Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10345–10349, November 1992
Biochemistry

DnaJ, DnaK, and GrpE heat shock proteins are required in *ori*P1 DNA replication solely at the RepA monomerization step

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Communicated by Nicholas R. Cozzarelli, June 24, 1992

ABSTRACT **We have found that three *Escherichia coli* heat shock proteins, DnaK (the hsp70 homolog), DnaJ, and GrpE, function in *ori*P1 DNA replication *in vitro* solely to activate DNA binding by the replication initiator protein RepA. Activation results from the conversion of P1 or P7 RepA dimers to monomers that bind with high affinity to the origin of replication of plasmid P1. Thus, the essential role of these three heat shock proteins in this replication system is to change the quaternary structure of a single protein, RepA.**

DnaK is a major heat shock protein of *Escherichia coli* and the homolog of eukaryotic heat shock protein hsp70 (for review, see refs. 1 and 2). These proteins protect cells against the stress of heat shock and repair damage done by heat. It has been proposed (3–5) that hsp70 binds to unfolded regions of proteins that have been exposed by the heat shock. hsp70 then releases the bound protein in a reaction coupled to ATP hydrolysis. The released protein can refold properly. Consistent with this model, DnaK has been shown to reactivate specific proteins. Heat-inactivated RNA polymerase is reactivated and disaggregated by DnaK in an ATP-dependent reaction (6). Aggregates of DnaA protein that are inactive in *oriC* DNA replication reactions with purified proteins are disaggregated and reactivated by DnaK in an ATP-dependent reaction (7).

DnaK, in addition to its role in the heat shock response, is essential for normal growth in nonstress conditions (1, 2). It comprises ≈1% of the total cell protein. Mutations in *dnaK* have pleiotropic effects on DNA and RNA synthesis, cell division, proteolysis, and phosphorylation of other proteins. Mutations in two other heat shock genes, *dnaJ* and *grpE*, have similar phenotypes. All three proteins are involved in the *in vivo* replication of phage λ and plasmids mini-P1 and mini-F and in the *in vitro* replication of *ori*λ and *ori*P1 DNA (1, 8–14).

We found (15) that DnaJ and DnaK activate the sequence-specific DNA binding of the P1 initiator protein RepA. RepA is a dimer in solution and forms a stable complex with DnaJ, containing a dimer of RepA and a dimer of DnaJ (14). DnaK, in an ATP-dependent reaction, stimulates the specific DNA binding activity of RepA by ≈100-fold (15). This reaction does not require DNA. We discovered that activation converts RepA dimers to monomers and that the monomer form binds with high affinity to *ori*P1 DNA (16). DnaJ and DnaK are not bound to the *ori*P1 DNA. The enzymatic activation can be mimicked by converting RepA dimers to monomers by urea denaturation at low protein concentration followed by renaturation.

We proposed (16) that, in normal growth conditions, native proteins are identified as targets for DnaK by a protein tag. In our system, DnaJ is the protein-specific tag. The RepA–DnaJ tetramer targets RepA for DnaK action. DnaK recognizes the RepA–DnaJ complex and either RepA or DnaJ acts as an allosteric effector of the DnaK ATPase activity. This produces a conformational change in DnaK that releases active monomeric RepA protein.

We investigated in this study whether the DnaJ- and DnaK-catalyzed activation of RepA DNA binding was required for *ori*P1 DNA replication *in vitro* and whether GrpE was also involved at this step. We used the RepA protein encoded by P7, a plasmid prophage closely related to P1, because it has a higher specific activity than P1 RepA in catalyzing *ori*P1 DNA synthesis in *in vitro* replication reactions. P7 RepA is identical to P1 RepA except for a single Asn → Lys substitution at position 142 (17). P7 RepA complements P1 *repA* mutants for mini-P1 replication and confers a high-copy phenotype for mini-P1 (17).

