Purification and Properties of the dnaJ Replication Protein of *Escherichia coli*

Maciej Zylicz, Tomoko Yamamoto, Niki McKittrick, Susan Sell, and Costa Georgopoulos

From the Department of Cellular, Virology, and Molecular Biology, School of Medicine, University of Utah, Salt Lake City, Utah 84132

The *Escherichia coli* dnaJ gene was originally discovered because mutations in it blocked bacteriophage λ DNA replication. Some of these mutations were subsequently shown to interfere with bacterial growth at high temperature, suggesting that dnaJ is an essential protein for the host as well. The first step in purifying the dnaJ protein was to overproduce it at least 50-fold by subcloning its gene into the pMOB45 runaway plasmid. The second step was the development of an in *vitro* system to assay for its activity. A Fraction II extract from dnaJ/259 mutant bacteria was shown to be unable to replicate λ DNA unless supplemented with an exogenous source of wild-type dnaJ protein. Using this complementation assay we purified the dnaJ protein to homogeneity from the membrane fraction of bacteria. The purified dnaJ protein was shown to be a basic (pl 8.5), yet hydrophobic, protein of M, 37,000 and 76,000 under denaturing and native conditions, respectively, and to exhibit affinity for both single- and double-stranded DNA. Using a partially purified λDNA replication system dependent on the presence of the λO and P initiator proteins and at least the host dnaB, dnaG, dnaJ, dnaK, single-stranded DNA-binding protein, gyrase, RNA polymerase holoenzyme, and DNA polymerase III holoenzyme, we have shown that the dnaJ protein is required at a very early step in the DNA replication process.

From both *in vivo* and *in vitro* experiments it is well established that early λ DNA replication starts at the cis-acting oriλ site, proceeds bidirectionally, and is entirely dependent on the presence of the λO and P initiator proteins as well as on a variety of host enzymes (for review see Ref. 1). The dnaJ gene product was originally identified as essential for λ DNA replication because: 1) mutations in it did not allow λ DNA replication at any temperature (2, 3) and 2) λ mutants able to overcome the block were shown to map in the λ P replication gene. This last observation suggested a functional interaction between the host and viral gene products. Mutations in the dnaJ gene were subsequently shown to interfere with host DNA synthesis as well (4), thus the name dnaJ. The dnaJ gene was shown to map at 0.3 min on the *Escherichia coli* genetic map (5) and to form an operon with the dnaK gene, the order of the loci being promoter-dnaK-dnaJ (6). Subsequent studies by Itikawa and co-workers (7, 8) have demonstrated a pleiotropic effect of dnaK and dnaJ mutations on both host RNA and DNA synthesis at the nonpermissive temperature.

The dnaJ protein was originally identified as a polypeptide of M, 37,000 produced after infection of UV-irradiated bacteria (9) or minicells (10) by λdnaJ* but not by a λdhnaJ derivative of the transducing phage.

Recently, Puller et al. (11) pioneered the use of an *in vitro* extract, called Fraction II, capable of replicating oriC-containing plasmids. Fraction II was subsequently shown to be capable of replicating λDNA plasmid DNA provided that the λO and P replication proteins were supplied (12–14). By simply preparing a Fraction II extract from dnaJ/259 bacteria, we were able to establish an *in vitro* λDNA replication complementation assay for dnaJ protein activity. As a source of dnaJ protein we used the membrane fraction of bacteria carrying the dnaJ gene on the runaway plasmid pMOB45, which resulted in at least a 50-fold overproduction of the protein.

**MATERIALS AND METHODS**

*Bacterial and Phage Strains—*The isogenic bacterial strains C600, C600ΔdnaJ259, and C600ΔdnaK756 and the transducing phage λdnaJ* and λdnaK*ΔdnaJ* have been previously described (2, 8, 15). Strain B178ΔdnaB538 has been previously designated as gemP538 (16); Strain dnaC308 was obtained from Dr. Cynthia Lark (Department of Biology, University of Utah).

*Plasmids—*The “runaway” plasmid pMOB45 (17), carrying the tet<sup>+</sup>, cam<sup>+</sup> genes, was obtained from Dr. Vepnek (Department of Molecular and Population Genetics, University of Georgia). Other plasmids are described in the text.

*Media, Growth, and Storage of Cells—*Bacteria were grown in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.4) supplemented with 0.4% glucose. One hundred µg/ml of ampicillin was included in the medium to assure maintenance of the appropriate plasmid in the bacterial strains. The bacterial cultures were centrifuged in a Beckman JA-10 rotor at 7,000 rpm for 15 min at 2°C; the bacterial pellets were rinsed with 10% (w/v) sucrose in 50 mM Tris/HCl, pH 8.0, and stored at -70°C.

