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Three *Escherichia coli* heat shock proteins are required for P1 plasmid DNA replication: Formation of an active complex between *E. coli* DnaJ protein and the P1 initiator protein

(DnaK/GrpE/*oriλ*/*oriC*)

SUE H. WICKNER*

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **DNA containing the plasmid origin of bacteriophage P1 is replicated *in vitro* by a protein fraction prepared from uninfected *Escherichia coli* supplemented with purified P1 RepA protein. It has previously been shown that the reaction required the *E. coli* DnaA initiator protein, the DnaB helicase, DnaC protein, RNA polymerase, and DNA gyrase. I show here that three *E. coli* heat shock proteins, DnaJ, DnaK, and GrpE, are directly involved in P1 plasmid replication. Purified DnaJ, DnaK, and GrpE proteins were required to stimulate P1 plasmid *ori* DNA-dependent replication in *in vitro* complementation assays in which the host protein fractions were prepared from cells mutated in the corresponding gene. I have also found that the DnaJ and RepA proteins form a complex. This complex exists in crude cell extracts and can be isolated as a molecular species of about 160,000 Da containing one dimer of DnaJ protein and one dimer of RepA. The complex can also be reconstituted by mixing purified DnaJ and RepA proteins. These results imply that the DnaJ–RepA complex, DnaK, and GrpE are directly involved in P1 plasmid replication.**

The *dnaJ*, *dnaK*, and *grpE* genes of *Escherichia coli* were first identified by the isolation of mutants that were unable to replicate bacteriophage λ DNA (1–3). Interestingly, these genes are members of the group of *E. coli* heat shock genes (reviewed in ref. 4). The heat shock genes are found throughout nature, and DnaK protein in particular is highly homologous in amino acid sequence to the Hsp70 proteins of eukaryotes. While the roles of DnaK, DnaJ, and GrpE proteins in *E. coli* cell growth are unknown, they are essential for growth at high temperatures (1–3, 5–8). Recently, it has been shown that a deletion of the *grpE* gene without compensating mutations is lethal at all temperatures (8). It has also been shown that a deletion of the *dnaK* gene is conditionally lethal at both low and high temperatures (6). In addition, a *dnaK* mutant has been isolated that is defective in initiation of replication at the normal *E. coli* origin at high temperatures (9). However, the function of DnaK in initiation may be indirect, because plasmid DNA containing the *E. coli* origin can be replicated *in vitro* with purified proteins in the absence of DnaK as well as in the absence of DnaJ and GrpE proteins (10).

Phage λ DNA replication *in vitro* requires DnaJ and DnaK proteins under all conditions tested and requires GrpE protein under certain reaction conditions (11–14). All three proteins have been purified by using *in vitro* complementation assays for λ replication (11–13). Purified DnaJ protein binds nonspecifically to single- and double-stranded DNA (11). Purified DnaK protein contains weak DNA-independent ATPase activity and autophosphorylating activity (15). Although no activity has been found associated with GrpE protein, it forms a protein complex with DnaK protein that is dissociated by ATP (13). Analysis of protein–DNA complexes by electron microscopy and gel electrophoresis has shown that these proteins function in assembling initiation complexes on the λ origin of replication (16, 17). First, λ O protein binds to specific sites in the λ origin. Then λ P protein and DnaB protein join the complex, forming a larger protein–DNA complex. The addition of DnaJ, DnaK, *E. coli* single-stranded DNA-binding protein (SSB) and ATP to the complex results in a structure in which the DNA in the origin is unwound, presumably by the DnaB helicase (18). It has been suggested that the role of DnaJ and DnaK is to release λ P protein from DnaB and thus activate the DnaB helicase activity (14, 16, 17). Since GrpE lowers the amount of DnaK required, it is thought that GrpE is also involved in releasing λ P protein from DnaB (14).