Our experiments show that the monomeric form of RepA bypasses the requirement for DnaJ, DnaK, and GrpE for the *in vitro* replication of *ori*P1 DNA. Therefore, the essential function of these heat shock proteins is to convert RepA from a dimer to a monomer that binds with high affinity to *ori*P1 DNA. Surprisingly, they are not required at subsequent steps in *ori*P1 DNA replication.

MATERIALS AND METHODS

Bacterial Strains, Phage, and Plasmids. The following strains were used for preparing crude protein fractions for *in vitro* complementation assays: PK102, *dnaJ* deletion (18); C600*dnaK103* (obtained from K. Tilly, National Institutes of Health), DA258, *grpE* deletion (19); and PK101, *dnaJ* and *dnaK* deletion (18). SG4148, *lon* deletion [derived from SG4144 (20)] was used for preparing wild-type extracts. MJH4 (21), a M13 phage that contains an insert of the P1 origin, was used to prepare M13*ori*P1 replicative form I DNA for *in vitro* replication reactions.

Proteins, Enzymes, DNA, and Chemicals. DnaJ, DnaK, and P1 and P7 RepA were purified as described (14). DnaA and GrpE were purified as reported (22, 23). The host protein fractions for *in vitro* complementation assays were prepared as described (14).

*ori*P1[^{3}H] DNA fragments (15) and M13*ori*P1 replicative form I DNA (21) were prepared as described.

[^{3}H]dTTP was from Amersham. Unlabeled ribo- and deoxyribonucleoside triphosphates were from P-L Biochemicals. Polyvinyl alcohol type II, Hepes, creatine kinase, and creatine phosphate were from Sigma.

Activation of RepA. Activation of P1 and P7 RepA by DnaJ and DnaK was as described (16) and was measured by nitrocellulose filter binding (16). Reaction mixtures (20 μl) contained 20 mM Tris·HCl (pH 7.5), 40 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 50 μM ATP, bovine serum albumin (50 μg/ml), calf thymus DNA (50 μg/ml), 40 fmol of *ori*P1[^{3}H] DNA fragment, 50 ng of DnaJ, 500 ng of DnaK, and 50 ng of P1 or P7 RepA. Incubations were for 30 min at 24°C. Reaction mixtures were

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then filtered through nitrocellulose filters and radioactivity was measured.

For urea activation, P1 or P7 RepA was diluted to 10 μg/ml in 5% (vol/vol) glycerol/20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.2 M NaCl/0.5 mM EDTA/8 M urea (buffer A). After 20 min at 24°C, the sample was dialyzed for 2 hr at 0°C against buffer A without urea and then bovine serum albumin (100 μg/ml) was added to the sample. DNA binding by activated RepA was measured by nitrocellulose filter binding as described (16).

***ori*P1 DNA Replication *in Vitro*.** *In vitro* complementation assays for DnaJ, DnaK, and GrpE were as described (14). Reaction mixtures (25 μl) contained 40 mM Hepes (pH 7.5), 20 mM KCl, 1 mM dithiothreitol, 7 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.2 mM UTP, 10 mM $MgCl_2$, 50 mM creatine phosphate, creatine phosphokinase (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 DNA, 100 ng of DnaA, 250 μg of a protein fraction from the wild-type strain or the strains noted above for the measurement of DnaJ, DnaK, or GrpE activity. Where indicated, untreated P1 or P7 RepA, urea-treated P1 or P7 RepA, DnaJ, DnaK, and GrpE were added. After 20 min at 37°C, acid-insoluble radioactivity was measured.

RESULTS

Comparison of P1 RepA and P7 RepA. Crude protein fractions from wild-type cells catalyze *ori*P1 DNA synthesis when supplemented with P1 RepA. We observed by measuring the stimulation of *ori*P1 DNA synthesis in crude extracts that P7 RepA had a 5- to 8-fold higher specific activity than P1 RepA (Fig. 1). This probably explains the high copy number phenotype observed with P7 RepA *in vivo* (17).

