The DnaK-DnaJ-GrpE Chaperone System Activates Inert Wild Type $\pi$ Initiator Protein of R6K into a Form Active in Replication Initiation*

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The plasmid R6K is an interesting model system for investigating initiation of DNA replication, not only near the primary binding sites of the initiator protein $\pi$ but also at a distance, caused by $\pi$-mediated DNA looping. An important milestone in the mechanistic analysis of this replicon was the development of a reconstituted replication system consisting of 22 different highly purified proteins (Abhyankar, M. A., Zzaman, S., and Bastia, D. (2003) J. Biol. Chem. 278, 45476–45484). Although the in vitro reconstituted system promotes ori $\gamma$-specific initiation of replication by a mutant form of the initiator called $\pi^*$, the wild type (WT) $\pi$ is functionally inert in this system. Here we show that the chaperone DnaK along with its co-chaperone DnaJ and the nucleotide exchange factor GrpE were needed to activate WT $\pi$ and caused it to initiate replication in vitro at the correct origin. We show further that the reaction was relatively chaperone-specific and that other chaperones, such as ClpB and ClpX, were incapable of activating WT $\pi$. The molecular mechanism of activation appeared to be a chaperone-catalyzed facilitation of dimeric inert WT $\pi$ into iteron-bound monomers. Protein-protein interaction analysis by enzyme-linked immunosorbent assay revealed that, in the absence of ATP, DnaJ directly interacted with $\pi$ but its binary interactions with DnaK and GrpE and with ClpB and ClpX were at background levels, suggesting that $\pi$ is recruited by protein-protein interaction with DnaJ and then fed into the DnaK chaperone machine to promote initiator activation.

The plasmid R6K contains three origins of DNA replication called $\alpha$, $\beta$, and $\gamma$ and two sequence-specific terminators (Ter sites) (1–9). The plasmid requires both the plasmid-encoded initiator protein $\pi$ and the host-encoded DnaA for replication initiation from the replication origins $\gamma$ and $\alpha$, whereas initiation from ori $\beta$ requires $\pi$ but can occur without DnaA (10–12). The WT $\pi$ initiator protein binds to seven tandem iterons at ori $\gamma$, and the binding of the dimeric proteins at $\gamma$ (13, 14) promotes DNA looping and binding to a single iteron at $\alpha$ and to the half-iteron at ori $\beta$ (see Fig. 1A) (15, 16). Thus the seven iterons are not only needed for initiation from $\gamma$ but also from the distant $\alpha$ and $\beta$ origins (2, 10, 17). The single iteron at $\alpha$ and the half-iteron at $\beta$ are essential for initiation from those two origins, respectively (10, 17). In vitro data are consistent with the notion that $\alpha$-$\gamma$ and $\beta$-$\gamma$ looping not only activates $\alpha$ and $\beta$, respectively, but looping also keeps ori $\gamma$ repressed (17). Thus R6K not only provides an opportunity to investigate the details of the mechanism of local and distant origin activation by looping but also the details of origin repression.

To dissect the biochemical steps of replication initiation locally and at a distance and of ori repression, we have developed a reconstituted in vitro replication system consisting of 22 purified proteins (18). In the in vitro system, a mutant form of the monomeric initiator called $\pi^*$ (P42L/P106L/F107S), but not the usually dimeric wild type $\pi$, is active in replication initiation. Although the monomeric form of $\pi$ promotes initiation from $\gamma$, it is unable to activate the distant $\alpha$ and $\beta$ origins because of its inability to loop DNA (17, 19). Therefore, it stands to reason that both the monomeric and the oligomeric forms of $\pi$ are probably involved in the activation of the distant origins, whereas monomeric initiator is sufficient for initiation at $\gamma$ (Fig. 1B). Furthermore, it is reasonable to expect that there should be a mechanism in the cell to maintain the requisite equilibrium between monomeric and dimeric $\pi$ so that the copy number is maintained and conditions are created that facilitate DNA looping and activation of replication from the two major origins ($\alpha$ and $\beta$) (see Fig. 1B).

