard conditions) contained 15 units of *dnaB* protein (Fraction V). Indicated amounts of γ-globulin were added and the mixtures incubated for 15 min at 0° before addition of φX SS, spermidine, rifampicin, $MgCl_2$, rNTPs, and dNTPs. *B*, varying amounts of *dnaB* protein were treated with γ-globulin for 15 min at 0°; after addition of other components as in *A*, incubation was for 10 min at 30°.

FIG. 9 (*right*). Effects of antibodies on replication intermediate formation and activity. A standard two-stage replication mixture (20-times scale, ATP increased 3-fold) was incubated at 30°, and, at intervals indicated, 20-μl aliquots (20 μl) were mixed with 1 μl of either assay buffer (no antibody) or the indicated antiserum. Mixtures were then incubated to a total of 20 min at 30° to complete the first stage and finally supplemented with second stage components (in 5 μl) and kept 2 min longer at 30°.

(BT1029) for φX SS to RF conversion, but *dnaC* protein was occasionally also necessary for maximal restoration of replication activity, as described previously (data not shown) (11).

Inhibition of dnaB Protein Activity by Specific Antibody—Antibody produced against purified *dnaB* protein inhibited φX DNA replication (Fig. 8*A*). At 1 μg, anti-*dnaB* γ-globulin neutralized about 10 units of *dnaB* protein activity. The slight activation by the control γ-globulin may be due to protection of the system from inactivation during the preliminary incubation. Inhibition by anti-*dnaB* antibody was partially reversed by the addition of excess *dnaB* protein (Fig. 8*B*). Failure by even large amounts of *dnaB* protein to reverse it fully is probably due to the inhibitory effect of excess *dnaB* protein noted earlier.

Inhibition of Replicative Intermediate Activity by Anti-dnaB Antibody—The *dnaB* protein becomes part of a replication intermediate required by primase in its priming action (39, 40). Addition of anti-*dnaB* antibody at any point during or after formation of the intermediate produces profound inhibition (Fig. 9). By contrast, action of protein i, also essential for producing the replication intermediate (19), becomes resistant to anti-protein i antibody once the intermediate is formed (Fig. 9). Actions of antibodies against other participants in forming the replication intermediate fall into two classes (39, 40): Class 1, proteins such as *dnaB* and DBP, which participate stoichiometrically and appear to become part of the intermediate complex, were inhibited by their respective antibodies after, as well as before, formation of the complex; Class 2, proteins i and n, which appear to act catalytically (19), were susceptible to antibody only before the complex was formed.

Association of ^{3}H-labeled dnaB Protein with Intermediate—Direct demonstration of *dnaB* protein in the replication intermediate was made with ^{3}H-labeled *dnaB* protein. The protein by itself was fully retained upon filtration through a column of Bio-Gel A-5m; after formation of the intermediate the radioactive protein was excluded from the gel along with the replicative activity indicative of the intermediate (Fig. 10*A*). Without incubation to produce the intermediate, only one-fourth as much labeled protein was found in the excluded fraction (Fig. 10*B*).

Inhibition of Primer RNA Synthesis by Anti-dnaB Antibody—The *dnaB* protein bound in replication intermediate participates in primer RNA synthesis as suggested by experiments with anti-*dnaB* antibody (Table II). Primer synthesis by primase was profoundly inhibited; by contrast, anti-protein i antibody had no effect. Incorporation under these conditions shows extent rather than rate of RNA synthesis.

DNA Elongation Step Not Inhibited by Anti-dnaB Antibody—Although anti-*dnaB* antibody inhibited formation of the replication intermediate (Fig. 9) and its function in the synthesis of primer (Table II), it had no significant effect on the final stage of DNA synthesis (Table III). Anti-protein i antibody, which inhibited the first stage, had no effect on the next two stages. Thus *dnaB* protein is not required for DNA elongation once the primer is formed. The fate of the bound

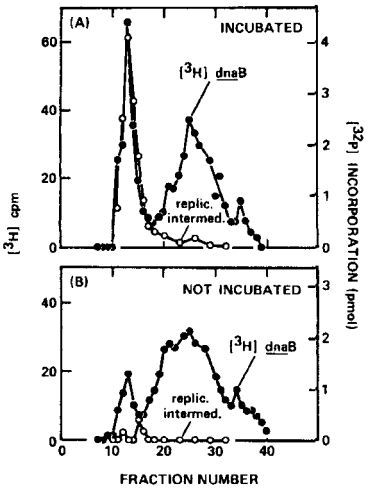


FIG. 10 (*left*). Association of [^{3}H]*dnaB* protein with the replication intermediate. ^{3}H-labeled *dnaB* protein (2000 cpm, about 4 units) was mixed with other components of the first stage mixture (7-times scale, 91 μl). After incubation for 15 min at 30° (in *A*) and 0 min (in *B*), 13 μl were analyzed for replicative activity. The rest of the mixture was applied to a Bio-Gel A-5m column (0.7 × 8 cm), then eluted at 24° as described ("Materials and Methods"). Replicative activity was assayed by incubating (3 min at 30°) with second stage components containing [α-^{32}P]dNTPs. Recoveries of ^{3}H and replicative activity were >90%.