Bacteriophage P1 replicates in a different manner from that of λ (reviewed in ref. 19). It has both a lytic and a lysogenic origin of replication. Unlike λ, P1 is maintained as a plasmid in the lysogenic state. The P1 plasmid origin of replication is contained on a 246-base-pair piece of DNA (20). At one end there are five direct repeats of a 19-base-pair sequence to which the P1 initiation protein RepA binds. In the middle there is a region slightly rich in adenine and thymine, and at the other end there are two 9-base-pair DnaA protein binding sites. Wickner and Chattoraj (21) have shown that DNA containing the P1 plasmid origin of replication could be replicated *in vitro* by the RepA protein in combination with *E. coli* proteins, under conditions similar to those described for the replication of *oriC* (22) and *oriλ* (23, 24) DNA. By using inhibitors, antibodies, and *in vitro* complementation tests, we showed that the reaction requires DnaA protein, DnaB helicase, DnaC protein, DnaG primase, RNA polymerase, and DNA gyrase (ref. 21; unpublished observations).

I show in this communication that the DnaJ, DnaK, and GrpE proteins are required for replication *in vitro* of DNA carrying the P1 origin. I have looked for protein–protein interactions and have found that DnaJ and RepA form a complex that can be isolated from crude lysates or reconstituted with purified proteins. The possible role of the heat shock proteins in P1 replication is discussed.

MATERIALS AND METHODS

Bacterial Strains, Phage, and Plasmids. The *E. coli lonΔ* strain SG4148 (25) was used to prepare the wild-type host replication factors. The *dnaJ259* strain MF634 (3), the *dnaK756* strain GR756 (26), the *grpE280* strain DA16 (5), the

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Abbreviation: SSB, *E. coli* single-stranded DNA-binding protein.

*To whom reprint requests should be addressed at: Laboratory of Molecular Biology, National Cancer Institute, Building 37, 2E14, National Institutes of Health, Bethesda, MD 20892.

*dnaJ*Δ, *dnaK*Δ strain PK101 (P. J. Kang and E. Craig, University of Wisconsin, Madison, WI), and the *dnaJ*Δ strain PK102 (P. J. Kang and E. Craig) were used for complementation assays for DnaJ, DnaK, and GrpE proteins.

The *ori*P1 plasmid DNA (to be described elsewhere) was M13 containing the P1 plasmid *ori* region from position 287 to 610 (20).

Proteins, Chemicals, and Enzymes. P1 RepA protein was purified by a procedure derived from that of Abeles (27). The DnaA protein used was fraction IVB (28). DnaJ and DnaK proteins were purified as described (refs. 11 and 12, respectively), with the exception that the final preparations were subjected to chromatography on an HPLC gel-filtration column (Bio-Sil TSK-250 column, 600 × 21.5 mm) in 10% glycerol, 0.2 M Na_2SO_4, 1 mM EDTA, 1 mM dithiothreitol, and 0.05 M Tris·HCl (pH 7.5). GrpE protein was purified by a procedure to be published elsewhere. All of these proteins were >95% pure as judged by PAGE followed by staining with Coomassie blue (29).

[^{3}H]dTTP was from DuPont/New England Nuclear. Unlabeled ribo- and deoxyribonucleoside triphosphates were from P-L Biochemicals. Polyvinyl alcohol type II, Hepes, and lysozyme were from Sigma. Creatine kinase and creatine phosphate were from Boehringer Mannheim.

DNA Procedures. M13 replicative form I DNA was isolated as previously described (30).