Which additional proteins might be needed to activate the inert dimeric $\pi$? A reasonable possibility is suggested by the known roles of chaperones in the replication of other plasmid and phage chromosomes and from the consideration that monomeric $\pi$ is active in the initiation of ori $\gamma$ in vitro, whereas dimeric $\pi$ is inert (19). Chaperones, originally discovered as heat shock proteins, are normally present in the cell at lower concentrations, but their synthesis is enhanced in response to a thermal shock. The Hsp70 class of heat shock proteins, to which DnaK belongs, are known to be involved in ATP-dependent protein disaggregation, unfolding for transport through the membrane, and activation of already folded proteins by promoting monomerization of the dimeric proteins (20). The DnaK chaperone works in conjunction with the DnaJ co-chaperone and the nucleotide exchange factor GrpE to promote phage $\lambda$ replication by dissociating the DnaB helicase from the phage-encoded $P$ protein (21–25). The DnaK chaperone system is also known to be required for the activation of the wild type RepA initiator protein of plasmids P1 and F (26–28). In contrast, replication of plasmid RK2 requires the ClpX chaperone (29).

The DnaK (Hsp70) chaperone system consists of DnaK, DnaJ, and GrpE proteins. DnaK contains 638 amino acids and has a N-terminal ATPase domain followed by a linker domain.
that in turn is followed by a substrate-binding domain and finally a C-terminal domain of unknown function. The ATP-bound DnaK chaperone has low affinity for its substrate and is converted to the ADP-bound high affinity form by interaction with the DnaJ co-chaperone that induces hydrolysis of the ATP bound to DnaK (20). The DnaJ protein (376 amino acids) consists of an N-terminal J domain followed by a GrpE motif, a linker domain, and a Zn\(^{2+}\) finger domain followed in turn by the C-terminal substrate-binding domain. DnaJ can act by itself as a chaperone (20). The GrpE protein (197 amino acids) binds to DnaK and acts as a nucleotide exchange factor that promotes the conversion of the ADP form of DnaK to the ATP form (30). It consists of an N-terminal domain of unknown function followed by an α-helical dimerization domain followed in turn by a C-terminal β-sheet domain. The last two domains are needed for binding to DnaK (20).

ClpA and ClpX, the ClpY family of proteins, are ATP-dependent proteases that have two components: 1) a chaperone component (such as ClpA and ClpX) and 2) a protease component (called ClpP and ClpQ) with which the chaperone component transiently associates and causes unfolding and degradation of damaged proteins (27, 31–35). The chaperone components, as described above, are also involved in initiator activation (29, 34, 36).

The DnaK chaperone system can collaborate with the ClpA family of chaperones in causing disaggregation and proper folding of aggregated proteins (37). In fact, ClpB can also collaborate with the DnaK system to activate the TrfA initiator of RK2, thus providing a second chaperone system for initiator activation in that plasmid (36). The ClpA chaperone can replace the DnaK system in the activation of the RepA protein of plasmid P1 (34).

Although the involvement of chaperones in plasmid replication is well documented, no information (either in vivo or in vitro) was available as to the identity of the chaperone system that might be needed to activate R6K-encoded \(\pi\) protein. Moreover, with the exception of phage \(\lambda\) replication (21, 22) and that of RK2 (29), no previous work has ever used purified chaperones in a reconstituted system to definitively establish that only the chaperones and no other associated protein(s) were involved in the activation of the plasmid-encoded initiator protein.

Keeping the above-mentioned questions in mind, we proceeded to purify several chaperones from the host \textit{Escherichia coli} and tested their ability to (i) enhance the binding of WT \(\pi\) to iteron DNA and (ii) enable the initiator to activate replication origins in the reconstituted system. In this paper, we reported on the development of such a system that includes the chaperone DnaK, the co-chaperone DnaJ, and the nucleotide exchange factor GrpE (38–40), along with 22 other purified proteins that include WT \(\pi\), to initiate origin-specific replication of an iteron DNA plasmid \textit{in vitro}. The results showed that the DnaK chaperone system activated WT \(\pi\) initiator by promoting a dimer to monomeric conversion of \(\pi\) upon binding to iteron DNA. We also found that the activation was relatively chaperone-specific in the sense that ClpB and ClpX chaperones were incapable of substituting for the DnaK-DnaJ-GrpE system to activate \(\pi\). The WT \(\pi\) physically interacted with DnaJ in the absence of ATP. The chaperone-activated \(\pi\) used the same replication origin \(\gamma\) to initiate replication, as did an active monomeric mutant form called \(\pi^*\) (P42L/P106L/F107S).