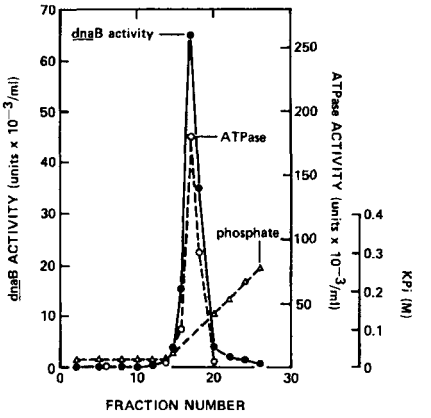


FIG. 11 (*right*). ATPase activity of *dnaB* protein. *dnaB* protein (Fraction IV, 750 μg) was applied to a hydroxyapatite column (0.7 × 2.6 cm), washed with 6 ml of 20 mM potassium phosphate (pH 6.5) containing 0.25 M KCl, 20% glycerol, and 1 mM EDTA, and eluted with a linear gradient (13 ml) of phosphate from 20 to 260 mM. Fractions (0.9 ml) were collected and assayed for *dnaB* protein and ATPase activity. Phosphate was determined by conductivity.

TABLE II

Inhibition of primer RNA synthesis by anti-dnaB antibody

Primer RNA was synthesized on the replication intermediate (formed by incubating the first stage components for 15 min at 30°) by further incubation with the second stage components for 10 min at 30°. Conditions are given under "Materials and Methods," except that NTP concentrations were: 80 μM ATP, 40 μM each of GTP and CTP, and 4 μM [5,6-^{3}H]UTP (10 Ci/mmol). γ-Globulins were added in the second stage as specified.

γ-Globulin	[^{3}H]UMP incorporated
	pmol
None	0.362
Nonimmune (8 μg)	0.368
Anti-protein i (8 μg)	0.357
Anti-*dnaB* (4 μg)	0.072
Anti-*dnaB* (14 μg)	0.059
Anti-protein i (8 μg) + anti-*dnaB* (14 μg)	0.050

TABLE III

Effects on DNA replication of anti-dnaB antibody added at three stages

See "Three-stage DNA Replication" under "Materials and Methods;" γ-globulins were added at stages specified.

γ-Globulins[a]	Added at stage			DNA synthesis
	1 Preprim-ing	2 Priming	3 Replica-tion	
				pmol
Experiment 1				
None				54.9
Experiment 2				
Anti-*dnaB*	+			0.6
Anti-protein i			+	
Anti-*dnaB*		+		0.9
Anti-protein i			+	
Anti-*dnaB*			+	41.7
Anti-protein i			+	
Experiment 3				
Anti-protein i		+		37.7
Anti-protein i			+	47.1
Experiment 4				
Nonimmune	+			45.5
Anti-protein i			+	
Nonimmune		+		39.3
Anti-protein i			+	
Nonimmune			+	47.2
Anti-protein i			+	

[a] Amounts of γ-globulins used were: nonimmune, 8 μg; anti-protein i, 8 μg; and anti-*dnaB*, 14 μg.

dnaB protein has not been determined.

Requirement for dnaB Protein in E. coli DNA Replication in vitro – E. coli dnaB mutants are unable to elongate DNA at the replication fork (1-5). In lysates of the mutants, using the cellophane-disc system, semiconservative DNA replication at pre-formed forks can be measured (29) and the requirement for *dnaB* gene product shown. Our preparation of pure *dnaB* protein and its specific antibody has permitted us to demonstrate directly the place of *dnaB* protein in the replication of the bacterial chromosome. Antibody against *dnaB* protein profoundly inhibited DNA synthesis in the cellophane-disc lysate system, but control γ-globulin did not (Table IV). The inhibition was partially reversed when purified *dnaB* gene product was mixed with anti-*dnaB* γ-globulin prior to adding it to the lysate.

ATPase in dnaB Protein – Ribonucleoside triphosphatase activity of *dnaB* protein, partially dependent on single-stranded DNA, has been previously reported (9, 14). We confirmed these observations by examining purified *dnaB* protein and the effects of antibody. Throughout purification of *dnaB* protein from HMS83 as well as H560 cells, NTPase activity was found associated with *dnaB* protein activity.

TABLE IV

Inhibition of Escherichia coli DNA replication by anti-dnaB antibody in cellophane-disc system

See "Materials and Methods." γ-Globulins and *dnaB* protein were added to the lysate 10 min before initiation of DNA synthesis.