Purified P7 RepA could be activated by the same conditions that activate P1 RepA. DNA binding was stimulated by incubation of P7 RepA with DnaJ, DnaK, and ATP (Fig. 2*A*). Both heat shock proteins and ATP were required (data not shown). Chemical dissociation of P7 RepA by 8 M urea at low protein concentration followed by dialysis to remove the denaturant also stimulated DNA binding (Fig. 2*B*). The low protein concentration during this procedure is necessary to prevent dimers from reforming. The specific *ori*P1 DNA binding activities of activated P7 RepA and P1 RepA were similar. This is in contrast to the higher replication activity of P7 RepA compared to that of P1 RepA. P7 RepA, like P1 RepA, was a dimer in solution as determined by gel filtration chromatography and urea-treatment simultaneously converted P7 RepA into the monomer form and activated origin DNA binding (data not shown). Thus, monomer P7 RepA is

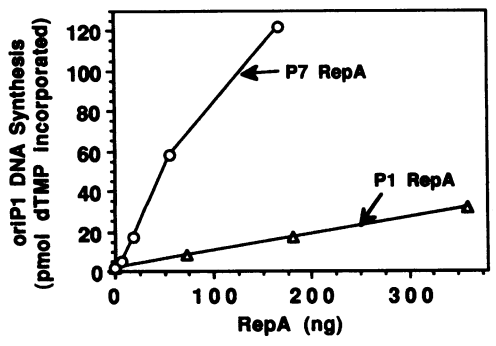

FIG. 1. *ori*P1 DNA synthesis by P1 RepA and P7 RepA. *ori*P1 DNA synthesis catalyzed by crude protein fractions of wild-type cells supplemented with P7 RepA (○) or P1 RepA (△) was measured.

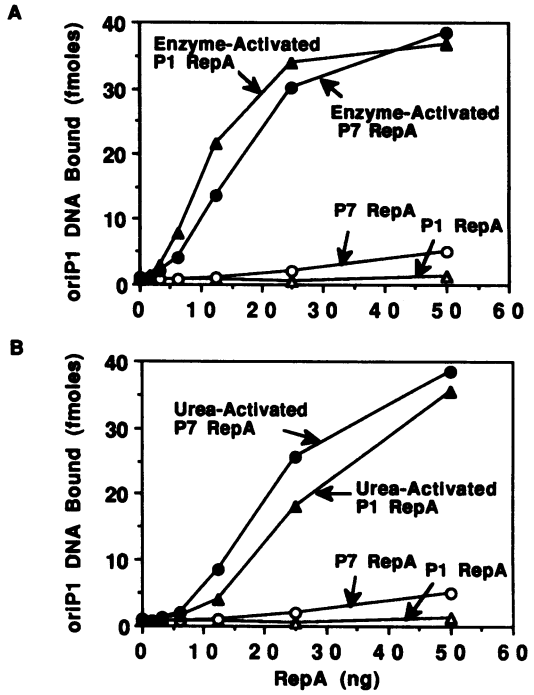

FIG. 2. Activation of the DNA binding function of P1 RepA and P7 RepA. (*A*) P1 or P7 RepA was activated by incubation with DnaJ and DnaK. (*B*) P1 or P7 RepA was activated by treatment with urea. *ori*P1 DNA binding was measured by retention of *ori*P1[^{3}H] DNA on nitrocellulose filters. In both *A* and *B*, DNA binding by untreated P7 RepA (○), untreated P1 RepA (△), activated P7 RepA (●), and activated P1 RepA (▲) is shown.

the form of the protein active in *ori*P1 DNA binding, just as it is for P1 RepA.

***In Vitro ori*P1 DNA Synthesis Requires DnaJ and DnaK Only for RepA Activation.** Wickner (14) showed by *in vitro* complementation assays that *ori*P1 DNA synthesis initiated by P1 RepA required DnaJ and DnaK. These two proteins are also required with P7 RepA. This was determined by measuring *ori*P1 DNA synthesis catalyzed by *dnaJ* and *dnaK* mutant extracts supplemented with P7 RepA, both with and without DnaJ or DnaK (Fig. 3).