**MATERIALS AND METHODS**

**Bacterial Strains**—The \textit{E. coli} strain DH5\(\alpha\) (\(\phi\)end 1, hsd R 17 (rK\(\lambda\)Em\(^{+}\)) strain B121 (DE3) was used for the cloning experiments, whereas the \textit{E. coli} strain BL21 (DE3) was used for the expression of all of the proteins. DNA template used in an \textit{in vitro} assay (pMA1) as described earlier (18).

Overexpression plasmids for the production of DnaK, DnaJ, ClpB, ClpX, and GrpE were generated by PCR amplification of the reading frames whose coordinates and the oligonucleotides used are shown in Table 1. The amplified DNA was restriction-digested and cloned into pET15b and pTXb1 and resulted in DnaK-pDZK03, DnaJ-pDZJ03, ClpB-pDZB04, ClpX-pDZX04, and GrpE-pDJE03. All of the clones were sequenced at the DNA Sequencing Facility of Biotechnology Resource Laboratory, Medical University of South Carolina.

**Purification of Proteins**—All of the proteins were quantified by the Bradford colorimetric assay (41). The molar concentration of the proteins was calculated considering the monomeric molecular mass of each protein. The procedures for the purification of all of the proteins needed for \textit{in vitro} replication have been described previously (18). The chaperones were purified essentially through a single affinity column. The cells were grown to a density of 0.6 at 600 nm at 37 °C in L broth supplemented with ampicillin (100 \(\mu\)g/ml). Protein expression was induced at 30 °C for 2 h 30 min, so the cells were harvested by centrifugation for 10 min at 5000 rpm. The cell pellets were suspended in lysing buffer (50 mM Tris-HCl (pH 8.0), 10 mM imidazole, 10% glycerol) and lysed by sonication (3 × 30-s bursts with 5 min of cooling on ice between bursts). The supernatants were collected by centrifugation at 30,000 for 20 min at 4 °C. DnaK and DnaJ supernatants were loaded onto a cobalt-affinity resin (TALON, BD Biosciences) previously equilibrated in buffer A (25 mM Hepes/KOH (pH 7.6), 300 mM KCl, 2 mM \(\beta\)-ME, 10 mM imidazole, 10% glycerol) for DnaK (42) and buffer B (25 mM Hepes/KOH (pH 7.6), 400 mM KCl, 2 mM \(\beta\)-ME, 20 mM imidazole, 10% glycerol) for DnaJ (38). The columns were washed until the flow-through was clear. The protein was eluted with its respective buffer containing imidazole (100 mM). DnaK-His\(_\alpha\) and DnaK-His\(_\beta\)-eluted fractions were pooled and further purified by Superose 12 column with their respective buffer without imidazole. These positive fractions were pooled and dialyzed against storage buffer C (25 mM Hepes/KOH (pH 7.6), 1 mM EDTA, 2 mM \(\beta\)-ME, 400 mM KCl, 50% glycerol) and storage buffer D (25 mM Hepes/KOH (pH 7.6), 0.1 mM EDTA, 2 mM \(\beta\)-ME, 50 mM KCl, 50% glycerol), respectively.

The intein-chitin-binding domain fusion proteins (ClpB, ClpX, and GrpE) were purified on chitin-agarose affinity column (New England
The gels were dried and analyzed by autoradiography and quantified.

