Biochemical Investigations of Control of Replication Initiation of Plasmid R6K*

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The mechanistic basis of control of replication initiation of plasmid R6K was investigated by addressing the following questions. What are the biochemical attributes of mutations in the $\pi$ initiator protein that caused loss of negative control of initiation? Did the primary control involve only initiator protein-ori DNA interaction or did it also involve protein-protein interactions between $\pi$ and several host-encoded proteins? Mutations at two different regions of the $\pi$-encoding sequence individually caused some loss of negative control as indicated by a relatively modest increase in copy number. However, combinations of the mutation P42L, which caused loss of DNA looping, with those located in the region between the residues 106 and 113 induced a robust enhancement of copy number. These mutant forms promoted higher levels of replication in vitro in a reconstituted system consisting of 22 purified proteins. The mutant forms of $\pi$ were susceptible to pronounced iteron-induced monomerization in comparison with the WT protein. As contrasted with the changes in DNA-protein interaction, we found no detectable differences in protein-protein interaction between wild type $\pi$ with DnaA, DnaB helicase, and DnaG primase on one hand and between the high copy mutant forms and the same host proteins on the other. The DnaG-$\pi$ interaction reported here is novel. Taken together, the results suggest that both loss of negative control due to iteron-induced monomerization of the initiator and enhanced iteron-initiator interaction appear to be the principal causes of enhanced copy number.

Ever since the publication of the landmark “Replicon Model” 40 years ago by Jacob, Brenner, and Cuzin (1), molecular analyses of the regulation of copy control of plasmid DNAs have been and continue to be instrumental in providing key insights into the mechanisms of regulation of replication initiation. There are at least two principal mechanisms of copy control of plasmid chromosomes. In CoE1 type plasmids, negative regulation of initiation is exercised through RNA-RNA interaction between the primer RNA and an antisense RNA. A small basic protein called Rom enhances the RNA-RNA interaction that negatively controls copy number by preventing the processing of the primer precursor into a functional primer (2–5). A second, more ubiquitous mechanism of copy control is by interaction of the plasmid encoded initiator protein with short tandemly repeated sequences called iterons, which are present at the replication origins of bacterial and plasmid chromosomes (6–9).

The binding of monomeric forms of the initiator to the iterons at the ori promotes assembly of the replication complex and loading of the helicase (10–12). The synthesis of the initiator protein $\pi$ of R6K and other initiator proteins are autoregulated at the transcriptional level (13–15). However, autoregulation of initiator synthesis by itself cannot adequately explain copy control simply because an increase in chromosome copy number would titrate out the initiator and derepress the synthesis of the initiator thereby causing further increase in copy number (16–20). Using topoisomerase II-mediated formation of cotiledon dimers, we showed previously that initiator protein promotes pairing of two replication origins in vitro (21). Helinski and independently Chattoraj were the first to point out that the initiator protein-mediated pairing of plasmid DNA at the iterons in vivo either in cis or in trans causes turning off of replication initiation, and this mechanism of repression is called “handcuffing” (17, 18).

Whereas normal amounts of initiator positively control initiation, an excess of initiator can down-regulate replication presumably by (i) handcuffing origins and (ii) sequestering host-encoded replication proteins by protein-protein interaction (16, 18, 22). Although handcuffing is an attractive mechanism of replication control and is supported by various in vitro and in vivo evidence (18, 23), it may not be the only control mechanism (24, 25).

The essential features of the replication origins of plasmid R6K are shown in Fig. 1. ori $\gamma$ forms a central element that has 7 tandem 21-bp repeated sequences called iterons that bind to the $\pi$ initiator protein. In addition, the region has two DnaA binding sites, an AT-rich region, and an ihf site that binds to the host-encoded DNA-binding protein called integration host factor (IHF)1 (26, 27). ori $\alpha$ and ori $\beta$ are located ~4000 and 1200 bp away, respectively, from ori $\gamma$. DNA looping, promoted by $\pi$ bound to the $\gamma$ iterons, the single iteron at $\alpha$, or the half iteron at $\beta$, is a determinative event in the activation of these two distantly located origins (25, 28, 29). We postulate that activation of $\beta$ (or $\alpha$) not only requires a single dimeric $\pi$ but also monomeric $\pi$ proteins that are bound to the remainder of the $\gamma$ iterons and are involved in the formation of a preinitiation complex that includes DnaA (see Fig.LB).