*Nucleic Acids and Replication Enzymes—*DNA from plasmids pRLM4 (carries oriλ DNA) and pRLM5 (carries ori82 DNA) (both kindly provided by Dr. Roger McMacken, Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health) was prepared as previously described (18). The λ O replication protein was purified from RLM826 bacteria harboring plasmid pRLM73 (obtained from Dr. Roger McMacken) as described in Ref. 19. The P replication protein was purified from bacteria harboring

---

1 The abbreviations used are: tet<sup>+</sup>, cam<sup>+</sup>, kan<sup>+</sup>, and amp<sup>+</sup>, resistance to tetracycline, chloramphenicol, kanamycin, and ampicillin, respectively; sb, single-stranded DNA-binding protein; ss, single-stranded; ds, double-stranded; dNTPs, deoxynucleoside triphosphates; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
plasmid pIG254 (obtained from Dr. Izabella Gorska, Department of Microbiology, University of Gdansk, Poland) as previously described (18). The E. coli dnaB protein was purified from YS1 recA bacteria carrying plasmid pKA1 (obtained from Dr. Arthur Kornberg, Department of Biochemistry, Stanford University Medical Center) as described in Ref. 19. The various purification steps of the dnaB protein through its complete isolation, and its fraction II extract, prepared from dnaB558 bacteria, for dna replication (see below). The dnaB protein was concentrated using an Amicon Centricon membrane cone.

The E. coli dnaG protein was purified from RLM757 bacteria harboring the overproducing plasmid pRLM61 (obtained from Dr. Roger McMacken) essentially as described in the published procedures (20, 21). The dnaG replication activity was monitored through its ability to complement a Fraction II extract, prepared from dnaG308 bacteria, for dna replication (see below).

All enzyme preparations with the exception of gyrB and dnaG were estimated, by the silver staining technique, to be at least 95% pure. The gyrB and dnaG preparations were at least 70% pure.

Preparation of Crude Cell Extracts—Fraction II extracts were prepared from dnaK756, dna259, and dnaB558 bacteria grown at 30 °C to an absorbance of 1.0. Fraction II extracts were prepared from dnaK756 bacteria grown at 37 °C to an absorbance of 1.0. The Fraction II extracts were prepared as follows, essentially based on the procedure outlined in Ref. 11. The cells were grown in 12-liter flasks each containing 500 ml of L broth + 0.2% glucose and 50 μg/ml of thymidine. The cells were collected by centrifugation at 7,000 rpm for 15 min at 2 °C in a Beckman JA-10 rotor and the pellets (approximately 20 g) were rinsed with Buffer D and transferred to a polycarbonate tube. The cells were resuspended in 5 ml of Buffer D and frozen at −70 °C. The cell paste was slowly thawed in a 4-8 °C water bath. A 4 ml solution was added to give a final concentration of 200 mM potassium dithiothreitol added to give a 2 mM solution and 50 ml/ml of lysozyme added to give a 0.5 mg/ml (Sigma, 60,000 units/ml) solution. The suspension was mixed well and incubated on ice for 30 min, followed by a freeze-thaw cycle using liquid N2 and a 4-8 °C water bath. The cell debris were removed by centrifugation at 45,000 rpm for 30 min at 0 °C in a Beckman Ti-50.2 rotor. Granulated ammonium sulfate (Schwarz/Mann) (0.285 g/ml) was slowly added to the supernatant (10 ml) over a 10-min period with continuous stirring. Stirring was continued for an additional 20 min at 0 °C and the solution was centrifuged at 30,000 rpm for 15 min at 0 °C in a Beckman Ti-50.2 rotor. The supernatant was removed and the pellet was centrifuged again in the same rotor at 20,000 rpm for 10 min at 0 °C. The pellet was transferred to a dialysis bag with a cut-off of M, 12,000 to 14,000 (American Scientific Products), 100 μl of freshly made Buffer E was added, and the mixture was dialyzed against 200 ml of Buffer E for 20 min at 0 °C to a conductivity equivalent to that of 400 mM KCl. The resulting Fraction II was kept frozen at −70 °C and was shown to be stable during two to three cycles of freezing and thawing.