8

**Table 1**

| Plasmid construct | Vector | Tag | Protein | EcoGene accession no. | Primers |
|--------------------|--------|-----|---------|-----------------------|---------|
| pDZK03             | pET15b | HIS6 | DnaK    | EG10241               | Same as Ndel-BamHI |
| pDZJ03             | pET15b | HIS6 | dnaJ    | EG10240               | Same as Ndel-BamHI |
| pDZB04             | pTXb1  | CBD | ClpB    | EG10157               | Same as Ndel-SapI |
| pDZX04             | pTXb1  | CBD | ClpX    | EG10159               | Same as Ndel-SapI |
| pDJE03             | pTXb1  | CBD | GrpE    | EG10416               | Same as Ndel-SapI |

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Biolabs) preequilibrated with buffer E (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 20% glycerol, 5 mM MgCl\(_2\)) for ClpB (43), buffer F (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20% glycerol, 5 mM MgCl\(_2\), 1 mM EDTA, 0.015% TX-114, 50% glycerol), and storage buffer H, (20 mM Tris-HCl (pH 7.8), 200 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl\(_2\), 50% glycerol), storage buffer J (buffer G + 50% glycerol), respectively, and stored at -80 °C.

**In Vitro Replication**—The template used (see Fig. 11A) and the procedures for in vitro DNA replications with purified proteins were performed as described previously (18) with some modifications. Reaction buffer contained 40 mM Hepes/KOH (pH 8.0), 10 mM magnesium acetate, 5 mM dithiothreitol, 50 μg/ml BSA, and 10% glycerol. The reactions were done in two steps. The step I contained 2 mM ATP, 5 mM creatine phosphate, 20 μg/ml creatine kinase, 10 mM magnesium phosphate, 200 ng (5.55 pmol) of WT \(\pi\) protein, 1 μg (14.28 pmol) of DnaK, 150 ng (3.65 pmol) of DnaJ, and 50 ng (2.17 pmol) of GrpE protein in 10 μl of reaction buffer. These components were assembled on ice and, after the addition of the WT \(\pi\) initiator protein, preincubated for 60 min at 37 °C. Meanwhile, the step II components were assembled on ice in 20 μl and confirmed the buffer mentioned above along with 40 μM dNTPs, 3000 cpm/ml [\(^{32}\)P]dATP, 3 mM ATP, 500 μM each of CTP, UTP, and GTP, 5 mM creatine phosphate, 20 μg/ml creatine kinase type I (Sigma), DNA template pMA1 (200 ng), 1 μg of single strand-binding protein, 20 ng (0.25 pmol) of WT \(\pi\) protein, 1 μg (14.28 pmol) of DnaK, 150 ng (3.65 pmol) of DnaJ, and 50 ng (2.17 pmol) of GrpE protein in 10 μl of reaction buffer. These components were assembled on ice and, after the addition of the WT \(\pi\) initiator protein, preincubated for 60 min at 37 °C. Meanwhile, the step II components were assembled on ice in 20 μl and confirmed the buffer mentioned above along with 40 μM dNTPs, 3000 cpm/ml [\(^{32}\)P]dATP, 3 mM ATP, 500 μM each of CTP, UTP, and GTP, 5 mM creatine phosphate, 20 μg/ml creatine kinase type I (Sigma), DNA template pMA1 (200 ng), 1 μg of single strand-binding protein, 20 ng (0.25 pmol) of WT \(\pi\) protein, 1 μg (14.28 pmol) of DnaK, 150 ng (3.65 pmol) of DnaJ, and 50 ng (2.17 pmol) of GrpE protein in 10 μl of reaction buffer. These components were assembled on ice and, after the addition of the WT \(\pi\) initiator protein, preincubated for 60 min at 37 °C. Meanwhile, the step II components were assembled on ice in 20 μl and confirmed the buffer mentioned above along with 40 μM dNTPs.

**RESULTS**

**Purification of the Chaperones**—Keeping in mind that the WT dimeric \(\pi\) retains specific DNA-protein and protein-protein interactions and therefore is biologically active, we reasoned that some chaperones are probably involved in the activation of this initiator in vivo. The principal goals that prompted this work were (i) to identify a chaperone or chaperone system that would monomerize and activate WT \(\pi\) and enhance its binding to a single initiator and, more importantly, allow it to catalyze the initiation of replication in vitro and (ii) to determine definitively using an in vitro reconstituted system that only chaperones but no other protein components were involved in the activation process. Although chaperone-mediated in vitro replication of plasmid DNA has been previously reported (26, 46, 47), most of them were carried out using crude extracts replication systems and used antibodies to inhibit the individual chaperones. This approach, while succeeding in identifying the necessary chaperones, left open the possibility that some other yet unidentified additional proteins could be playing a role in this process. Furthermore, previously, there was no information available as to which of the many chaperones encoded in E. coli could activate \(\pi\), either in vivo or in vitro.