Many high copy number mutants of the $\pi$ initiator protein of R6K have been isolated (24, 25, 30, 31) and have been studied in vivo and in vitro in crude cell extracts (32–34). Because

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1 The abbreviations used are: IHF, integration host factor; ORF, open reading frame; WT, wild type; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; DTT, dithiothreitol; DR, direct repeat; IR, inverted repeat; ELISA, enzyme-linked immunosorbent assay; CD, circular dichroism; TM, triple mutant; SOS, DNA damage-induced inhibition of cell division.
chaperones appear to mediate conversion of inactive dimeric initiator to active monomers and because one does not know at this time the identity of the chaperones involved in R6K replication, the in vitro analysis needs to be carried out with a replication system reconstituted from highly purified proteins that strictly excludes any chaperones. We have recently developed such systems for R6K (12) and for the F factor. We wished to investigate the biochemical properties of the π protein that were altered by high copy mutations by asking the following questions. Is the high copy number (enhanced initiation frequency) solely due to altered interactions of the mutant forms of the π protein with the ori DNA and altered π-π interaction (changing the monomer-dimer equilibrium in the cell) or does it also involve altered interactions of π with the host-encoded replication proteins DnaA, DnaB, and DnaG that are known to be required for R6K replication?

We have investigated the above-mentioned questions in vivo and in vitro using a reconstituted replication system that lacked chaperones (12). It is important to use a replication system without chaperones that might introduce structural alterations (in oligomeric status) in the π protein(s). Our observations show that (i) the high copy mutations reduced negative control not only by reducing or eliminating handfudging (looping) but also by enhancing the interaction of the protein with ori DNA; (ii) the enhanced binding was probably caused by DNA-ligand-induced monomerization of the high copy mutant forms of π as contrasted with the WT protein that did not monomerize upon DNA binding; and (iii) there was no measurable differences in binary interactions between the wt π with host-encoded DnaA, DnaB helicase, and DnaG primase on one hand and between the mutant forms with the same host proteins on the other. Thus the principal control of replication initiation appears to be effected by the interaction of the π protein with ori DNA and by interaction of π with itself. The data suggest that initiator-ori interaction is the primary mechanistic step in copy control.

**MATERIALS AND METHODS**

**Strains, Plasmids, and Oligonucleotides—*Escherichia coli* strain DH5α was used for all the cloning experiments. The list of mutagenesis oligonucleotides used is given in Table I. Some of the oligonucleotides are mentioned in the text wherever they are used. The sequences of oligonucleotides will be provided upon request. All the strains were grown at 37 °C (unless mentioned otherwise). All the chemicals were purchased from Sigma Chemical Co., St. Louis, MO (unless mentioned otherwise). The custom oligonucleotides were purchased from Integrated DNA Technologies.**

**Cloning and Site-directed Mutagenesis of the π ORF—**Cloning and other manipulations of DNA were carried out according to standard procedures. Site-directed mutagenesis was carried out using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and custom oligonucleotide pairs (Table I) following manufacturer’s instructions. An NdeI restriction site was generated at the 5′-end of π ORF in pAM5383 by site-directed mutagenesis to generate pRSβWT. This was used as the wild type (WT) plasmid for introducing all of the mutations. Briefly, PCR was carried out in a 50-μl reaction volume using 50 pmol of each primer, 400 μM of each deoxynucleotide triphosphate, 50 ng of the template DNA, 4 units of Pfu DNA polymerase (Stratagene) in the reaction buffer provided by the manufacturer. The PCR profile was 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 4 min. A total of 25 cycles were carried out with a final extension at 72 °C for 7 min. The PCR product was digested with DpnI restriction enzyme for 3 h and used to transform DH5α competent cells. The transformants were analyzed by DNA sequencing. The resultant plasmids were called pRSβ-R6, pRSβ-113, pRSβ-R6-113, pRSβ-DM, pRSβ-R6DM, pRSβ-βTM, and pRSβ-βRTM for different mutants, respectively.