Preparation of Fraction III Extracts—Fractionation on an Amicon red-A agarose column was based on the information supplied in Ref. 22. The Fraction II extracts were preincubated at 0 °C and then applied to a 2-ml red-A agarose column equilibrated in Buffer H supplemented with 0.05 M KCl in Buffer H. Both the protein peak (as judged by absorption at 280 nm) and the void volume were precipitated separately by slowly adding solid ammonium sulfate (2 h at 0 °C) to a final concentration of 0.35 M. The pellets were resuspended in a minimal volume and dialyzed against Buffer H to a conductivity of 200 mM KCl (20-min dialysis time).

Replication Assay—The dna replication assay was as described in Ref. 12. The 25-μl assay mixture contained: 1.5 μl 1 M Hepes (Sigma)-KOH, pH 8.0, 12 mg/ml of BSA (Sigma), 0.3 μl of 2 mM magnesium acetate, 0.6 μl of 20 mM each of CTP, GTP, and UTP (Sigma), 0.5 μl of 100 mM ATP (Sigma), 1 μl of 2 mM creatine phosphate, pH 7.6 (Boehringer Mannheim), 1 μl of a mixture of 2.5 mM each of ATP, dCTP, dGTP, and [methyl-3H]dTTP (Amersham) (50 dpm/pmol of dNTP), 0.75 μl of pRLM4 plasmid DNA A$_{260}$ = 8.0, 300 ng of λ O protein, 20 ng of P protein, 5 μl of 30% (w/v) polyvinyl alcohol (Sigma), and 0.5-1.5 μl of Fraction II (50-150 ng) from the appropriate bacterial strain supplemented with 100 ng of gyrA and 400 ng of gyrB proteins. Incubations were carried out for 10-20 min (as indicated) at 30 °C and the reactions were stopped and counted as previously described (24, 25).

In the partially reconstituted system, the constituents were added to 600 pmol of λdu DNA in the following order: 250 ng of ssb, 200 ng of DNA polymerase III holoenzyme, 20 ng of dnaG, 100 ng of dnaB, 20 ng of λ P, 1 μg of dnaK, 250 ng of RNA polymerase, 100 ng of gyrA, 400 ng of gyrB, 200 μg of λ O, 7% (w/v) final concentration of polyvinyl alcohol, red-A agarose fractions (exact amounts shown in the legend to Fig. 8), and 100 ng of dnaJ.

Buffer A contained 2 M urea (Schwarz/Mann), 50 mM Tris/HCl, pH 7.8, 100 μg/ml of phenylmethylsulfonyl fluoride (Sigma), 5 mM dithiothreitol (Sigma), 5 mM EDTA, 10% (w/v) sucrose, and 1% (w/v) Triton X-100; Buffer B, 40 mM potassium phosphate, pH 6.8, 5 mM dithiothreitol, 0.1 mM KCl, 10% (w/v) gycerol, and 0.05% (w/v) Brij 58; Buffer C, 40 mM potassium phosphate, pH 6.8, 5 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM KCl, and 10% (w/v) gycerol; Buffer D, 25 mM Hepes/KOH, pH 7.6, and 1 mM EDTA; Buffer E, 25 mM Hepes/KOH, pH 7.6, 2 mM dithiothreitol, and 0.1 mM EDTA; Buffer H, 25 mM Hepes/KOH, pH 7.6, 2 mM dithiothreitol, 1 mM EDTA, and 15% (w/v) gycerol.

Two-dimensional Gel Electrophoresis—The technique used was essentially that described by O'Farrell (26). The first dimension consisted of isoelectric focusing to equilibrium (6400 V/h) using a 2% (w/v) Ampholine mixture (pH 8-10.5) (Pharmacia) in a 4% (w/v) polyacrylamide gel. The second dimension was run on a 12.5% SDS-polyacrylamide gel.

Protein Determination—The protein concentration was estimated using the Bio-Rad protein assay. A solution of a known concentration of BSA was used as a standard.

RESULTS

Fraction II extracts (11) prepared from dnaB558, dnaG308, dnaA259, and dnaK756 mutant bacteria were shown to be inactive in λdu DNA replication unless supplemented by a source of the corresponding wild-type protein. Fig. 1 shows the dependence of such extracts on the addition of exogenously purified proteins. The molar amounts of purified dnaB, dnaG, and dnaJ proteins required to restore the full level of activity are approximately 20-fold lower than those of the dnaK protein. It is not known why such high levels of dnaK protein are needed in this system. A similar effect was seen before in the dnaK-dependent M13 DNA replication system (24). In the specific case of the dnaJ protein, the λdu DNA replication assay responds linearly to the addition of up to 100 ng of purified protein. Various steps in the purification of the dnaJ protein were monitored using the linear range of the assay.