We next asked whether DnaJ and DnaK function in *ori*P1 DNA replication solely to activate RepA. The alternatives are as follows. (*i*) DnaJ and DnaK are required to activate RepA and in addition are required at a subsequent stage of DNA replication. (*ii*) RepA monomerization is required for *ori*P1 DNA replication but can be carried out by other proteins in the crude extracts. The heat shock proteins would then be required at another stage, as they are in *ori*λ DNA replication. (*iii*) RepA monomerization is not essential and the heat shock proteins are required at another step in *ori*P1 DNA replication.

If the DnaJ and DnaK were needed solely for activation of RepA, then RepA chemically dissociated by urea treatment should catalyze *ori*P1 DNA synthesis in complementation assays with *dnaJ* and *dnaK* mutant extracts without the addition of purified DnaJ or DnaK. We found that urea-treated P7 RepA alone stimulated DNA synthesis in both *dnaJ* and *dnaK* complementation assays (Fig. 4 *A* and *B*). The addition of DnaJ and DnaK did not further stimulate DNA synthesis. Moreover, urea-activated P7 RepA also promoted DNA synthesis in assays in which the crude protein fraction was prepared from a strain with a deletion of both *dnaJ* and *dnaK* (Fig. 4*C*).

Urea-activated P1 RepA also stimulated *ori*P1 DNA synthesis in the *dnaJ* and *dnaK* complementation assays. The stimulation, however, was less due to the lower specific

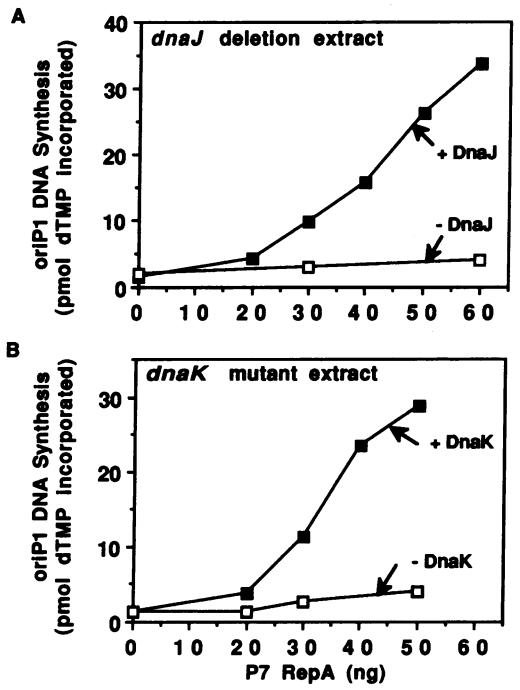

FIG. 3. *ori*P1 DNA synthesis by P7 RepA requires DnaJ and DnaK. *In vitro* complementation assays were carried out using *dnaJ* deletion (*A*) or *dnaK* (*B*) mutant extracts. P7 RepA was added as indicated. □, Reactions carried out in the absence of DnaJ (*A*) or DnaK (*B*); ■, reactions with a saturating amount of DnaJ (100 ng) (*A*) or with a saturating amount of DnaK (1 μg) (*B*).

activity of the P1 RepA protein, as discussed above (data not shown). Larger volumes of urea-activated P1 RepA could not be added due to the volume constraint of the reaction mixtures and more concentrated urea-activated P1 RepA could not be used without dimers reforming.

Thus, DnaJ and DnaK are required exclusively for the activation of RepA for *ori*P1 DNA synthesis *in vitro*. However, from these experiments it does not necessarily follow that the function of the dimer to monomer conversion is to create a pool of molecules that can bind with higher affinity to the P1 origin. Monomers may also be able to (*i*) associate better than dimers with some other component in the extract before DNA assembly, (*ii*) be modified, or (*iii*) isomerize more effectively before loading onto the DNA.