**Expression of π Protein—** The protein was expressed in the vector pTXB1 (New England Biolabs, Beverly, MA) under the control of T7 promoter using *E. coli* BL21(DE3) as the host strain. The resultant plasmids were designated as pTXB1-WT, pTXB1-R6 etc. for respective mutants. Upon induction with isopropyl β-D-thiogalactopyranoside (IPTG) π protein was expressed as a fusion with the chitin binding peptide separated by an intein sequence, which can be used to cleave off the fusion partner in the presence of dithiothreitol (DTT). The ORFs of WT and different mutant π proteins were amplified by PCR using oligonucleotide pairs, PfFFNde and PilnSalRIP. The PCR product was digested with NdeI and SalI, ligated between the NdeI and XhoI sites to pTXB1 vector, and used to transform *E. coli* BL21(DE3). This strain was used for production of π protein as well as for complementation of replication of γ ori in trans.

**Replication of γ ori in Vivo—** BL21(DE3)-competent cells containing the pTXB1-Pi (for all the mutants) were co-transformed with ppy-Kan and plated on LB agar containing 50 μg/ml ampicillin and 25 μg/ml kanamycin. The culture medium was supplemented with IPTG and DTT to final concentrations of 5 μM and 1 mM, respectively. The transformants were counted for each set and verified for presence of the plasmids.

**Activation of ori β (Looping Assay)—** The rationale for the assay is that ori γ, but not ori α and β, needs the DNA-bending protein IHF for efficient replication. Therefore, in an IHF3 host α and β, which depend on DNA looping with γ (but not localized DNA bending), are active, whereas γ replications are inactive and form no colonies. The competent cells of *E. coli* HN356 (IHF) and HN840 (IHFΔ) were transformed with different pRSβ plasmids (containing ori β-γ and various forms of π) and plated on LB agar containing ampicillin (for HN356) or ampicillin and tetracycline (for HN840). Colony formation in an IHF3 host was a measure of the efficiency of β-γ-looping. The average numbers of colonies from three separate experiments are shown in Table IV.

**Determination of Relative Copy Number—** The competent DH5α cells containing different pRSβ-π plasmids were transformed with pACYC184 (New England Biolabs) and plated on LB agar containing 50 μg/ml ampicillin and 17 μg/ml chloramphenicol. Individual double transformants were grown in 5 ml of LB broth at 37 °C with shaking at 250 rpm containing the above antibiotics to same density. Plasmids were prepared using the Qiaspin™ Miniprep kit (Qiagen, Chatsworth, CA) and finally eluted in the same volumes. Equal volumes of DNA samples were digested for 3 h with BamHI, which linearizes both the pRSβ and pACYC184 plasmids, and electrophoresed in a 1% agarose gel. The gel was stained with ethidium bromide and analyzed by the
Quantitation of the oligonucleotides would be made available upon request.

### RESULTS

#### In Vivo Characterization of High Copy Number Single Mutants and Combinations of Mutants of \(\pi\) — During the course of the last several years, we and others have isolated several copy up mutants in the \(\pi\) open reading frame (ORF) that individually but modestly enhance the copy number of the plasmid (24, 30, 31). We have previously isolated a mutant form of \(\pi\), P42L, that is defective in DNA looping and fails to activate the replicon is enhanced 2- to 3-fold by the mutation (25). Other high copy number mutations (e.g. P106L, P107S, and P113S) have been isolated in the region spanned by the residues 106–113, and each mutation caused a relatively modest enhancement of copy number (24, 30, 31). The mutant genotypes are shown in Table I. First, we wished to investigate the effect of these mutations individually and in various combinations on the copy number in vitro and to investigate possible growth changes induced in the protein structure by the mutations. Second, we also wished to characterize bio-