Our strategy was to purify different chaperones from E. coli, namely, DnaJ, DnaK, GrpE, ClpB, and ClpX, and systematically investigate their ability to activate WT \(\pi\). We tagged each
of the open reading frames (see Table I) of the chaperones with His<sub>6</sub> and chitin-binding domain tags. Proteins were expressed and purified through a Cobalt affinity and/or a chitin column and purified through a Cobalt affinity and/or a chitin column with or without a 1.4-fold excess of BSA (equivalent to the chaperone added in other experiments) generated two bands corresponding to the positions of monomeric and dimeric π-DNA complexes (Fig. 3, lanes 2 and 6). The intensities of the monomeric and dimeric complexes were approximately equal. Pretreatment with chaperones enhanced the monomeric complex by a factor of 5 (determined as average of four independent experiments; Fig. 3, lane 3). The monomeric π<sup>+</sup> yielded exclusively monomeric complexes (Fig. 3, lane 5). Incubation of the chaperones with DNA in the absence of π yielded, as expected, no DNA-protein complex (Fig. 3, lane 4). It should be noted that our previous work, using both gel filtration and electrophoresis, had established that the first and the second shifted bands corresponded to monomeric and dimeric protein-DNA complexes, respectively (19).

It should be noted that the WT protein preparations did not have any detectable amounts of denatured protein as indicated by the fact that both the dimeric π and monomeric π<sup>+</sup> had the same specific activity of interaction with DNA and with the host proteins DnaA, DnaB, and DnaG. In addition, incubation of the dimeric WT π with the DnaK chaperone system did not further increase the interaction of π with DnaB (data not shown). Thus, the chaperone effect was not due to global re-
folding of a denatured form of WT \( \pi \), rather it specifically converted the protein that bound to iterons as a monomer.

**Optimal Concentrations of DnaK, DnaJ, and GrpE Needed for Interaction of \( \pi \) with Iterons—**Several chaperones, namely ClpA (34), ClpB (in collaboration with the DnaK system) (36), and ClpX (29) have previously been implicated in the replication of plasmid and phage \( \lambda \) DNAs (21, 22, 48). Therefore, we wished to determine whether one or more of these chaperones might also be involved in the activation of WT \( \pi \).

We determined the optimal concentration of DnaK needed to convert WT dimeric \( \pi \) to a monomeric form as determined by a gel mobility shift assay. We kept the amount of DnaJ and GrpE fixed at 100 ng (2.43 pmol) and GrpE fixed at 150 ng (6.52 pmol), respectively, in a 10-\( \mu \)l reaction mixture that also contained 200 ng (5.55 pmol) of WT \( \pi \) and 40 fmol of \( ^{32} \)P-labeled single iteron DNA, and the concentrations of DnaK was varied from 0 to 3200 ng (0–45.7 pmol). The results of the gel shift assays are shown in Fig. 4B, and its quantification (from three independent experiments) is shown in Fig. 4C. The results revealed that 1600 ng (22.9 pmol) of DnaK (in the presence of the indicated amounts of DnaJ, GrpE, and ATP) was needed to get the maximal gel shift. Neither WT \( \pi \) by itself nor in the presence of solo DnaK, DnaJ, or GrpE (data not shown) detectably enhanced DNA binding (Fig. 4A), showing that the DnaK but not the individual proteins were needed for the activation of WT \( \pi \). Despite the fact that DnaJ is known to be a chaperone in its own right in other systems, it did not activate \( \pi \) without the presence of DnaK and GrpE (20). Solo additions of 1600 and 3200 ng of DnaK and ATP did not convert WT \( \pi \) to a monomeric form as determined by gel mobility shift (data not shown). Concentrations of DnaK <800 ng (11.4 pmol) were not effective in promoting the binding of \( \pi \) to iteron DNA under the experimental conditions. It should be noted from the known mobility of the shifted bands characteristic of binding 1 molecule of monomeric \( \pi \) per each molecule of the single iteron DNA. We will show in later experiments that the binding was absolutely dependent on ATP.