Preparation of Host Protein Fraction. Cells were grown to late exponential phase in Luria–Bertani broth, collected, resuspended in 20 ml of 10% (wt/vol) sucrose and 50 mM Tris·HCl (pH 7.5) per 10 g of packed cells, frozen in liquid nitrogen, and stored at −70°C. Cells from 5 liters of culture were found to be the minimum amount that could conveniently be used for preparing an extract. Cells were thawed and distributed to centrifuge tubes, and the suspension was adjusted to 0.05 M KCl, 2 mM dithiothreitol, hen egg white lysozyme at 0.4 mg/ml, and 10 mM EDTA. The tubes were inverted several times to assure mixing and then incubated on ice for 20 min. The lysed cells were centrifuged at 40,000 rpm for 1 hr in a type 60 Ti rotor. The crude extract was decanted, and 20% (wt/vol) streptomycin sulfate dissolved in H_2O was added to give a final concentration of 4%. After 5 min on ice, the solution was centrifuged at 15,000 rpm for 10 min in a JA-20 rotor. To each milliliter of supernatant 0.27 g of $(NH_4)_2SO_4$ was added. The mixture was stirred for 10 min on ice and centrifuged at 15,000 rpm for 10 min. The pellets were resuspended, but not necessarily dissolved, in a small amount of 50 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, and 5% glycerol (dialysis buffer), so that the protein concentration was 40–80 mg/ml. The resuspended pellets were dialyzed against 200 volumes of dialysis buffer for 2 hr (final salt concentration of 300–400 mM), and 100-μl portions were distributed into tubes, frozen rapidly in liquid nitrogen, and stored at −70°C. The fractions could be thawed and refrozen several times but lost activity if left on ice for more than a few minutes at a time.

***In Vitro* Assay for *ori*P1 DNA Replication.** Reaction mixtures were similar to those described earlier for the *in vitro* replication of *ori*P1 plasmid DNA (21). They contained (in 20 μl) 40 mM Hepes (pH 7.5), 40 mM KCl, 1 mM dithiothreitol, 7 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.2 mM UTP, 10 mM $Mg(CH_3COO)_2$, 50 mM creatine phosphate, creatine phosphokinase at 100 μg/ml, 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 50 μM [^{3}H]dTTP (1000 cpm/pmol), 6% (wt/vol) polyvinyl alcohol, 300 pmol of *ori*P1-containing DNA (deoxynucleotide equivalent), 100 ng of DnaA protein, 5 μl of a protein fraction from *E. coli* strain SG4148 (containing ≈50 mg of protein per ml), and 100 ng of RepA protein. Reaction mixtures were incubated at 38°C for 20 min, and acid-insoluble radioactivity was measured. For complementation assays, reactions contained protein fractions prepared from PK102, GR756, or DA16 in place of the SG4148 extract and purified DnaJ, DnaK, or GrpE protein, respectively.

RESULTS

Requirement for *DnaJ*, *DnaK*, and *GrpE* for *ori*P1 Replication. To determine whether DnaJ, DnaK, and GrpE proteins were required for *ori*P1 plasmid DNA replication *in vitro*, crude protein fractions were prepared from *dnaJ*Δ cells, *dnaK* mutant cells, and *grpE* mutant cells. Then I assayed the extracts for their ability to support replication of *ori*P1 plasmid DNA after the addition of RepA and DnaA protein plus and minus purified DnaJ, DnaK, and GrpE proteins. The complementation assays showed clearly that *ori*P1 DNA replication required DnaJ, DnaK, and GrpE proteins (Table 1). When the crude host protein fraction was prepared from a strain carrying a *dnaJ* point mutation (strain MF634, *dnaJ259*), there was residual *ori*P1 replication activity (4–8 pmol of dTMP incorporated) in the absence of added DnaJ protein. However, the extracts were always stimulated by the addition of DnaJ protein (16–25 pmol). The requirement for DnaJ protein was more complete with the deletion mutant as shown in Table 1. Using extracts prepared from a *dnaJ*, *dnaK* double-deletion strain, I observed that neither DnaJ nor DnaK protein alone was complementary for *ori*P1 replication, but the combination was, again demonstrating that both DnaJ and DnaK proteins were required (Table 1).

The amount of each protein required to restore full *ori*P1 replication activity to mutant extracts is shown in Fig. 1. It is puzzling why 10-fold higher molar amounts of DnaK protein [native molecular mass of 78,000 Da (12)] are needed than DnaJ [native molecular mass of 76,000 Da (11)] or GrpE [native molecular mass of 23,000 Da (13)]. However, the same result has been found for *ori*λ replication (11, 13). For *ori*λ replication *in vitro*, it has been observed that GrpE protein was dispensable if high amounts of DnaK were supplied (14). Therefore, I tested whether or not DnaK protein could substitute for GrpE protein in the *in vitro* complementation assay for *ori*P1 replication. I observed that DnaK with or without DnaJ could not substitute for GrpE protein (Table 1). These experiments show that GrpE has a separate function from DnaK in *ori*P1 replication, at least in a crude system.