| Name of mutant | Change in residue | (Oligonucleotide pair/template used for mutagenesis) |
|----------------|------------------|--------------------------------------------------|
| WT             | Wild type Pi (GenBank accession number X00320) | (\(\pi\) NdeTOP/\(\pi\) NdeBOT/pAM8388) |
| R6             | P42L             | (R6TOP, R6BOT/pRS8388) |
| 113            | P113S            | (\(\pi\) 113TOP, \(\pi\) 113BOT/pRS8388) |
| R6–113         | P42L, P113S      | (R6TOP, R6BOT/pRS8388–R6) |
| DM             | P106L, F107S     | (DMTOP, DMBOT/pRS8388) |
| R6DM           | P42L, P106L, F107S | (R6TOP, R6BOT/pRS8388–DM) |
| TM             | P106L, F107S, P113S | (TMTOP, TMBOT/pRS8388) |
| R6TM           | P42L, P106L, F107S, P113S | (R6TOP, R6BOT/pUC18-TM) |
chemically the mutant forms of the π protein in vitro using a reconstituted replication system (12), to gain additional insights into the initiation control mechanism.

The replication efficiency in vivo of the WT and the various mutant forms is shown in Table II. The data show that all of the various forms of π are almost equally competent to promote replication of the γ replicon in vivo.

The copy numbers of the various combinations of mutations are shown in Fig. 2. The measurements of copy number were normalized with respect to a resident pACYC184 plasmid that was used as an internal standard after linearizing the plasmids with a single restriction cut. Qualitatively (Fig. 2A) and quantitatively (Fig. 2B) it is clear that R6 (P42L) and 113 mutations individually caused a modest increase in copy number (between 1.25 and 1.5, considering the WT copy number as 1) in comparison with the WT form. However, a combination of R6 and 113 yielded the highest increase in copy number (~2.7-fold over the WT). The double mutant DM (P106L, F107S) was as effective as the single mutant 113 (P113S) in elevating copy number. A triple mutant R6DM further enhanced the copy number to 2.5. Similarly the triple mutant R6TM (P106L, F107S, and P113S) elevated the copy number to 2.0. The quadruple mutant R6TM caused a ~2.6-fold enhancement in copy number. Thus, a combination of the P42L mutation with one or mutations from the 106–113 region of the π ORF caused the maximum enhancement of copy number. The results suggest that π monomers interact with each other at two regions, at or near residue 42 and in the region of the residues 106–113. It is possible that the folding of the protein might bring the two regions into close proximity forming a single interaction domain.

**Binding Affinity of the WT and the Mutant Forms of π to a Single Iton**—We proceeded to characterize the WT and mutant forms of π by measuring the binding affinity with the objective of gaining insight into the mechanism of initiation control. We measured the binding constants of interaction between the WT and the various mutant forms of π with a single copy of the iteron by gel mobility shift experiments in nondenaturing polyacrylamide gels. The data presented are averages of four independent sets of experiments (Fig. 3A). The binding constants derived from protein concentration that promoted half-maximal binding are shown in Table III. Generally, there was a positive correlation between the copy number and the $K_d$ with one exception. The R6–113 double mutant form of π did not bind to a single iteron DNA as strongly as some of the other forms (e.g. R6-DM), but it caused the highest elevation in copy number. Keeping in mind that the affinity measurements are in vitro experiments, whereas copy number measurements are in vivo, it is possible that the R6–113 double mutant is more easily activated (monomerized) by chaperones in vivo thus showing higher copy number.

**FIG. 2.** Copy number increase caused by various mutant forms of π. A, photograph showing the increase in copy number of an ori γ plasmid with the WT or the various mutant forms of π. The resident pACYC184 plasmid served as an internal standard. B, the gels such as that shown in A were quantified by densitometry and plotted after normalization using the internal standard.

**FIG. 3.** Binding curves of a single iteron and inverted half iteron (operator) DNAs with WT and the various mutant forms of π. A, binding curves of iteron-protein complexes (curve for R6 is not shown). B, binding curves of inverted half repeat-protein complexes.

