**dnaB Protein of Escherichia coli**

PURIFICATION AND ROLE IN THE REPLICATION OF \(\phi X174\) DNA

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KUNIHKO UEDA,† ROGER MCMACKEN,§ AND ARTHUR KORNBERG

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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 ColEl plasmid carrying the dnaB gene. The protein is an oligomer of 35,000-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 \(\phi X174\) 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 \(\phi X174\) 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 the 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.

dnaB protein has also been shown to be necessary for conversion of phage \(\phi X\) 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 vitro, 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 \(\phi X\) 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 \(\phi X\) 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; [methy]-\(^3\)HdATP, [\(^5,6\)-\(^3\)H]UTP, [\(^\alpha\)\(^32\)P]GTP, [\(^\alpha\)\(^32\)P]ATP from New England Nuclear; Freund’s complete adjuvant from Difco Laboratories. The abbreviations used are: \(\phi X\), \(\phi X174\); 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.

\(^1\) The abbreviations used are: \(\phi X\), \(\phi X174\); 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 [32P]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. [32P]dX71 am13 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. Buffer C is 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 HMS88 (polA1, polB1, thy, his) is a strain of K12 constructed by Campbell et al. (22), was used for the main preparation of dnaB protein. H560 (F, polA1, endA1, and BT1029 (dnaB, polA1, endA1, thy) (originally isolated by F. Bonhoeffer and co-workers) were used for dnaB mutants following conjugation.

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. Buffer C is 50 mM Tris·Cl (pH 7.5), 10% sucrose, 20 mM dithiothreitol, and 0.2 mg/ml of albumin.

Preparation of Replication Proteins and DNA

Proteins necessary for dX SS to RF replication, assayed as previously described (11), and partially purified from E. coli HMS88 were: DBP (Fraction 3b, 1, 65 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, 87,000 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).

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 V, 90,000 units/mg) followed by a booster (70 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 dX 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 (0.5 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 dX DNA (SS), 0.1 µg of rifampicin, 120 nmol of MgCl2, 0.45 nmol of [3H]dTTP (350 Ci/mmol), and 1.2 nmol each of dATP, dGTP, and dCTP (total volume, 25 µl). The components, mixed at 0°C in this order, were incubated for 10 min at 30°C. 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) and washed three times with 1 ml of 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 (6.5 µg), holoenzyme (1.3 µg), and protein u (2.0 µg)), (b) dX SS was first treated 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°C for 10 min) of BT1029 with the dnaB sample, dX SS and the low weight molecular components of Assay A at 30°C.

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 dITTP 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°C) contained dnaB protein, dnaC protein, DBP, protein i, protein n, protein u, dX SS, spermidine, ATP, MgCl2, 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°C for 3 min after addition of anti-protein i γ-globulin (8 µg), and the amounts of primase, GTP, CTP, UTP, rifampicin, [3H]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°C. The third stage (DNA elongation) (3 min at 30°C) was performed by adding the remaining components of the two-stage replication (see above).

E. coli Chromosome Replication on Cellophane Discs

The procedure (28, 30) consisted of prelabeling H560 cells with [14C]thymidine (1.8 Ci/mmol) for 1 h at 37°C, 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°C in a DNA synthesis mixture which included [3H]thymidine (315 Ci/mmol). The amount of DNA synthesis was corrected for variation in the number of cells on a disc by the 14C value.

Assay of ATPase

The reaction mixture (25 µl) contained 15 µl of assay buffer, 120 nmol of MgCl2, 20 nmol of [γ-32P]ATP (2 to 6 Ci/mmol), 0.3 nmol of dX 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°C, and the reaction was terminated by addition of 100 µl of each of 7% perchloric acid and Norit (20% w/v) in H2O. The mixture was stirred on a Vortex mixer and centrifuged for 10 min at 5,000 x g. [32P]P was measured in a 25-µl aliquot of the supernatant. One unit is the release of 1 pmol of inorganic phosphate (measured as 32P 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 mM 2-mercaptoethanol, 0.1% bromophenol 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; 35 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 MgCl2. 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 mM 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 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.

**H-labeling of dnaB Protein**

Radioactive labeling of dnaB protein was performed at 0-2° by the 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, NaBH4 (160 ml, 6 CI/mmole, freshly dissolved in 0.01 M NaOH to 50 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 × 1010 cpm/µg (0.15 µCi/µg); only about 10% of the original dnaB activity was recovered. For preparation of replication intermediate, 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), catalyse 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 300-500-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).