**ClpX and ClpB Did Not Activate \( \pi \)—**The chaperone ClpX has been reported to activate the TrfA initiator protein of the broad host-range plasmid RK2 (29). We wished to find out whether this chaperone would also be effective in enhancing the iteron DNA binding activity of WT \( \pi \). We performed gel mobility shift experiments with the labeled single iteron DNA fragment that was incubated with \( \pi \) and also with and without ClpX in a
range of concentrations from 50 to 1600 ng (1.06–34.04 pmol). Trace amounts (<2 fmol) of a shifted band corresponding to the location of DNA-single π monomer complex were observed at the lower concentrations of ClpX, and a stronger shifted band (~4–6 fmol) was observed at 1600 ng (34 pmol) of ClpX per reaction (Fig. 5, lane 7). 800 ng (11.4 pmol) of DnaK, 100 ng (2.4 pmol) of DnaJ, and 150 ng (6.5 pmol) of GrpE (Fig. 5, lane 9) induced the same amount of the shift. However, upon supplementing ClpX with DnaK chaperone proteins in the reaction mixture, there was further enhancement of the gel shift (12–16 fmol; Fig. 5, lane 8). Although there was apparent cooperation between ClpX and the DnaK system in the enhancement of DNA binding, further experiments showed that the enhancement was not manifested in the activation of DNA replication in vitro as presented in Table II.

The ClpB chaperone has been reported to work in conjunction with the DnaK system to activate the dimeric inert TrfA initiator protein of RK2 (36). Furthermore, the functional cooperation of ClpB with the DnaK system has been reported to promote the reduction of aggregation and refolding of proteins (37). We attempted to determine whether ClpB would also provide an additional pathway of activation of WT π. We incubated 200 ng (5.55 pmol) of WT π with 40 fmol of labeled iteron DNA and ATP and supplemented the reaction further with 0–1600 ng (0–17 pmol) of purified ClpB. The reaction mixture was resolved in non-denaturing 6% polyacrylamide gels (Figs. 6 and 7). The results revealed that ClpB by itself had insignificant ability to activate DNA binding of WT π (Fig. 6, lanes 1–8). There was 2-fold enhancement of binding when the DnaK chaperone system was supplemented with 1600 ng (17 pmol) of ClpB (Fig. 6, lane 8). Once again, as presented in Table II, no physiologically significant cooperation was observed in terms of enhancement of in vitro replication catalyzed by WT π when ClpB was supplied in addition to the DnaK system. Systematic omission experiments showed that the enhancement in DNA binding of WT π to the iteron DNA was both chaperone- and ATP-dependent (Fig. 7).

Activation of the Initiator Activity of WT π by the DnaK Chaperone System in Vitro—WT π is known to be inert in initiating replication from ori γ in the purified replication system (18). We proceeded to measure the possible activation of the replication initiation activity of the WT protein by chaperones in vitro as follows. We attempted to optimize the reaction by preincubating 200 ng (5.6 pmol) of WT π with ATP and fixed amounts of DnaJ (150 ng, 3.6 pmol) and GrpE (50 ng, 2 pmol) and a range of concentrations of DnaK (0–1500 ng, 0–21 pmol) for 60 min at 37 °C. After preincubation, the remainder of the components was added and the reaction was allowed to continue as described in the preceding section. The maximal activity resulted from preincubation with 1 μg (14.0 pmol) of DnaK and the stated levels of the co-chaperones. The DnaK chaperone system, under these conditions, promoted vigorous replication yielding up to ~250 pmol of reaction products (Fig. 8).

Optimization of DnaJ and GrpE Concentrations—We attempted to work out the optimal concentrations of DnaJ co-chaperone and the GrpE nucleotide exchange factor by keeping...
DnaK concentration fixed at 1000 ng (14.3 pmol) and using a fixed concentration of the second component while systematically changing that of the third protein. The data showed that the optimal concentrations of DnaJ and GrpE under the experimental conditions were 100 ng (2.4 pmol) and 50 ng (2.2 pmol), respectively (Fig. 9).