**Binding to Inverted Half Iterons**—The inverted half iteron (IR) constitute the operator of the π ORF, and binding of π protein to this site autoregulates the initiator at the transcrip-
We determined the molecular mass of the WT and the various mutant forms for the inverted repeat. The data were quantified with a PhosphorImager and the average of three independent experiments, are presented in Fig. 3B. The results showed no significant differences in binding affinities of the WT and the various mutant forms for the inverted repeat. The average $K_d$ was $2 \times 10^{-14}$ mol/liter. The results are consistent with the observation presented below that, in the absence of a denaturing agent such as guanidium chloride, the WT and all of the mutant forms existed as dimers in solution. It was interesting that no difference in affinity of binding between the WT and those of the high copy mutant forms to the IR sequence was observed. The results suggest that, unlike the direct repeat iteron, the IR sequence did not cause monomerization of the mutant forms of $\pi$. In fact the gel mobility shift data (not shown) did not show any difference between the WT and the mutant forms of $\pi$ binding to the IR sequence.

**Ligand-induced Monomerization of the Mutant Forms of $\pi$—** We determined the molecular mass of the WT and the various mutant forms of $\pi$ at neutral pH, in a buffer containing 2 M guanidium chloride, by gel filtration through a Sephadex G-75 column. Carbonic anhydrase and bovine serum albumin were used as molecular mass standards. Under these conditions, the WT $\pi$ migrated as a dimer with a relative molecular mass of 72 kDa, whereas the mutant form R6TM eluted as a monomer (Fig. 4A). Equal amounts of the WT and the mutant forms were mixed and subjected to gel filtration in 2 M guanidium chloride to confirm that the WT protein remained as a dimer and the mutant was monomerized (Fig. 4A). In contrast, when the gel filtration was carried out in the absence of guanidium chloride, both the WT and the R6TM forms eluted as dimers (Fig. 4C). We prepared $^{32}$P-labeled, single iteration double-stranded DNA and performed high resolution gel mobility shift experiments and discovered that the WT form bound to the iteron DNA mostly as a dimer and a minor form as a monomer (Fig. 4B). In contrast, the R6TM (Fig. 4B) and R6DM (not shown) were completely monomerized upon binding to a single iteron. The 113 and R6 forms showed a higher level of monomers than the WT protein but lower than those of the R6TM/R6DM forms (Fig. 4D). The data showed that the high copy forms appeared to be predisposed to iteron DNA ligand-induced monomerization. This property was most pronounced in the R6DM and R6TM mutant forms (Fig. 4E) that have a higher copy number phenotype. The latter mutants also show the highest affinity for the iteron DNA (Table III). The relative amounts of the dimeric and monomeric forms of iteron-bound $\pi$ is plotted in Fig. 4E.

Comparing the CD spectra of WT $\pi$ protein with R6TM mutant form clearly indicated the overall structural similarity of these two forms. The minima at 222 nm and 209 nm, the maximum at 195 and the zero crossings at 203 nm are iden-

**Fig. 4. Monomerization of the various forms of $\pi$ after binding to a single iteron, double-stranded DNA fragment.** A, elution profiles of the WT and the R6TM protein from a Sephadex G-75 column (dimensions 55 $\times$ 1.5 cm) that was equilibrated in 2 M guanidinium chloride. B, electrophoretic gel mobility shift patterns of WT and the R6TM protein to $^{32}$P-labeled single iteron DNA. Note the positions of monomeric shift versus dimeric shift. Note that binding to iteron has completely monomerized the R6TM protein, whereas the WT protein appears to remain mostly as a dimer. C, elution profiles of the WT and the R6TM proteins from a Sephadex G-75 column in the absence of guanidinium chloride. D, electrophoretic gel mobility shift patterns of R6 and 113 proteins. E, quantification of the monomers versus the dimers by PhosphorImager analysis of the gels such as shown in B and D.