To address this question, we prebound urea-activated RepA or DnaJ- and DnaK-activated RepA to *ori*P1 plasmid DNA, isolated the RepA–DNA complexes by gel filtration, and asked whether the isolated complexes supported DNA synthesis. We have shown (15, 16) that protein–DNA complexes isolated from activation reactions contain RepA but not DnaJ and DnaK. We found that complexes formed with RepA activated by either DnaJ plus DnaK or urea were effective substrates for DNA synthesis by *dnaK* mutant extracts (Fig. 5). The addition of DnaK to the isolated activated RepA–DNA complexes did not further stimulate DNA synthesis by the *dnaK* mutant extracts. In a control experiment, unactivated RepA was incubated with *ori*P1 plasmid DNA and subjected to gel filtration (Fig. 5). The DNA in the excluded volume was not a template for DNA synthesis by the *dnaK* mutant extract with or without the addition of DnaK, indicating that unactivated RepA had not bound to the DNA. However, the DNA was a template for DNA synthesis with the addition of both RepA and DnaK. These experiments show that the RepA–DNA intermediate isolated free from DnaJ and DnaK is effective in DNA synthesis and does not require DnaJ and DnaK for later steps in replication. Very likely, the only role of DnaJ and DnaK

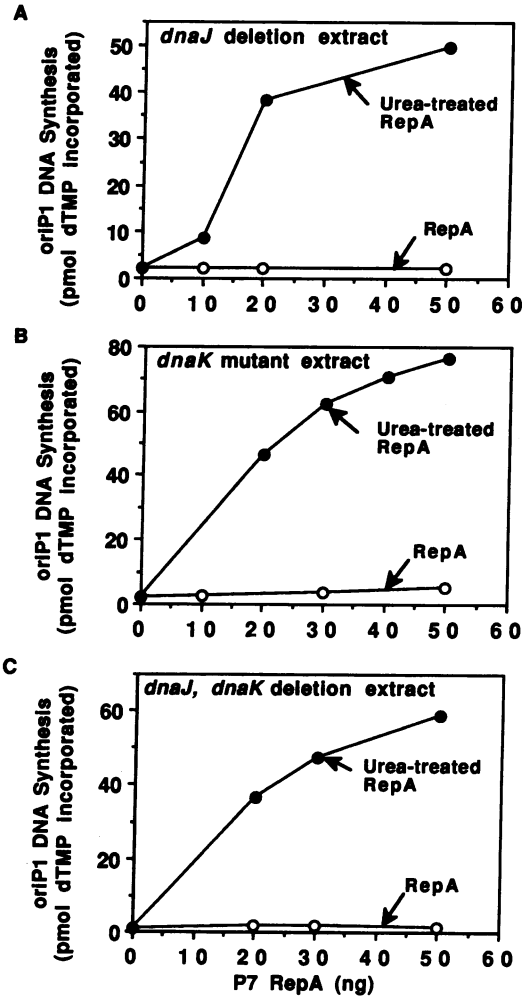

FIG. 4. RepA dissociated by urea treatment bypasses the requirement for DnaJ and DnaK. P7 RepA was treated with urea or diluted to the same protein concentration and similarly dialyzed for 2 hr. The urea-treated RepA (●) and the untreated RepA (○) were assayed for their ability to stimulate *ori*P1 DNA synthesis in the following assays: (*A*) Complementation assays using *dnaJ* deletion extracts without added DnaJ. (*B*) Complementation assays using *dnaK* mutant extracts without added DnaK. (*C*) Complementation assays using *DnaJ, DnaK* deletion extracts without added DnaJ and DnaK. The extent of DNA synthesis with the *dnaJ* and *dnaK* mutant extracts supplemented with urea-treated RepA was nearly identical to that seen with mutant extracts supplemented with untreated RepA and purified DnaJ or DnaK.

in *ori*P1 DNA replication is to convert RepA into a monomer form that binds to *ori*P1 DNA with higher affinity than the dimer.