Cloning of the dnaJ Gene—Because of the low number of dnaJ protein molecules/cell (see below) we decided to overproduce the dnaJ protein prior to attempting its purification. Because of our previous success in overproducing various proteins on the runaway plasmid pMOB45 (17), we cloned the dnaJ gene into it as follows. DNA from the transducing phage λadn+$mKC (6) was partially digested with the restriction endonuclease Sau3A. The resulting fragments were...
the membrane fraction of bacterial spheroplasts lysed by our technique (25). This result is in agreement with the localization of the dnaJ protein in the membrane fractions of minicells (10). By lysing the spheroplasts in the presence of 1 M KCl and 0.1% Triton X-100, up to 50% of the dnaJ activity could be released in the soluble fraction. However, a M, 39,000 protein was also released and remained closely associated with the dnaJ protein during all subsequent steps of purification attempted. Because of this, we extracted dnaJ protein from crude membrane fractions using 2 M urea and 0.1% Triton X-100. This resulted in the release of at least 90% of the dnaJ activity. 2 M urea was also included in the next two steps of purification, DEAE-Sephacel, and ammonium sulfate precipitation. Using these conditions we were able to successfully separate the dnaJ protein from the M, 39,000 contaminating polypeptide. The subsequent steps of hydroxylapatite and P-11-cellulose phosphate chromatography assured the purification of the dnaJ protein to more than 95% purity (Table I).

During our first attempts at purification we found that when fractions containing dnaJ protein activity were applied onto a P-11-cellulose phosphate column previously equilibrated with low salt (<100 mM KCl), it was impossible to recover either the protein or the activity, even after subsequently washing the column with 1 M KCl. We presume that this was due to precipitation of the dnaJ protein on the column in the absence of high salt and/or Brij 58. However, ligated into the unique BamHI site of pMOB45. DNA from such ligations was used to transform dnaJ259 bacteria to camR at 30 °C. Since the wild-type dnaJ gene product is dominant over the mutant dnaJ259 protein (2), plasmid clones carrying the dnaJ+ gene were selected on the basis of their ability to allow λ phage growth. Approximately 10 of such plasmid-carrying bacterial transformants were tested for overproduction of the dnaJ protein, after 3 h of growth at 42 °C, in the following two ways. First, the proteins from the bacterial extracts were separated on SDS-polyacrylamide gels and the amount of dnaJ protein, at its expected M, 37,000 position in the gel, was visually inspected. Second, the bacterial extracts were tested for ability to complement a dnaJ259 Fraction II for λDNA DNA replication. A clone was found, pMOB45dnaJ+12, which on the basis of both criteria over-

| Purification step | Protein | Activity | Specific activity | Yield |
|------------------|---------|----------|------------------|-------|
|                   | mg      | units    | units x 10^-4/mg | %     |
| 1. 2 M urea membrane extract | 612.0 | 521* | 85 | 100 |
| 2. DEAE-Sephacel | 82.7 | 499* | 60.7 | 96 |
| 3. Ammonium sulfate | 34.5 | 248 | 72.0 | 48 |
| 4. Hydroxylapatite | 10.9 | 102 | 93.6 | 20 |
| 5. P-11-cellulose phosphate | 8.1 | 80 | 98.4 | 15 |

* Dialyzed for 8 h at 0 °C against 40 mM Hepes/KOH, 100 mM KCl, 0.1 mM EDTA, and 5 mM dithiothreitol.