**Component Omission Experiments**—We wished to determine whether the addition of either DnaJ and DnaK or GrpE and DnaK resulted in ~30% replication in comparison with the complete system, the presence of all three chaperone components, namely DnaK, DnaJ, and GrpE, was needed for optimal replication. Even though DnaJ is known to be a chaperone in its own right (20), it could not promote detectable activation of \( \pi \) unless DnaK was also present during preincubation. ATP was essential for the preincubation reaction, and the activity of mutant form \( \pi^* \) (R6DM) (for review see Refs. 18, 19, and 45), which by itself promotes vigorous replication, was not further enhanced by the preincubation with the chaperones and ATP (Fig. 10B).

We attempted to determine whether the addition of either ClpB or ClpX to the preincubation mixture containing the DnaK chaperone system had any further effect on the extent of activation of WT \( \pi \). This experiment was prompted by our observations presented above that some synergy between the chaperone systems was observed while measuring the DNA binding activity of \( \pi \). The results showed no significant enhancement of replication initiation activity of WT \( \pi \) by either ClpB or ClpX (Table II). In fact, the addition of ClpX and ClpB inhibited the reaction (the reason for this inhibition is not apparent at this time).

**Site of Replication Initiation by the Activated WT \( \pi \)**—We wished to determine whether the chaperone-activated WT \( \pi \) initiated replication at the correct origin of the template pMA1 DNA by carrying out replication at increasing ratios of ddTTP/TTP in an attempt to trap the initiation bubble as close to the origin as possible. We had previously mapped the initiation site using the reconstituted system that included the mutant form of the initiator called \( \pi^* \) (18). The results (obtained from three independent experiments) showed that the 1.2-kb-long HindIII-ScaI restriction fragment had the highest specific activity at 75 \( \mu \)M ddTTP. We knew from our previous work that \( \text{ori}\gamma \) is located close to the junction between the 1.2- and the 0.9-kb fragments (Fig. 11). Thus the data were consistent with the interpretation that the activated WT \( \pi \) was initiating replication at or close to the \textit{in vivo} location of \( \text{ori}\gamma \) and therefore the replication was deemed to be origin-specific. We have analyzed the topology of the replication intermediates by two-dimensional gel electrophoresis and observed the expected \( \Theta \)-shaped intermediates that were consistent with Cairns-type replication that have been observed \textit{in vivo} (1) and \textit{in vitro} (12, 18, 49) (data not shown).
Physical Interaction of WT $\pi$ with DnaJ—How did the DnaK system activate WT $\pi$? What might be the initial step in the reaction pathway? To address these questions, we attempted to analyze possible direct physical interaction between WT $\pi$ and the various chaperones in the absence of ATP. We immobilized the component chaperones on the plastic surfaces of microtiter plates, and after blocking the unoccupied surfaces with BSA, we incubated the immobilized proteins with WT $\pi$ protein in solution. The amount of $\pi$ binding to the immobilized chaperones was measured after reacting the bound protein with its primary antibody detection of the primary antibody with reporter enzyme-linked secondary antibodies and developed with a chromogenic substrate. Quantification was done with an ELISA plate reader. The results revealed that $\pi$ protein readily interacted with DnaJ while showing only low background levels of binding to the other chaperones. Our previous work (41, 50, 51) has shown that $\pi$ interacts with DnaA, DnaB, and DnaG and also with DnaJ, as shown here. It should be kept in mind that the possibility of conjoint interaction of WT $\pi$ with DnaJ and DnaK could not be ruled out by the results.

