Mechanistic studies of initiator–initiator interaction and replication initiation

Ya-Bin Lu, Hirock J.Datta and
Deepak Bastia¹

Department of Microbiology, Duke University Medical Center,
Durham, NC 27710, USA

¹Corresponding author
e-mail: bastia@abacus.mc.duke.edu

Unlike the chromosome of *Escherichia coli* that needs only one replication initiator protein (origin recognition protein) called DnaA, many plasmid replicons require dual initiators: host-encoded DnaA and a plasmid-encoded origin recognition protein, which is believed to be the major determinant of replication control. Hitherto, the relative mechanistic roles of dual initiators in DNA replication were unclear. Here, we present the first evidence that DnaA communicates with the plasmid-encoded π initiator of R6K and contacts the latter at a specific N-terminal region. Without this specific contact, productive unwinding of plasmid ori γ and replication is abrogated. The results also show that DnaA performs different roles in host and plasmid replication as revealed by the finding that the ATP-activated form of DnaA, while indispensable for oriC replication, was not required for R6K replication. We have analyzed the accessory role of the DNA bending protein, integration host factor (IHF), in promoting initiator–origin interaction and have found that IHF significantly enhances the binding of DnaA to its cognate site. Collectively, the results further advance our understanding of replication initiation.

Keywords: DnaA/IHF/ π protein/protein–protein interaction/replication control

Introduction

Despite extensive work on the enzymology and molecular biology of DNA replication in prokaryotic and eukaryotic systems, several critical aspects of the mechanism of initiation of DNA replication and its control still remain obscure. One significant gap in our knowledge is a lack of understanding of the biological roles of multiple initiators required by a large group of replicons. Consistent with the classical replicon model (Jacob *et al.*, 1964), some chromosomes (e.g. *Escherichia coli*, SV40 etc.) require a single initiator protein encoded in the replicon. However, others require at least two or more initiators (e.g. plasmids R6K, pSC101, RK2, yeast etc.; see Hasunuma and Sekiguchi, 1977; Gaylo *et al.*, 1987; MacAllister *et al.*, 1991; Bell and Stillman, 1992).

Interestingly, some of the plasmid replicons that require dual initiators for autonomous replication no longer require the host-encoded initiator when present in an integrated state

as part of the host chromosome. The ori of the integrated plasmid drives the replication of the host chromosome in the complete absence of the host-encoded initiator (DnaA), a phenomenon known as integrative suppression (Hansen and Yarmolinsky, 1986; Bernander *et al.*, 1992).

The observations referred to in the preceding paragraphs beg the following questions. What are the relative mechanistic contributions of host-encoded DnaA and plasmid-encoded initiator to replication initiation? Do these proteins communicate with each other in catalyzing the initiation reaction or do they work independently of each other at separate steps of the reaction? In this paper we report experiments designed to address these interesting questions using the plasmid R6K as a model system.

Replication of plasmid R6K is initiated at one of three origins called α , β and γ (Lovett *et al.*, 1975; Croso *et al.*, 1976, 1980). The major origins ori α and ori β are located ~4000 and ~1200 bp away, respectively, from a centrally located ori γ . To be functional, both α and β origins have an absolute requirement for the ori γ sequence *in cis* (Croso *et al.*, 1978; Kolter and Helinski, 1978; Kolter *et al.*, 1982; Shon *et al.*, 1982; Stalker *et al.*, 1982a,b; Figure 1).

Initiation from ori α and ori γ requires both the plasmid-encoded π and the host-encoded DnaA proteins, whereas initiation from ori β can take place in the complete absence of DnaA (Kelley and Bastia, 1992; Kelley *et al.*, 1992). In addition to the DnaA box and the π -binding iterons (Germi and Bastia, 1983a,b; Filutowicz *et al.*, 1985), the γ origin region has a binding site for the host-encoded DNA bending protein called integration host factor (IHF). ori γ requires IHF for maintenance at a detectable copy number (Filutowicz and Appelt, 1988; Kelley and Bastia, 1991). The IHF-binding site (ihf) is located between the single DnaA-binding site (dnaA) and the seven tandem iterons. We have postulated that the only function of IHF at ori γ is to bring into contact DnaA and π proteins that are bound to physically separated sites, by bending the intervening DNA (Miron *et al.*, 1992; Figure 1).