These experiments also show that there is no observable difference in the replication efficiency of the RepA–DNA complex formed with RepA that has been chemically dissociated by urea and the one formed with RepA activated by the heat shock proteins. Thus, by the DNA replication assay as well as by the DNA binding assay, urea-activated RepA is indistinguishable from heat-shock-protein-activated RepA, suggesting that DnaJ and DnaK do not modify RepA but simply dissociate it into monomers.

***In Vitro ori*P1 DNA Synthesis also Requires GrpE at the Step of RepA Activation.** GrpE was required for *ori*P1 DNA synthesis initiated by P7 RepA, as we had shown for P1 RepA (Fig. 6). Complementation of *grpE* deletion extracts was specific for purified GrpE; DnaJ, DnaK, or the combination of DnaJ and DnaK did not stimulate DNA synthesis, even at protein concentrations that were 10-fold higher than required

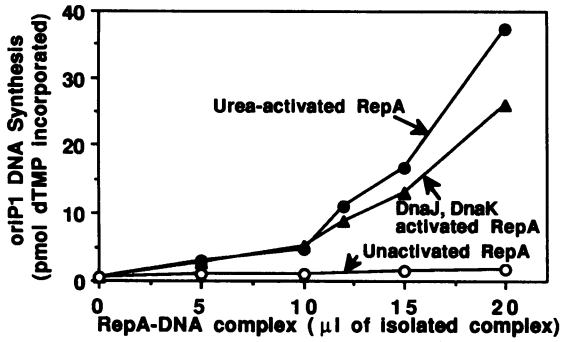

FIG. 5. DNA synthesis by isolated complexes of activated RepA and *ori*P1 DNA. In a first reaction, P7 RepA was activated by urea treatment or by incubating RepA (1 μg), DnaJ (1 μg), and DnaK (10 μg) in a 150-μl activation reaction mixture for 20 min at 24°C. In a second reaction, RepA was bound to *ori*P1 plasmid DNA by incubating 10 min at 0°C (*i*) 100 μl of urea-treated RepA (10 μg/ml), (*ii*) 150 μl of the DnaJ and DnaK activation reaction mixture, or (*iii*) 100 μl of untreated RepA (10 μg/ml) in a 0.2-ml DNA binding reaction mixture containing 20 mM Tris·HCl (pH 7.5), 0.1 M NaCl, 5% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 6 nmol of *ori*P1 plasmid DNA. The mixtures were applied to a 0.7 × 9 cm column of Sepharose 6B equilibrated with the DNA binding reaction buffer. Void volume fractions (containing RepA–DNA complexes) were incubated in replication reaction mixtures with *dnaK* mutant extracts without additional DNA, RepA, or DnaK. ▲, Complexes made with DnaJ- and DnaK-activated P7 RepA; ●, isolated complexes made with urea-activated P7 RepA; ○, isolated DNA that had been incubated with untreated P7 RepA.

to saturate the DnaJ or DnaK complementation assay (Table 1). This result allowed us to ask whether P7 RepA activated by DnaJ and DnaK in a separate reaction with pure proteins could bypass the GrpE requirement for *ori*P1 DNA replication in a complementation assay with a *grpE* deletion extract. P7 RepA was activated by incubation with DnaJ, DnaK, and ATP at 24°C, and then, the reaction mixture was added to the GrpE complementation assay and *ori*P1 DNA synthesis was measured. We found that replication was independent of GrpE (Fig. 7*A*). The GrpE requirement was not bypassed when monomerization was prevented by carrying out the first incubation at 0°C. Thus, GrpE has a key role in the activation of RepA *in vivo* and in crude extracts, but this role can be bypassed in the model activation system with pure proteins.