**DISCUSSION**

The principal goal of this work was to identify additional proteins that might convert the inert dimeric WT $\pi$ initiator of R6K into a form that is capable of initiating replication in a reconstituted system. The results showed that the DnaK chaperone, DnaJ-co-chaperone, and the GrpE nucleotide exchange factor and ATP hydrolysis were all needed for optimal activation of the WT $\pi$ initiator. Previously, the same chaperone system was known to be involved in phage $\lambda$ replication (21) and in the replication of plasmid P1 (52). In bacteriophage $\lambda$, the phage-encoded P protein recruits the DnaB helicase to the origin region but the P protein shuts down the helicase activity of DnaB in the helicase-P complex. The DnaK chaperone system dissociates the P protein from the complex, thus restoring the helicase activity needed for initiation of replication (22, 40).

In plasmid P1, not unlike in the present system, the dimeric inert RepA protein is converted to an active monomeric form by the action of the DnaK system (43, 53–55).

Why is it that the dimeric initiator apparently predominates in the cell milieu, whereas monomers are the forms actually used for initiation in most plasmid systems? The biological rationale for the existence of the dimers seems to be at least 4-fold, all having to do with control of replication initiation. First, the dimeric initiator binds to the inverted repeat operator sequence of the initiator cistron, thereby autoregulating its expression at the transcriptional step (56, 57). Second, the dimerization also controls the frequency of initiation by allowing for equilibrium to exist between the monomeric and dimeric forms in the cell milieu (58). Third, the dimeric form also promotes the pairing or “handcuffing” of iteron arrays that results in the shutting down of the paired origins (59, 60).

Fourth and finally, in addition to the monomers, the dimeric form of $\pi$ is also needed for initiation from the distant $\alpha$ and $\beta$ origins because only the dimeric form can promote looping of DNA between the $\gamma$-$\alpha$ or $\gamma$-$\beta$ sequences. The looping not only activates initiation from $\alpha$ and $\beta$ but also represses replication at ori $\gamma$ (16, 17, 61). The identification of the DnaK as the required activating chaperone system in R6K should now help in further dissecting the mechanism of replication initiation.

In broad host-range plasmids such as RK2, the initiator protein must be susceptible to activation by at least one chaperone system present in each of the permissive hosts. Whereas it is possible that the evolutionary conservation of a chaperone system such as the Hsp70 (DnaK) could allow such interactions to occur, the ability to be activated by multiple chaperone systems might be beneficial in allowing replication to occur in diverse hosts of broad host-range plasmids (36, 62, 63). In this context, it is perhaps interesting that R6K-encoded $\pi$ initiator apparently had chaperone specificity at least under the present conditions of the experiments. The implication of this apparent specificity could be that R6K may have a narrower host range than RK2.

The results presented in this paper show that the chaperone-mediated activation did not change the origin specificity of the $\pi$ protein, and the initiation site as detected by ddNTP incorporation was indistinguishable between initiation site of WT $\pi$ and $\pi^*$. What might be the sequence of events involving the activation of WT $\pi$ by the DnaK system? While further work addressing this and other related questions are in progress, a provisional outline of a pathway has been formulated by taking into consideration the published information on the DnaK chaperone system (64, 65) and the observation presented here showing direct protein-protein interaction between DnaJ and $\pi$ (Fig. 12A). It appears from published work that the ATP-bound low
affinity form of DnaK and DnaJ binds conjointly to the substrate protein and that contact between DnaJ and DnaK stimulates the ATPase activity, converting DnaK to the high affinity ADP form that binds to the substrate tightly (65). The bound protein is then remodeled and released. Contact between GrpE and the ADP form of DnaK results in the ejection of the bound ADP and its replacement by ATP (see Fig. 12B). We have previously reported that several mutant forms of $$\pi$$ behave as monomers during gel filtration but bind to DNA as monomers (19). DnaK is also known to convert the dimeric TrfA initiator of Rk2 and the RepE of F before binding to the iteron DNA (47, 62). It should be pointed out that, whereas $$\pi$$ interacted with DnaJ but not with DnaK in the absence of ATP, we have observed that the RepE initiator of the F plasmid interacted both with DnaJ and DnaK.2

In summary, this paper presented significant new results on the activation of the $$\pi$$ initiator protein in vitro in a reconstituted system. Future work using this system along with the determination of the crystal structure of this protein bound to an iteron DNA that is in progress in our laboratory should provide valuable tools for a more complete understanding of replication initiation in this model system.

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