**TABLE I**

**Purification of dnaJ protein from E. coli B178 (pMOB45dnaJ+12) bacteria**

E. coli B178(pMOB45dnaJ+12) bacteria were grown in L broth supplemented with 0.2% glucose and 20 μg/ml of chloramphenicol at 30 °C to A660 nm = 0.5. The culture was shifted to 42 °C for 3 h and harvested by centrifugation in a Beckman J-10 rotor at 7,000 rpm for 20 min at 30 °C. The bacterial pellet (28 g) was resuspended in 40 ml of 50 mM Tris/HCl, pH 8.0, 10% (v/v) sucrose and lysed by sonication (3 x 1 min at 0 °C, 70 watts). The membrane fraction was pelleted by centrifugation in a Beckman R35 rotor at 30,000 rpm for 60 min at 0 °C, resuspended in 40 ml of Buffer A, and gently mixed for 1 h at 0 °C. The extract was subsequently centrifuged in a Beckman R35 rotor at 30,000 rpm, for 1.5 h at 0 °C, and the 40-mL supernatant (step 1) was applied directly onto a DEAE-Sephacel column (2.5 x 12 cm), equilibrated with Buffer A at a flow rate of 2 ml/min. The first peak of absorption at 280 nm was collected (step 2) and precipitated with 0.28 g/ml of ammonium sulfate. The pellet was washed once in 1/10 volumes of 0.28 g/ml of ammonium sulfate in Buffer B and resuspended in 5 ml of Buffer B containing 0.1 M KCl (step 3). The fraction from step 3 was applied directly onto a hydroxylapatite (Bio-Rad) column (2.5 x 4 cm), equilibrated with Buffer B at a flow rate of 2 ml/min. The column was washed first with 100 ml of Buffer B, then with 100 ml of Buffer B containing 100 mM potassium phosphate, followed with 50 ml of Buffer B containing 500 mM potassium phosphate. The fractions with dnaJ activity were pooled (step 4), diluted to 50 ml with Buffer B without KCl to a conductivity equivalent to that of Buffer C, and applied onto a P-11-cellulose phosphate column (Whatman) (1.5 x 7 cm), pre-equilibrated with Buffer C at a flow rate of 2 ml/min. The column was washed with 100 ml of Buffer C and the dnaJ activity was eluted using a 100-ml linear gradient of 0-100 mM KCl in Buffer C. The active fractions (step 5), eluting around 400 mM KCl, were frozen at -70 °C. The dnaJ activity has been stable at -70 °C for at least one year and can withstand repeated freezing and thawing cycles.
when the P-11-cellulose phosphate column was equilibrated with 100 mM KCl plus 0.05% Brij 58, most of the dnaJ activity could be eluted with 400 mM KCl.

The protein composition of the various fractions in the purification of the dnaJ protein are shown in Fig. 2. Fig. 3 shows the co-purification of the dnaJ protein and the dnaJ259 extract-complementing activity assayed during the last two steps of the purification procedure.

Properties of the Purified dnaJ Protein—The purified dnaJ protein was shown to exhibit a M, of 37,000 under denaturing conditions (Fig. 4). Under equilibrium isoelectrofocusing conditions (28), the dnaJ protein migrated with an isoelectric point (pI) of approximately 8.5. The amino acid composition of the purified protein is shown in Table I. Since the exact number of glutamic acid and aspartic acid residues is not known, we cannot predict the pI of the dnaJ protein. However, the sequence of the first eight N-terminal amino acids of the purified protein is identical to that predicted by an open reading frame found a few bases beyond the TAA termination triplet of the adjacent dnaK coding region.23 This, coupled with the genetic analysis of the dnaJ and dnaK loci, suggests that we indeed purified the dnaJ protein.

Both the dnaJ protein (monitored by SDS-polyacrylamide gel electrophoresis) and the dnaJ replication activity sedimented in glycerol gradients at 4.7 ± 0.1 S, a position between the BSA and transferrin marker proteins (Fig. 5), suggesting that the active dnaJ form is a dimer. The protein behaved in an identical manner in metrizamide gradients (results not shown). A similar conclusion was drawn from the gel filtration data presented in Fig. 6. The Stokes radius of the nondenatured dnaJ protein was estimated to be 38.0 ± 0.5 Å. Using this information as well as the known sedimentation coefficient, the native molecular weight of the dnaJ protein was estimated to be approximately 76,000 (Table III).

The dnaJ protein was shown to bind strongly to a phenylagarose affinity column in the presence of 3 M KCl (results not shown). This fact, coupled with the amino acid composition of the dnaJ protein, suggests the presence of large hydrophobic regions in the dnaJ protein.

Fig. 7 shows that the dnaJ protein binds to a ss cally thymus DNA affinity column and elutes around 0.2 M KCl. Both the phenyl-agarose and DNA affinity columns are excellent purification steps for the dnaJ protein. In preformed metrizamide gradient experiments (18, 30), it was found that the dnaJ protein also binds to both ss M13 DNA and ds λdv DNA in the presence of 25 mM Hepes/KOH, pH 7.6, 5 mM dithiothreitol, 80 mM KCl, and 10 mM magnesium acetate (results not shown). These observations may not be surprising in view of the dnaJ protein’s basic isoelectric point.