Addition	Incubation	Normalized DNA synthesis
		cpm ^3H/*cpm* ^{14}C
Experiment 1		
None	−	3.7
	+	22.5
Anti-*dnaB* γ-globulin (4 μg)	−	2.5
	+	5.5
dnaB protein (55 units)	−	4.4
	+	23.5
Anti-*dnaB* γ-globulin (4 μg) + *dnaB* protein (55 units)	−	3.9
	+	13.3
Experiment 2		
None	+	15.2
Nonimmune γ-globulin (4.5 μg)	+	16.7
Anti-*dnaB* γ-globulin		
(0.4 μg)	+	9.7
(1.7 μg)	+	7.4
(4.2 μg)	+	5.8
dnaB protein (12 units)	+	24.2
Anti-*dnaB* γ-globulin (1.7 μg) + *dnaB* protein (40 units)	+	24.8

After the phosphocellulose step, the ratio of NTPase (with SS DNA present) to *dnaB* protein activity reached 2.5 and thereafter remained nearly constant. In the elution profile of highly purified *dnaB* protein from hydroxyapatite, the ATPase coincided with *dnaB* activity (Fig. 11).

ATPase showed an 8- to 10-fold stimulation by SS DNA. The K_m for ATP in the presence of φX SS DNA was 50 μM. Both DNA-independent and -dependent ATPase activities were inhibited by about the same percentage by anti-*dnaB* antibody (14 μg of antibody inhibited 50 units of *dnaB* ATPase activity 76% and 86%, respectively); antibody effectiveness was only about half as great as against the *dnaB* protein replicative activity. ATPase activity was present in the replication intermediate and inhibited by anti-*dnaB* γ-globulin, indicating that *dnaB* ATPase activity is fully functional in the intermediate (39).

Cellular Content of dnaB Protein

Approximately 20 *dnaB* oligomers were calculated to be present per HMS83 or H560 cell, and about 100 in the plasmid-bearing cell (RLM365 (pLCll-9)). The greater abundance of *dnaB* protein in the latter had no apparent effect on its growth, unlike the inhibition observed by excess protein *in vitro* (Fig. 7A).

DISCUSSION

Purification of the *dnaB* protein was undertaken to enlarge our understanding of its crucial role in DNA replication. It had been known from studies *in vivo* that temperature-sensitive *dnaB* mutants raised to a restrictive temperature immediately stopped making DNA (1-5), but it was unclear at which stage in DNA synthesis *dnaB* gene product acted.

With the pure protein in hand and with a highly specific and active antibody against it, we have conducted studies reported here and elsewhere (39) which place the *dnaB* protein in a pivotal role in the initiation of DNA chains. Synthesis of a replication intermediate of the φX viral circle requires the participation of DNA-binding protein to coat the single strand, and *dnaC* protein, proteins i and n, and ATP to fix a *dnaB* molecule in the intermediate (19, 39). We show here that anti-*dnaB* antibody prevents formation of the intermediate and also neutralizes the activity of the intermediate in supporting primer formation by primase. Once primer is produced, subsequent elongation by DNA synthesis is not affected by anti-*dnaB* antibody. This indicates that *dnaB* protein does not participate in elongation (whether attached to the DNA or dissociated from it) or, less likely, that it has become unavailable for interaction with the antibody.

We have presented a model (39) in which *dnaB* protein acts as a "mobile promoter" for primer synthesis by primase to initiate the growth of a DNA strand at the chromosome origin as well as at the replicating fork. In view of the small number of *dnaB* protein molecules in a cell and the complex nature of generating the nucleoprotein replication intermediate, it seems plausible that *dnaB* protein would not dissociate upon promoting primase action but rather remain attached to the template. It could move along the template in the direction of replicating fork movement to provide fresh loci for primase action. Energy to propel the *dnaB* protein along the template may be provided from hydrolysis of ATP which the protein itself can manage.

In addition to the demonstration that *dnaB* protein participates in *E. coli* DNA replication by the lysate-cellophane disc system, much remains to be learned about the physical and functional properties of *dnaB* protein *in vitro* and how they are correlated with events inferred from *in vivo* studies. Defects attributed to *dnaB* mutants range from failure in DNA replication (1-5, 15, 16, 41) to changes in membrane structure (42) and possibly altered incompatibility with F-factor (43, 44). Multiple allelic forms of this oligomeric protein may very well account for the large variety of responses observed with different mutants (15, 16). The relationship between *dnaB* protein and the deficiency of a protein of similar subunit size in membranes of *dnaB* mutants (42) also needs to be explained.

The complex formed between *dnaB* and *dnaC* proteins in the presence of ATP would appear to be an important step in formation of the replication intermediate but neither this reaction nor the functions performed by proteins i and n are understood. The mission of *dnaB* protein in the replication intermediate would appear to be to signal primase action, possibly by creation of secondary structure in the template strand or through direct protein-protein interactions. Thus clarification of what *dnaB* protein does must encompass its interactions with other replication proteins and with the template, and its inherent capacity to utilize ATP.

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