Table 1. *In vitro* complementation assay for DnaJ, DnaK, and GrpE proteins for *ori*P1 plasmid DNA replication

E. coli crude protein fraction	Protein(s) added	DNA synthesis, pmol of dTMP incorporated
Wild-type	None	23.8
*dnaJ*Δ	None	1.0
	DnaJ	14.8
dnaK756	None	0.4
	DnaK	24.6
grpE280	None	1.5
	GrpE	15.4
	DnaK	0.2
	DnaJ + DnaK	0.3
*dnaJ*Δ, *dnaK*Δ	None	0.7
	DnaJ	0.7
	DnaK	0.8
	DnaJ + DnaK	17.7

Reactions were carried out as described in *Materials and Methods* for *ori*P1 replication; the host protein fractions were prepared from strains SG4148, PK102, GR756, DA16, and PK101. One hundred nanograms of RepA and 100 ng of DnaA protein were added to each reaction mixture. Where indicated, 170 ng of DnaJ protein, 1.4 μg of DnaK protein, and 350 ng of GrpE protein were added.

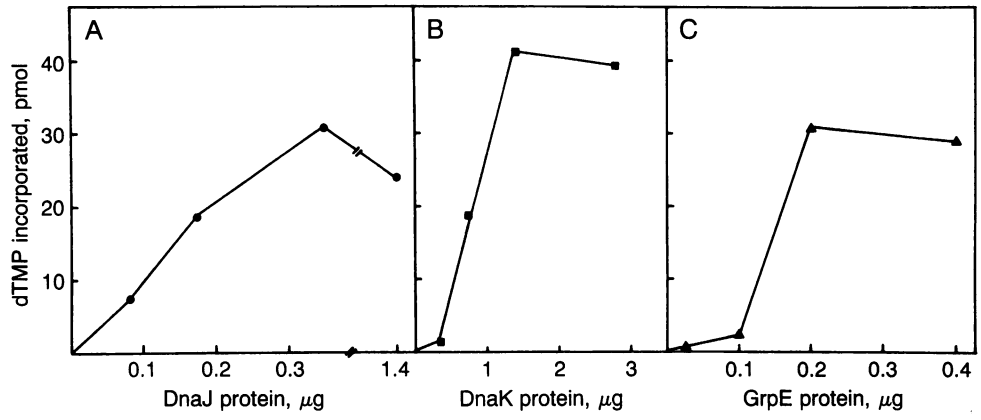

FIG. 1. *In vitro* complementation of *dnaJ*, *dnaK*, and *grpE* extracts for *ori*P1 replication. Complementation assays were carried out with extracts prepared from *dnaJ*Δ strain PK102 (*A*), *dnaK756* strain GR756 (*B*), and *grpE280* strain DA16 (*C*). Purified DnaJ, DnaK, and GrpE proteins were added in the amounts indicated.

Isolation of DnaJ–RepA Protein Complex. The molecular mechanism of heat shock proteins is unknown. I discovered an important clue to the role of DnaJ during the purification of RepA. Most of the RepA chromatographed on HPLC gel filtration with a native molecular mass of 70,000 Da (Fig. 2) and a subunit molecular mass of 32,000 Da (ref. 27; Fig. 3). I observed that a small amount of RepA activity, as measured in the *in vitro ori*P1 DNA replication assay, chromatographed with an apparent molecular mass of 160,000 Da (Fig. 2). SDS/PAGE of this protein fraction showed that it contained two protein species. One had a molecular mass of 32,000 Da as expected of RepA. The other had a molecular mass of 40,000 Da and comigrated with authentic DnaJ protein in SDS/polyacrylamide gels (Fig. 3). From densitometer scans of the gels, I calculated that the molar ratio of DnaJ protein to RepA in the complex is 1.07:1. Therefore, most likely, the complex contains one dimer of DnaJ and one dimer of RepA.