Purified dnaJ protein did not exhibit ATPase activity (less than 0.1 molecule of ATP hydrolyzed per min/dnaJ monomer at 30°C) under the conditions used to demonstrate DNA-dependent ATPase activity of the dnaB protein (31) or under our assay conditions for ATPase activity of the dnaJ protein (24) (results not shown). In an endonuclease activity test, using supercoiled λdv DNA as substrate, the dnaJ protein did not exhibit any detectable activity (no relaxed or linear DNA species were observed after incubation of 1 mg of λdv DNA with 1 μg of dnaJ protein for 30 min at 30°C; results not shown).

dnaJ Protein Is Necessary for λdv Replication—In order to better understand the steps involved in λdv DNA replication in general, and the role of the dnaJ and dnaK proteins in particular, we are attempting to reconstitute an in vitro λdv DNA replication system composed entirely of purified components. We found that when λdv DNA was incubated together with the phage λ O and P replication proteins, E. coli RNA polymerase holoenzyme, dnaB, dnaG, dnaJ, dnaK, gyrA, gyrB, ssb, and DNA polymerase III holoenzyme proteins, rNTPs, dNTPs, an ATP regeneration system, and 7% polyvinyl alcohol, some limited replication was observed (approximately 100 pmol out of 600 pmol of substrate DNA). Maximal DNA replication activity (over 600 pmol) was observed when the purified system was supplemented with either a diluted Fraction II extract (twenty times less than the normal amount, which by itself has no DNA replication activity) or by various Amicon red-A agarose affinity column fractions of Fraction II extracts (see “Materials and Methods”). Using such red-A agarose fractions (called Fraction III) we showed that the replication observed is totally oriV-specific since a plasmid bearing the heterologous lambdoid oriR2 (pRLM5) will not serve as substrate. In addition to the dnaJ and dnaK proteins, the DNA replication activity in this partially reconstituted system is dependent on the additional presence of the Λ O and P proteins and the host dnaB, dnaG, and DNA polymerase III holoenzyme proteins and is stimulated by...
Fig. 3. Co-purification of dnaJ activity and dnaJ protein. The DNA replication activity (●) and protein concentration (○) after chromatography on A, hydroxylapatite and B, P-11-cellulose phosphate columns. The chromatographic steps were carried out as described in the legend to Table I. . . . . indicates the concentration of KCl estimated by conductivity measurements.

Additional gyrase and RNA polymerase enzymes (data not shown). The absolute requirement for gyrase and RNA polymerase is suggested by the complete sensitivity of the reconstituted system to the addition of nalidixic acid or rifampicin (inhibitors of gyrase and RNA polymerase, respectively). When Fraction III was prepared from dnaK756 or dnaJ259 bacteria, the reconstituted system was shown to be totally dependent on the subsequent addition of the corresponding wild-type proteins. In both cases, however, if the dnaJ or dnaK proteins were not preincubated for 10 min prior to the addition of dNTPs, a lag of 1.0-1.5 min in the commencement of DNA synthesis was observed (Fig. 8 and data not shown). The demonstration of such a lag suggests that a rate-limiting step, dependent on both the dnaJ and dnaK proteins, is required before initiation of DNA synthesis can take place.

DISCUSSION

Previous genetic analysis has indicated that λ DNA replication in vitro depends not only on the presence of the phage-coded initiation proteins λO and P, but also on an array of host-coded replication factors (1). Some of these host factors, namely dnaB, dnaJ, dnaK, grpD, and grpE, were originally discovered because mutations in them resulted in a block to λ DNA replication (2, 3, 16, 32). Additional host factors include the dnaG primase protein, the DNA polymerase III holoenzyme, and the RNA polymerase holoenzyme (1).

The recently developed in vitro λdu replication system (12-14), using E. coli Fraction II extracts (11), has already been used to demonstrate that the λO and P replication proteins are required for this process. In this work, by employing Fraction II extract preparations from the appropriate mutant bacterial strains, we have provided direct evidence that λdu
FIG. 5. Glycerol gradient sedimentation. Purified dnaJ protein (15 μg; 1,464 replication units; step 5) was sedimented through a 5.0-ml linear 20–35% glycerol gradient (25 mM Hepes/KOH, pH 7.6, 0.2 M KCl, 0.1 mM EDTA, 2 mM dithiothreitol) in a Beckman SW50.1 rotor at 48,000 rpm, for 26 h at 2°C. Fractions (125 μl) were collected from the bottom. A portion of each fraction (6 μl) was used to assay for replication activity (■) and the rest was used to determine protein concentration (■) using the Bio-Rad assay. In a parallel experiment, the dnaJ protein was sedimented along with protein markers. The positions of the various protein species was monitored by SDS-polyacrylamide gel electrophoresis. The arrows point to the positions of 1, fibrinogen (7.9); 2, transferrin (5.1); 3, BSA (4.3); and 4, ovalbumin (3.6), respectively.