The requirement for purified GrpE in the *in vitro grpE* complementation assay was also bypassed by P7 RepA that had been chemically dissociated by urea treatment (Fig. 7*B*). The amount of DNA synthesis was the same with heat-shock-protein-activated P7 RepA or with urea-activated P7 RepA. Similarly, P1 RepA that was urea-activated also bypassed the requirement for GrpE (data not shown). Therefore, GrpE is

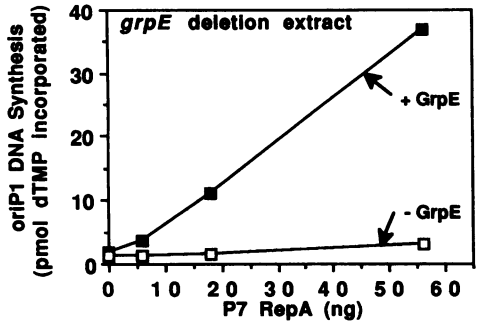

FIG. 6. *ori*P1 DNA synthesis by P7 RepA requires GrpE. *ori*P1 DNA synthesis was measured in complementation assays with *grpE* deletion extracts in the absence of GrpE (□) and in the presence of saturating amounts of GrpE (100 ng) (■).

Table 1. Specificity of the complementation assay for GrpE activity

Addition(s)	*ori*P1 DNA synthesis, pmol of dTMP incorporated
None	1.1
+ GrpE	36.2
+ DnaJ	0.9
+ DnaK	1.6
+ DnaJ + DnaK	1.6

Complementation assays using *grpE* deletion extracts were performed with 100 ng of P7 RepA. GrpE (100 ng), DnaJ (1 μg), and DnaK (2 μg) were added, where indicated. Similar results were obtained when lower amounts of DnaJ and DnaK were used.

required for activation of the DNA binding activity of RepA for *ori*P1 DNA replication. It is not required at any later steps of DNA replication.

DISCUSSION

Our model for the initiation of *ori*P1 DNA replication is shown in Fig. 8. We demonstrated that DnaJ, DnaK, and GrpE all function at an early stage in this process. These three heat shock proteins catalyze the monomerization of RepA, and RepA monomers bind with high affinity to the five direct 19-base-pair repeated sequences in the P1 origin. Although, with our standard reaction conditions, GrpE is not required for the *in vitro* activation of RepA, we have discovered recently that it is required with other reaction conditions. DnaA binds to the two direct repeats of the 9-base-pair DnaA binding site in *ori*P1 (unpublished results). DnaA and RepA may interact, as has been shown for DnaA and Rep protein of plasmid R1 (24). HU, which is required for the *in vivo*

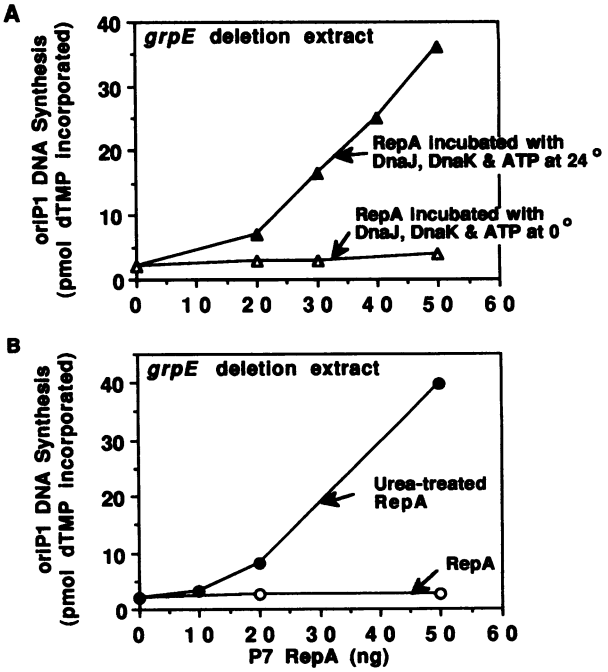

FIG. 7. RepA activated by DnaJ and DnaK or by urea treatment bypasses the requirement for GrpE. (*A*) P7 RepA was incubated in activation reaction mixtures with DNA omitted at 24°C (▲) or at 0°C (△) for 30 min. Portions of the reaction mixtures were then assayed for their ability to support *ori*P1 DNA synthesis in complementation assays using *grpE* deletion extracts in the absence of added GrpE. (*B*) Urea-treated (●) and untreated (○) P7 RepA proteins were assayed for their ability to stimulate *ori*P1 DNA synthesis in complementation assays using *grpE* deletion extracts without added GrpE.