I also assayed the DnaJ–RepA complex for activity in *ori*P1 replication reactions by using a host protein fraction prepared from cells deleted for *dnaJ*. I found that the complex completely satisfied the requirement for both DnaJ protein and RepA (Table 2). Furthermore, this fraction had DnaJ activity in an *in vitro* complementation assay for *ori*λ replication, while the RepA fraction had none (Fig. 2).

The isolated DnaJ–RepA complex was very stable. Even after the complex had been stored frozen for 6 months, it eluted at the same position as the original complex when reapplied to the gel-filtration column. The complex still contained equimolar amounts of DnaJ and RepA.

Reconstitution of dnaJ–RepA Protein Complex. I have formed a complex of DnaJ and RepA *in vitro* by simply mixing the two purified proteins. With the protein preparations I used, I found that purified RepA protein eluted from a HPLC gel-filtration column as a dimer of 70,000 Da and DnaJ protein eluted as a mixture of dimers of 85,000 Da and higher molecular mass aggregates (Fig. 4 *A* and *B*). When the two proteins were mixed in the column buffer and then injected onto the column, a protein species of 160,000 Da was seen (Fig. 4 *C* and *D*). SDS/PAGE of the fractions from the column confirmed that the new peak did indeed contain both DnaJ protein and RepA (Fig. 5). When the molar ratio of DnaJ to RepA was 2.3:1, most of the RepA was in the complex with DnaJ (Fig. 4*D*). When the ratio was 0.8:1, some RepA was in the complex and some was free (Fig. 4*C*). Reaction conditions were not varied further, so it is not known if some of the DnaJ protein was inactive or if the reaction was incomplete.

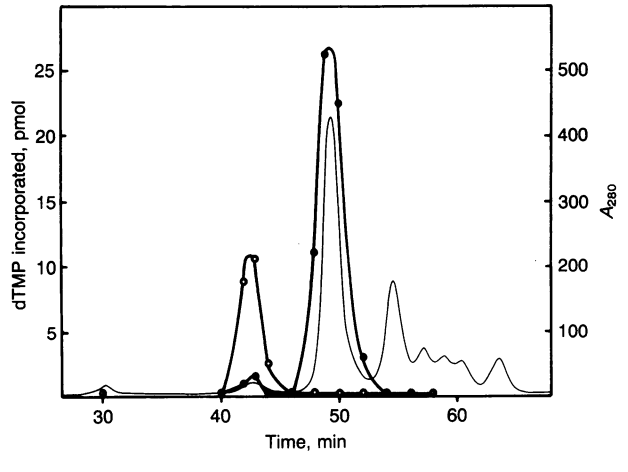

FIG. 2. Isolation of DnaJ–RepA protein complex using HPLC gel filtration. A partially purified fraction of RepA was adjusted to 0.2 M Na_2SO_4, 1 mM EDTA, 1 mM dithiothreitol, 50 mM Tris·HCl (pH 7.5), and 10% glycerol. The sample was injected onto a Bio-Sil TSK-250 gel-filtration column (600 × 21.5 mm) equilibrated with the same buffer. The column was developed with the same buffer with a flow rate of 0.75 ml/min, and fractions were collected. Fractions were assayed for RepA activity in the *in vitro* P1 replication system (●) and for DnaJ activity in the *in vitro* complementation assay for λ replication (○). DnaJ complementation assays with *ori*λ DNA were identical to those with *ori*P1 DNA with the following changes: 100 ng of λ P protein (31) and 600 ng of λ O protein (31) were substituted for RepA, and *ori*λ DNA was substituted for *ori*P1 DNA. The absorbance at 280 nm (in absorbance units × 10^3) is indicated by the thin line. Molecular mass standards run separately on the column eluted in the following positions: thyroglobulin (670,000 Da), 31.7 min; IgG (158,000 Da), 43.4 min; ovalbumin (44,000 Da), 53.4 min; myoglobin (17,000 Da), 59.7 min; and cyanocobalamin (1350 Da), 69.2 min.