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**TABLE III**

| Protein | $K_d$ (pmol/liter) |
|---------|-------------------|
| WT      | 68.0 +/- 1.8      |
| R6      | 57.8 +/- 2.6      |
| 113     | 68.0 +/- 1.2      |
| R6–113  | 49.1 +/- 1.1      |
| DM      | 40.3 +/- 1.4      |
| TM      | 34.2 +/- 1.2      |
| R6DM    | 5.19 +/- 0.4      |
| R6TM    | 1.93 +/- 0.1      |

*Interaction of the Various Forms of $\pi$ Proteins with DnaA, DnaB, and DnaG—*This set of experiments was designed to...
determine whether the phenotype of the mutant form as contrasted with that of the WT could also be attributed to differences in the interaction with host-encoded replication proteins that are known to act at the plasmid ori. DnaB helicase and DnaA protein were known to physically interact with \( \pi \) and probably participate in helicase loading (37, 41). We have discovered that DnaG also physically interacts with \( \pi \) (see below), although in this case the mechanistic implications of the interaction are not presently known. We have raised polyclonal, monospecific antibodies to DnaA, DnaB, and DnaG and the antibodies specifically immunoprecipitate the antigen (not shown). The antibodies provided us with a reliable, quantitative, and reproducible method for measuring protein-protein interactions by performing ELISA (39).

We separately immobilized purified DnaA, DnaB, and DnaG proteins to plastic wells of microtiter plates, blocked protein free surfaces with bovine serum albumin and then loaded the wells with a range of increasing concentrations of the WT or mutant \( \pi \). After incubation and washing, secondary antibody coupled to a chromogenic substrate for alkaline phosphatase was added and the generation of color by hydrolysis of the indicator was measured in a plate reader. Three independent sets of experiments were performed, and the data were averaged. With the exception of DnaA (that gives high background when added in solution to immobilized proteins), we have also performed the reciprocal experiments where we immobilized the \( \pi \) in the plates and overlaid that with purified DnaA, DnaB, and DnaG. Bovine serum albumin-coated wells were used as a control to measure background binding. The data show that DnaA (Fig. 6), DnaB (Fig. 7), and DnaG (Fig. 8) bound equally well to the WT and the various mutant forms of \( \pi \). In the past we have performed binding assays using labeled protein with a second protein immobilized in an affinity matrix and surface plasmon resonance measurements but have found ELISA with monospecific antibodies to be the superior method.3

In Vitro Replication in a Reconstituted System—What is the impact of high copy number mutations on the efficiency of replication initiation? This question is best addressed using a fully reconstituted in vitro replication system that did not include any chaperones. We have developed such a system consisting of 22 purified proteins (12). The plasmid pMA1 that contained ori \( \gamma \) cloned in a pUC19 vector was used as the template to investigate the effect of the high copy mutations on in vitro replication. It should be noted that the reconstituted system did not include DNA polymerase I, and consequently, initiation from the vector ori that needed polymerase I was prevented and all of the replication initiated from ori \( \gamma \) (12). The reactions were carried out for selected mutant forms of \( \pi \) over a range of protein concentrations, keeping the template concentration constant (Fig. 9A). We also present the picomoles of template replicated at the concentrations of each type of \( \pi \) that yielded the maximal replication for that type (Fig. 9B). The data showed that the WT, R6, 113, R6–113 forms were relatively ineffective in catalyzing replication. The DM and TM forms promoted some replication with TM being more effective than DM. However, the R6DM also called \( \pi^* \) (12) and R6TM promoted vigorous replication. It should be noted that there is a direct correlation between the relative abundance of the

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monomeric form generated upon DNA binding (Fig. 4E) with the efficiency of replication (Fig. 9).