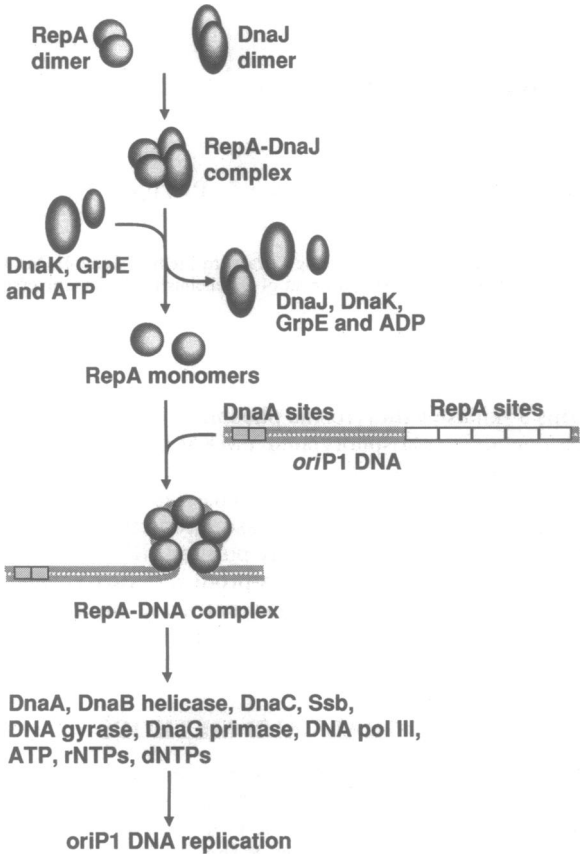

FIG. 8. Model of initiation of *ori*P1 DNA replication. The diagram is not meant to suggest the molecular structures of the individual proteins, except for DnaJ–RepA complex formation.

replication of mini-P1 (25), may also be involved in forming the initiation complex. By analogy with *ori*C and *ori*λ replication (26, 27), the binding of RepA and DnaA to the origin probably results in localized unwinding of the DNA. It is likely that initiation proceeds as has been proposed for *ori*C (26): DnaB helicase is transferred to the origin from DnaC; the origin is further unwound by DnaB helicase (28) helped by DNA gyrase and stabilized by Ssb; DnaG synthesizes primers; and, DNA polymerase III catalyzes DNA elongation.

The role of DnaJ, DnaK, and GrpE in *ori*P1 DNA replication is apparently in sharp contrast to their role in *ori*λ replication. For λ replication, these heat shock proteins act at a later step in initiation (1, 12, 13, 29, 30). λO initiator protein specifically binds to the λ origin without the aid of the heat shock proteins. λP protein forms a protein complex with DnaB and the complex is positioned in the origin through an interaction of λP with λO. DnaB helicase is inactive in this complex with λP, λO, and DNA. The λ replication data are consistent with our model for the action of DnaJ and DnaK: DnaJ tags the DnaB–λP–λO–DNA complex for recognition by DnaK. DnaK in an ATP-dependent reaction activates the DnaB helicase by catalyzing the release of λP from the initiation complex. *ori*P1 replication, like *ori*C replication, does not require DnaJ and DnaK at this step to activate DnaB helicase, probably because these replicons use DnaC protein to transfer DnaB to the origin.

In summary, we have demonstrated that the precise function for the three heat shock proteins, DnaJ, DnaK, and GrpE, in *ori*P1 DNA replication is to activate the RepA initiator protein by converting dimers to monomers that bind with high affinity to *ori*P1 DNA. They are not required at subsequent steps in the initiation reaction.

We thank N. Cozzarelli for helpful discussions and M. Botchan for helpful suggestions.

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