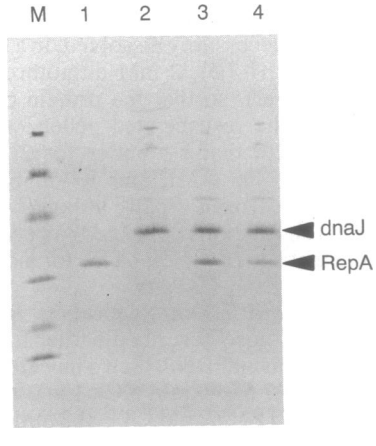

FIG. 3. SDS/PAGE of DnaJ–RepA complex. The fraction containing the DnaJ–RepA protein complex from the column shown in Fig. 2 was subjected to SDS/PAGE side by side with known proteins. Lane M, molecular mass markers: phosphorylase b, 97,400 Da; bovine serum albumin, 66,200 Da; ovalbumin, 42,699 Da; carbonic anhydrase, 31,000 Da; soybean trypsin inhibitor, 21,500 Da; and lysozyme, 14,400 Da. Lanes: 1, purified RepA protein; 2, purified DnaJ protein; 3, fraction eluting at 48.7 min from the column shown in Fig. 2; 4, mixture of the samples (1:1) applied to lanes 2 and 3.

Table 2. Replication activity of DnaJ–RepA protein complex

Addition(s)	DNA synthesis, pmol of dTMP incorporated
None	<0.2
RepA	1.2
DnaJ	<0.2
DnaJ + RepA	28.3
DnaJ–RepA complex	25.3

Reactions were carried out as described in *Materials and Methods*; the host protein fraction was prepared from strain PK102 (*dnaJ*Δ), and 100 ng of DnaA protein was added to each reaction mixture. Where indicated, 170 ng of DnaJ protein, 100 ng of RepA, or 250 ng of DnaJ–RepA complex (isolated from cell extracts during the purification of RepA as described in Fig. 2) was added.

Complex formation was also detected in buffer containing 100 mM Na_2SO_4. Since the salt concentration in the replication reactions was about 150 mM, the DnaJ–RepA complex most likely exists in the reactions.

I have looked for complexes of other combinations of replication proteins by identical HPLC gel-filtration methods. I have not seen complexes of RepA and DnaA protein or RepA and DnaK protein in the presence or absence of ATP and Mg^{2+} or complexes of DnaJ and DnaA protein or DnaJ and DnaK protein in the absence of ATP.