**in vitro** replication is also dependent on the presence of the dnaB, dnaG, dnaK, and dnaJ proteins. Furthermore, we have taken advantage of this **in vitro** complementation system to purify to homogeneity all four of these proteins, by following the corresponding complementing activity throughout all steps of the purification procedure. Thus, the **in vitro** *E. coli* DNA replication system constitutes a novel and facile way to monitor the purification of these, and potentially other, DNA replication proteins.

The purified *E. coli* dnaJ protein has been shown to possess a dimeric form under native conditions, the denatured polypeptide exhibiting a *M* of 37,000. The dnaJ protein exhibits a salt-dependent affinity for the *E. coli* membrane which made its original purification laborious. It is not known whether this apparent association with the membrane is physiologically significant or whether it represents an artifact of the lysis procedure. For example, the dnaJ protein could be associating with it through its demonstrated nonspecific binding to *E. coli* DNA fragments present in the crude membrane fractions. However, the highly hydrophobic behavior of this protein as well as its exclusive presence in the membrane fraction prepared from minicells (10) (which presumably is free of host DNA) argue in favor of a physiological role for the dnaJ protein’s association with the membrane.

It is still not clear what role the dnaJ protein has in *E. coli* DNA replication. Two facts suggest that the dnaJ protein is a pre-priming protein required at an early step in DNA synthesis: 1) the dnaJ activity is essential during the formation of a pre-priming intermediate, a step preceding that of the formation of primer RNA in the dnaB, dnaG, dnaJ, ssb, DNA polymerase III, λ O, and λ P purified system; and

*J.* LeBowitz, M. Zylicz, C. Georgopoulos, and R. McMacken (1985) Proc. Natl. Acad. Sci. U. S. A., in press.
TABLE III
Physical properties of the purified dnaJ protein

The details of the determination of the various physical properties of the dnaJ protein are given in the text. The amino-terminal sequence of purified dnaJ protein was done in the laboratory of Dr. W. Gray, Department of Biology, University of Utah. The stepwise degradation (28) was carried out in a Beckman 890 D spinning cup instrument, using a 1 m Quadrol program. 3-Phenyl-2-thioxanthostatin derivatives were identified by high performance liquid chromatography on a column (0.46 × 15 cm) of Ultrasphere ODS, using a gradient of acetonitrile in 0.05 M sodium acetate, pH 4.5.

Native $M_r$ (by sedimentation and diffusion) 76,000 ± 4,000
Denatured $M_r$ (on 12.5% SDS-polyacrylamide gels) 37,000 ± 2,000
Sedimentation coefficient ($s_{20, w}$) 4.70 ± 0.1
Stokes radius (Å) 38.0 ± 0.5
Amino-terminal sequence:
Ala-Lys-Gln-Asp-Tyr-Tyr-Glu-Ile

Fig. 7. DNA affinity chromatography. A denatured calf thymus DNA column (1.0 × 2.0 cm) was prepared as described by Alberts and Herrick (29) and equilibrated with 25 mM Hepes/KOH, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol, and 10% (v/v) glycerol. Purified dnaJ protein (0.6 mg; 59,040 DNA replication units; step 5) was dialyzed overnight against the same buffer and applied onto the column at a flow rate of 1.5 ml/20 min. The column was washed with 10 ml of the same buffer and eluted with a 40-ml linear gradient of 0.05-0.5 M KCl. Fractions (0.75 ml) were collected, a small portion (2 μl) was used to assay for DNA replication activity (O—O), and the rest was used to determine protein concentration (O—O) using the Bio-Rad assay.

2) a 1-2-min lag period is detected in the λdv DNA replication system when the functional dnaJ protein is not present during the preincubation (using a Fraction II prepared from dnaJ259 bacteria) but is supplied later along with the dNTPs.

The dnaJ and dnaK genes form an operon (4, 6) which is under heat shock regulation (33). Their gene products are essential for host viability at high temperature, since certain mutations in either gene result in an inability to form colonies at 43°C (2, 4, 7, 8, 16, 32). These mutations have been shown to exhibit diverse pleiotropic effects on host macromolecular synthesis, including effects on RNA and DNA synthesis (3, 4, 7, 8) and the ability to turn off the heat shock response in the case of dnaK protein (34). The following observations suggest that the dnaJ and dnaK proteins are necessary for host DNA synthesis in vitro: 1) Fraction II extracts prepared from either dnaJ756 or dnaJ259 bacterial mutants are partially active in the oriC, dnaA-dependent (11) replication system. Subsequent addition of the corresponding purified protein stimulates oriC replication, and 2) purified IgG preparations against either protein inhibit the ability of Fraction II extracts from wild-type bacteria to carry out oriC replication unless excess purified dnaJ and dnaK proteins are simultaneously added to the corresponding mutant extracts.