**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 (1/3 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) (1/3 volume of Fraction I), centrifuged (31,000 × g for 15 min) and dissolved in a minimal volume of Buffer B containing 1 mM dithiothreitol (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, 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
to a phosphocellulose column (1 x 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 x g for 25 min. Precipitate was dissolved in 1.9 ml of Buffer B containing 0.1 M KC1 and clarified by centrifugation for 30 min at 30,000 g for 25 min. Precipitate was dissolved in 1.9 ml of Buffer B for 2 h and again for 6 h. The dialyzed material was applied to a second phosphocellulose column (1 x 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 KC1 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 KC1 (Fraction IV, 14 ml) (Fig. 1A).

Hydroxyapatite—A portion of Fraction IV (0.5 mg of protein) was applied to a hydroxyapatite column (0.7 x 3 cm) equilibrated with Buffer B containing 0.25 M KC1. 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 (18 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. 1B). 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—dnaB protein was also purified from E. coli strains H560 and RLM365 (pLC11-9). 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 (pLC11-9), the strain with 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 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. 5A) 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 MgCl2, essentially no dnaB activity was recovered from the gradient (Fig. 5B), suggesting that Mg2+ 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 MgCl2. In its presence, dnaB protein emerged with hemoglobin, suggesting a monomeric or an asymmetric form penetrating the gel. In the absence of MgCl2, dnaB activity appeared in two peaks corresponding to molecular weights of 220,000 and 110,000 (Fig. 6B), suggestive of tetrameric and dimeric forms.

Functional Properties

Requirement of dnaB Protein for Conversion of δX SS to RF—The requirement for dnaB protein (6, 9, 11, 18) was
**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. 7A). 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. 7B).

**Complementation of dnaB Extract** - Purified dnaB protein was capable of complementing extracts from the dnaB mutant 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. 7A). 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. 7B).

**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 MgCl₂. 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.
**DNA Replication Complex Formation**

Inhibition of dnaB Protein Activity by Specific Antibody—

Antibody produced against purified dnaB protein inhibited φX DNA replication (Fig. 8A). 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. 8B). Failure of 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 3H-labeled dnaB Protein with Intermediate—

Direct demonstration of dnaB protein in the replication intermediate was made with 3H-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. 10A).

Without incubation to produce the intermediate, only one-fourth as much labeled protein was found in the excluded fraction (Fig. 10B).

**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
In vitro—E. coli dnaB mutants are unable to elongate DNA. dnaB protein has not been determined. The product was mixed with anti-dnaB y-globulin prior to adding it to the lysate system, but control y-globulin did not (Table IV). The inhibition was partially reversed when purified dnaB gene product was mixed with anti-dnaB y-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 HS80 cells, ATPase activity was found associated with dnaB protein activity.

**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-'H]UTP (10 Ci/mmol). γ-Globulins were added in the second stage as specified.

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<tr>
<th>γ-Globulin</th>
<th>['H]UMP incorporated pmol</th>
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<tr>
<td>None</td>
<td>0.362</td>
</tr>
<tr>
<td>Nonimmune (8 μg)</td>
<td>0.368</td>
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<tr>
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</tr>
</tbody>
</table>

dnaB protein has not been determined.

**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.

<table>
<thead>
<tr>
<th>γ-Globulins</th>
<th>Added at stage</th>
<th>DNA synthesis pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Prepriming</td>
<td>2 Priming</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>None</td>
<td>54.9</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Anti-dnaB</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Anti-protein i</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>Anti-dnaB</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Anti-protein i</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>Nonimmune</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Nonimmune</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Anti-protein i</td>
<td>+</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Addition</th>
<th>Incubation</th>
<th>Normalized DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm /Hmgm /°C</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Anti-dnaB γ-globulin (4 μg)</td>
<td>+</td>
<td>22.5</td>
</tr>
<tr>
<td>dnaB protein (55 units)</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>Anti-dnaB γ-globulin (4 μg) + dnaB protein (55 units)</td>
<td>+</td>
<td>4.4</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>15.2</td>
</tr>
<tr>
<td>Nonimmune γ-globulin</td>
<td>+</td>
<td>16.7</td>
</tr>
<tr>
<td>Anti-dnaB γ-globulin (0.4 μg)</td>
<td>+</td>
<td>9.7</td>
</tr>
<tr>
<td>Anti-dnaB γ-globulin (1.7 μg)</td>
<td>+</td>
<td>7.4</td>
</tr>
<tr>
<td>Anti-dnaB γ-globulin (2 μg)</td>
<td>+</td>
<td>5.8</td>
</tr>
<tr>
<td>dnaB protein (12 units)</td>
<td>+</td>
<td>24.2</td>
</tr>
<tr>
<td>Anti-dnaB γ-globulin (1.7 μg) + dnaB protein (40 units)</td>
<td>+</td>
<td>24.8</td>
</tr>
</tbody>
</table>

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 hydroxypatite, the ATPase coincided with dnaB activity (Fig. 11).

ATPase showed an 8- to 10-fold stimulation by SS DNA. The $K_a$ 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 (RLM385 (pLCII-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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