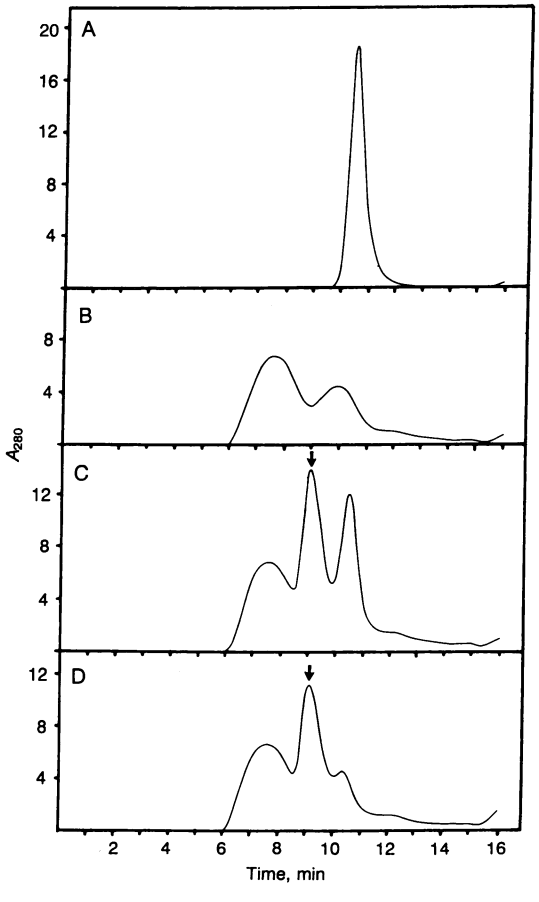

FIG. 4. Formation of DnaJ–RepA complex from purified proteins. RepA and DnaJ protein were incubated, either separately or together, in 50-μl reaction mixtures containing 0.2 M Na_2SO_4, 1 mM dithiothreitol, 1 mM EDTA, 50 mM Tris·HCl (pH 7.5), and 10% glycerol for 5 min at room temperature. The reaction mixtures were injected separately onto a Bio-Sil TSK-250 column (300 × 7.5 mm) equilibrated with the same buffer, and the column was developed with the same buffer at a flow rate of 0.75 ml/min. The A_{280} (in absorbance units × 10^3) is indicated. (*A*) Thirty micrograms of RepA. (*B*) Thirty-five micrograms of DnaJ protein. (*C*) Thirty micrograms of RepA mixed with 35 μg of DnaJ protein. (*D*) Ten micrograms of RepA mixed with 35 μg of DnaJ protein. The arrow indicates the 160,000-Da protein species containing the DnaJ–RepA complex.

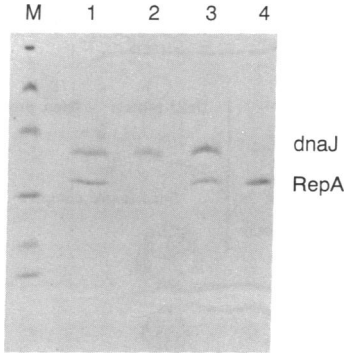

FIG. 5. SDS/PAGE of reconstituted DnaJ–RepA complex. A portion of the sample applied to the gel-filtration column described in Fig. 4*C* and fractions collected from the column were analyzed by SDS/PAGE. Lanes: M, molecular mass markers as described in the legend to Fig. 3; 1, reaction mixture containing DnaJ and RepA that was applied to the column; 2, fraction eluting at 7.5 min; 3, fraction eluting at 9.2 min; 4, fraction eluting at 10.4 min.

DISCUSSION

I have demonstrated that *ori*P1 DNA replication *in vitro* utilizes three heat shock proteins, DnaJ, DnaK, and GrpE. During the course of this work, Tilly and Yarmolinsky (32) and Bukau and Walker (33) found that mini-P1 plasmids are not stably maintained in cells that have mutations in the *dnaJ*, *dnaK*, and *grpE* genes, suggesting that these proteins are also involved in mini-P1 replication *in vivo*. This is the second replicon to be shown to require these proteins for replication, the first being phage λ (1–3). *E. coli* chromosome replication does not appear to absolutely require DnaJ, DnaK, or GrpE. However, Sakakibara (9) and Zylicz *et al.* (11) have seen a requirement for DnaK and DnaJ in *in vitro* complementation assays for *oriC* replication. I have also measured *oriC* replication in complementation assays for DnaJ, DnaK, and GrpE proteins and have obtained similar results. I found that, while there was not an absolute dependence on added DnaJ, DnaK, and GrpE proteins, there was a 2- to 8-fold stimulation of each assay by the addition of the appropriate purified protein (unpublished observations). Therefore it is tempting to speculate that these three proteins are of general importance in *E. coli* DNA replication.

My results also demonstrate that DnaJ protein forms a stable complex with RepA. At this time, the function of the DnaJ–RepA complex is not known. DnaJ might alter RepA, rendering it functionally active for P1 initiation. It is also possible that RepA, through its recognition of both the P1 origin and DnaJ, might direct DnaJ to plasmid P1 replication. Interactions of DnaJ with other host proteins might then promote the assembly of an initiation complex at the P1 origin.