**In Vivo DNA Looping Analysis**—The looping analysis is based on the observation that \( \pi \)-mediated looping of ori \( \gamma \) to \( \alpha \) or \( \beta \), is required to activate the latter origins and thus the distantly located ori \( \alpha \) or \( \beta \) activation is a measure of looping proficiency of \( \pi \). Furthermore, initiation from ori \( \gamma \) (not from \( \alpha \) or \( \beta \)) requires the DNA-bending protein IHF, and therefore maintenance of R6K in an ihf\^-host, where \( \gamma \) remains inactive, is a measure of initiation activity of ori \( \alpha \) or \( \beta \) (25). The data presented in Table IV show that all of the high copy mutant forms, including R6, are of the non-looping form. It should be kept in mind, as shown by our previous work (25), the mutants that cause defect in \( \pi \)-mediated DNA looping are also defective in handcufing. Therefore, measurements of the looping efficiency are also an acceptable measure of the extent of handcufing. Therefore, from the data presented in Table IV, we infer that all of the high copy mutants are also defective in handcufing.

**DISCUSSION**

The evidence presented in this report show that the high copy number mutations in the ORF of \( \pi \) caused elevation of copy number not only by abolishing negative control by handcufing (looping) but also by promoting stronger interaction of the mutant forms of the protein with iteron DNA that in turn enhanced the initiation frequency. The high copy mutations were manifested in the monomerization of the normally dimeric \( \pi \) protein upon binding to iteron DNA. Our previous in vitro reconstitution experiments had shown that the WT dimeric \( \pi \) was inert in catalyzing replication in vitro, whereas a mutant form called \( \pi^* \) catalyzed vigorous replication (12). The present work further extended and generalized that observation by showing that there was a positive correlation between the relative abundance of the monomers and the efficiency of replication in vitro.

The present work showed that the iteron DNA ligand apparently acted as an allostERIC effector and caused monomerization of the high copy mutant forms of \( \pi \). Interestingly, CD analysis showed no gross structural changes in the mutant forms in comparison with WT \( \pi \). Gel filtration carried out in the absence of denaturing agents showed that not only the WT \( \pi \) but also all of the mutant forms were dimeric in solution. While this work was in progress, iteron-induced monomerization of an initiator protein encoded by a plasmid from a different bacterial system was reported (42). DNA ligand-induced monomerization has the advantage that only a subpopulation of initiator that is bound to the ori will be in the active monomeric form. This line of thinking raises the interesting question as to how can negative regulation by handcufing occur if iteron binding causes

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**FIG. 8.** ELISA analysis of DnaG-\( \pi \) interaction.

**FIG. 9.** Replication of an ori \( \gamma \) template in an in vitro, reconstituted replication system. A, extent of replication catalyzed by the different forms of \( \pi \) as a function of initiator protein concentration. B, maximal synthesis catalyzed by various forms of \( \pi \) in 60 min at 37°C.

**TABLE IV**

| Type of \( \pi \) | Number of colonies growing in IHF | IHF\^- |
|-----------------|-------------------------------|--------|
| WT | 1848 | 1088 |
| R6 | 1176 | 3 |
| 113 | 1464 | 10 |
| R6–113 | 1152 | 5 |
| DM | 1072 | 0 |
| R6-DM | 1016 | 3 |
| TM | 792 | 0 |
| R6-TM | 814 | 0 |

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dissociation of the initiator into monomers? One should keep in mind the observation reported here that WT π dimers do not monomerize when bound to the iteron DNA.

Because WT π is active in vivo and in crude extract replication systems in vitro (32–34, 43), it is reasonable to suspect that chaperones present in the cell milieu participate in predisposing a certain percentage of the WT π to be monomerized upon iteron binding. A monomer-dimer equilibrium in the cell milieu would provide both forms of π to mediate the diverse functions noted above and would maintain proper copy number control. The dimeric protein would be available for autoregulation of π synthesis and promoting looping-dependent activation of α and β origins (25, 28). Published observations along with data presented here show that the activation of the distantly located α and β origins requires DNA looping by dimeric (or oligomeric) form of π (24, 25, 28, 29). Although a dimeric protein would be needed to loop α or β to a single iteron of γ (Fig. 1B), the remainder of the iterons at γ probably interact with and wind around monomeric π to assemble a pre-initiation complex and transfer it to the distant origins (see Fig. 1B). We have discovered that α (or β)-γ looping needed for origin activation does not just transfer the π protein from its central location at the seven iterons of γ, but it probably delivers an initiation complex formed at γ to ori α or β.4 The dissection of DNA looping-dependent origin activation would require further work using the in vitro reconstituted system (12).