Acknowledgments—We would like to thank those mentioned in the text, especially Dr. Roger McMacken, for enzymes and bacterial and plasmid strains, and Jerri Cohenour for preparation of the manuscript.

REFERENCES
1. Furth, M. E., and Wickner, S. H. (1983) in Lambda II (Hendrix, R. W., Roberts, J. W., Stahl, F. W., and Weisberg, R. A., eds) pp. 145–174, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Sunshine, M., Feiss, M., Stuart, J., and Yochem, J. (1977) Mol. Gen. Genet. 113, 1-25
3. Saito, H., and Uchida, H. (1977) J. Mol. Biol. 113, 1-25
4. Saito, H., and Uchida, H. (1978) Mol. Gen. Genet. 164, 1-8
5. Bachmann, B. (1983) Microbiol. Rev. 47, 180-230
6. Yochem, J., Uchida, H. Sunshine, M., Saito, H., Georgopoulos, C. P., and Feiss, M. (1978) Mol. Gen. Genet. 164, 9-14
7. Iikawa, H., and Ryu, J. (1979) J. Bacteriol. 138, 339-344
8. Wada, M., Kadokami, Y., and Itikawa, H. (1982) Jpn. J. Genet. 57, 407–413
9. Georgopoulos, C. P., Lundquist-Heil, A., Yochem, J., and Feiss, H. (1980) Mol. Gen. Genet. 178, 583–588
10. Zylicz, M., Nieradko, J., and Taylor, K. (1983) Biochem. Biophys. Res. Commun. 110, 176–180
11. Fuller, R. S., Kaguni, U. M., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7370–7374
12. 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
13. Anderl, A., and Klein, A. (1982) Nucleic Acids Res. 10, 1733–1740
14. Tsurimoto, T., and Matsubara, K. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7639–7643
15. Georgopoulos, C. P., Lam, B., Lundquist-Heil, A., Rudolph, C. F., Yochem, J., and Feiss, M. (1979) Mol. Gen. Genet. 172, 143–149
16. 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
17. Bittner, M., and Vapnek, D. (1981) Gene 15, 319–329
18. Zylicz, M., Gorska, L., Taylor, K., and Georgopoulos, C. (1984) Mol. Gen. Genet. 196, 401–406
19. Roberts, J. D., and McMacken, R. (1983) Nucleic Acids Res. 11, 7435–7452
19a. Ueda, K., McMacken, R., and Kornberg, A. (1978) J. Biol. Chem. 253, 261–269
20. Wickner, S., Wright, M., and Hurwitz, J. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1613–1618
21. Rowen, L., and Kornberg, A. (1978) J. Biol. Chem. 253, 758–764
22. Mizuuchi, K., Mizuuchi, M., O’Dea, M. H., and Gellert, M. (1984) J. Biol. Chem. 259, 9199–9201
23. Dixon, N. E., and Kornberg, A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 424–428
24. Zylicz, M., LeBowitz, J. H., McMacken, R., and Georgopoulos, C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6431–6435
25. Zylicz, M., and Georgopoulos, C. (1984) J. Biol. Chem. 259, 8820–8825
26. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
27. Spackman, D. H., Stein, W. H., and Moore, S. (1958) Anal. Chem. 30, 1190–1206
28. Edman, P., and Begg, G. (1977) Eur. J. Biochem. 1, 80–91
29. Alberts, B., and Herrick, G. (1971) Methods Enzymol. 21, 198–217
30. Zylicz, M., and Taylor, K. (1981) Eur. J. Biochem. 113, 303–309
31. Arai, K., and Kornberg, A. (1981) J. Biol. Chem. 256, 5253–5259
32. Georgopoulos, C. P. (1977) Mol. Gen. Genet. 151, 35–39
33. Georgopoulos, C., Tilly, K., Drahos, D., and Hendrix, R. (1982) J. Bacteriol. 149, 1175–1177
34. Tilly, K., McKittrick, N., Zylicz, M., and Georgopoulos, C. (1983) Cell 34, 641–646