A model for the initiation of P1 replication is presented in Fig. 6 that incorporates many features of the models for *oriC* (34) and *ori*λ (14, 17) replication. However, P1 replication is unique in that it utilizes DnaA and DnaC like *oriC* as well as utilizing its own initiator protein and DnaJ, DnaK, and GrpE like *ori*λ. Initiation of replication at a molecular level is the generation of the first primers for DNA elongation. The DnaG primase needs an extensive single-stranded region, which is provided by the helicase activity of DnaB (18) with the help of DNA gyrase and SSB. DnaB protein in turn needs a single-stranded entry point, which is provided by local unwinding. The position of the local unwinding is directed by origin-specific DNA binding proteins. Thus the first step in

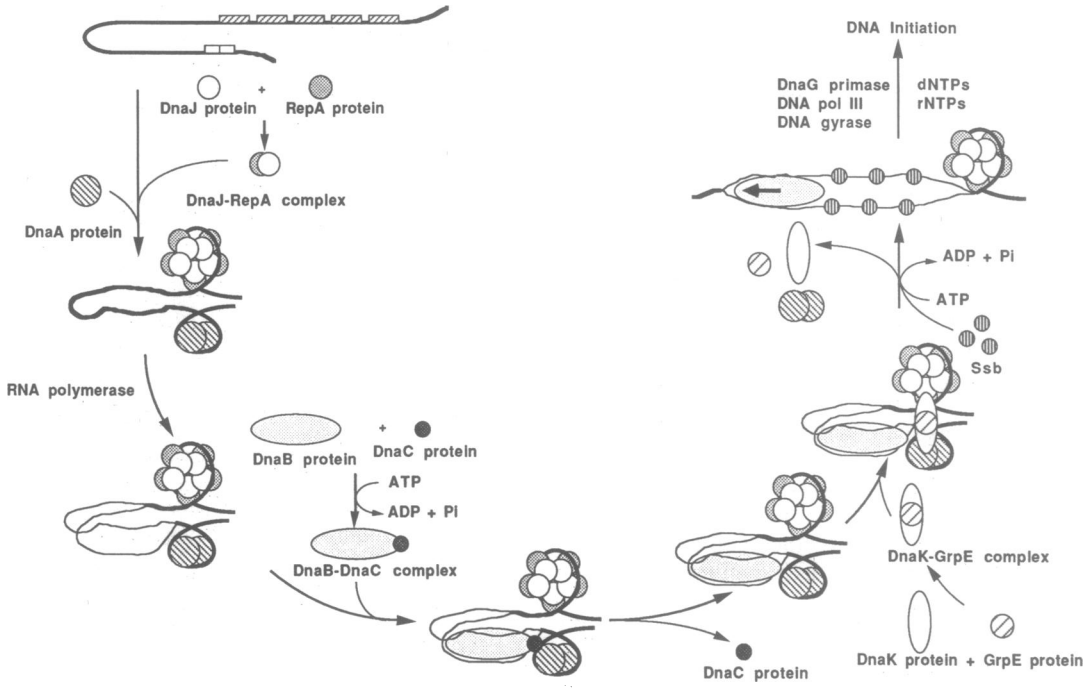

FIG. 6. Model of initiation of *ori*P1 DNA replication. See *Discussion* for description of model.

initiation of P1 replication is the binding of DnaJ–RepA complexes to the RepA binding sites and DnaA protein to the DnaA binding sites in the origin. Possibly DnaK and GrpE function here in conjunction with DnaJ. The binding of the proteins unwinds the DNA to a limited degree. Transcription in the vicinity of the origin may also aid in the reaction. DnaB is transferred to the single-stranded DNA by DnaC protein in an ATP-dependent reaction. This transfer might involve an interaction between DnaC and DnaA, DnaJ, or RepA. The next step in initiation is the local unwinding by DnaB. It is possible that DnaK and GrpE partially dissociate or reorganize the initiation complex such that the DnaB helicase is free to unwind DNA, as proposed for λ initiation (14, 17). The unwinding may be aided by DNA gyrase and stabilized by SSB. The single-stranded DNA, with DnaB and SSB associated with it, is then available for priming by DnaG, very likely through an interaction of DnaG with DnaB. With the first primers synthesized, the initiation phase of replication is complete.

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