Perhaps the high copy mutant forms of π could be considered as intermediates in the pathway of a chaperone-mediated conversion to monomers. The chaperone-π interaction could generate pre-monomers that upon interaction with the iterons are finally converted to active monomers. Which set of chaperones are involved in the activation of π? The answer to this question is not known at this time and is a subject of our ongoing work. However, the chaperones DnaJ, DnaK, and GrpE are known to activate the RepA protein of P1 (44–46) and ClpA (47) and ClpX (48) are also known to activate plasmid initiator proteins in other systems. It would be interesting to determine whether chaperone action is necessary and sufficient to monomerize π or is iteron binding also necessary to complete the process.

The π protein is known to interact with several host-encoded replication proteins, e.g. DnaA, DnaB and as reported here, also with DnaG. The interaction with DnaA is crucial for origin opening and helicase loading (41, 49). The ATP-bound form of DnaA is not needed for replication of R6K and pSC101 (38, 41). Interaction of π with DnaB is probably needed to recruit the DnaB helicase to the plasmid ori (39). There are other examples of the need for direct interaction between the plasmid-encoded initiator and the host-encoded helicase for loading the latter on to the ori. We have shown previously that a mutant form of the initiator RepA of the plasmid pSC101 that fails to interact with DnaB also fails to load the helicase to the plasmid ori (49). The larger form of the TrfA initiator protein of plasmid RK2 interacts with DnaB helicase in Pseudomonas to load the helicase onto the plasmid ori (40, 50).

In this report we have shown that the DnaG primase physically interacted with π. The precise elucidation of the physiological role of the latter interaction would require isolation and biochemical characterization of non-interacting mutants and such work is in progress. However, there is already some evidence that is consistent with in vivo interaction between DnaG and a plasmid-encoded initiator protein. For example, overproduction of the RepA initiator protein of the plasmid pSC101 causes induction of SOS response and arrest of cell division. The SOS checkpoint is relieved by overproduction of DnaG (carried in a compatible plasmid) in the cell (22). The results have been interpreted as follows. Overproduction of RepA titrates out DnaG causing arrest of forks that activates the SOS checkpoint. Overcoming the deficiency of DnaG in the cell milieu helps to restart arrested forks and relieves the checkpoint arrest (22).

Given the interactions described above, in principle, it was not unreasonable to expect that enhancements or attenuation of the strengths of interaction between the plasmid encoded initiator and the host-encoded replication protein could have had an impact on the frequency of initiation and hence on the copy number. With this thought in mind, we systematically examined the interaction of the WT π with the host-encoded proteins on one hand and that between the high copy mutant forms of π with the same host proteins on the other but found no measurable differences. CD analysis did not reveal any major structural change caused by the high copy mutations in comparison with the WT π. In reaching this conclusion, one should be cautious and keep in mind that the protein-protein interactions have been investigated in the absence of ori DNA. The binding of the π protein to the ori iterons might modulate interaction with the host proteins. We attempted to investigate the protein-protein interactions reported here in the presence of iteron DNA but so far have failed in these attempts due to technical problems such as high background. However, in this context, it is worth remembering that despite several decades of work by a large number of laboratories, no one has ever isolated and characterized (perhaps with one exception) autologous mutations in a host-encoded replication protein that altered plasmid copy number. The rare exception is an IHFα mutant that causes ori γ replicons to be maintained at very low copy numbers (27). However, there is no evidence that IHF interacts with π (41). Barring the isolation of host mutants in the future that affect plasmid copy number, one would cautiously conclude that, at present, there is no evidence for host protein-π interactions playing a major role in copy control.

Finally, the work presented here suggests several experiments to be carried out in the immediate future. Determination of the physiological role of DnaG-π interaction, the mechanism of initiation in vitro of replication from ori α and β, solving the crystal structure of monomeric and dimeric π as a complex with iteron DNA are major areas of work that are now in progress.

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