Due to the considerable physical separation between γ and α on one hand, and γ and β on the other, the natural flexibility of DNA allows π -mediated α – γ and β – γ looping without the need for additional DNA bending by IHF (Mukherjee *et al.*, 1988a,b). Consequently, ori α and β can initiate replication in the absence of IHF (Filutowicz and Appelt, 1988; Kelley and Bastia, 1991; Miron *et al.*, 1992). The γ – α and γ – β looping activates ori α and β , respectively, but represses ori γ (Mukherjee *et al.*, 1988a,b; Miron *et al.*, 1992).

The DnaA protein is known to exist in an ATP-bound form that is essential for the activation and unwinding of oriC of *E.coli* (Gille and Messer, 1991; Hwang and Kornberg, 1992). The ATP-bound form is gradually hydrolyzed to an ADP-bound form that is inert in initiating replication at oriC (Sekimizu *et al.*, 1987). If DnaA played

Fig. 1. The replication origins of R6K. (A) The centrally located ori γ consists of ~420 bp and is separated from the ori α and ori β by ~4000 and ~1200 bp, respectively. Note the presence of a single iteron (▶) and a DnaG site (●) at ori α and a half iteron (▶) at ori β . H = HindIII, B = BglII. (B) Initiation from ori α and β require π -mediated looping with γ . An expanded view of ori γ shows the critical elements: the DnaA box (■), an IHF box (▨), an AT-rich segment, seven iterons (▶), and a critical sequence called χ with unknown function. ori γ is hypothesized to be activated by DnaA- π contact brought about by IHF-mediated DNA bending (see lower part of figure). (C) The π ORF is divided into a 116 amino acid N-terminal and a 168 amino acid C-terminal peptide. The alanine scanning mutations m1 to m4 at the N-terminus are shown.

a similar role in the activation of the plasmid ori, one would expect to find a need for the ATP-bound form of DnaA for plasmid replication.

In this paper we have investigated the role of DnaA in the initiation of replication of plasmid R6K and show that the ATP-activated form of DnaA is dispensable for ori γ replication. Unwinding at ori γ requires the activities of three proteins, namely IHF, π and DnaA. We show that a mutant form of DnaA that was almost completely defective in ATP binding was unable to unwind oriC, but was as effective in ori γ unwinding as wild-type DnaA. We provide the first evidence for a direct physical interaction between two initiators, the N-terminal segment of π and DnaA, and show further that a mutant form of π that was defective in this interaction failed to support the unwinding of ori γ *in vitro* and its replication *in vivo*. We report binding experiments that show co-operation of interaction between IHF and DnaA that greatly enhances the binding of DnaA to ori γ .

Results

The ATP-bound form of DnaA is not needed for initiation from ori γ

If the mechanistic role of DnaA is different in plasmid replication as compared with that in host replication, it is

Fig. 2. Biochemical characterization of the mutant form of DnaA in which the GKT motif known to be involved in ATP binding was mutated to GAT. (A) Nitrocellulose filter binding assay showing the binding of [γ - 32 P]ATP to wild-type and the mutant form of DnaA. Note that the mutant has suffered a >90% loss in its ability to bind to ATP. (B) The mutant form of DnaA is competent in promoting lagging-strand DNA replication in an ABC primosome system. The template that is single stranded and has a hairpin sequence containing a DnaA box is shown at the top of the figure. The reaction mixture contained purified DnaA (mutant and wild-type), DnaB, DnaC, DnaG, pol III* and the β sliding clamp. Note that there are no significant differences between the wild-type and the mutant form of DnaA in catalyzing lagging-strand synthesis.

conceivable that the obligatory requirement for the ATP-activated form of DnaA for oriC replication might be dispensable for initiation from ori γ of R6K. We wished to investigate this possibility by isolating a mutant form of the protein that did not bind ATP. We converted the consensus ATP-binding amino acid motif GKT of DnaA to GAT by site-directed mutagenesis, purified the mutant form of the protein to near homogeneity and compared its ATP binding activity with that of the wild-type protein by a nitrocellulose filter binding assay. The result showed that the wild-type DnaA readily bound to labeled ATP whereas the mutant form was almost completely defective in ATP binding (Figure 2A).

All plasmid constructions used in this work are listed in Table I. The mutant form (pYBL320, Cm^R) was unable to support oriC (pYBL400, Tc^R) replication, but was capable of supporting ori γ (pYBL750, Amp^R) replication *in vivo* when expressed in a DnaA Δ strain of *E. coli*. As expected, the wild-type DnaA (pYBL310, Cm^R) in control experiments supported the maintenance of both oriC and ori γ in the same DnaA Δ host (Table II).

Table I. Plasmid constructs

Plasmid	Description	Reference
pYBL110	wild-type dnaA cloned in pET15b between the <i>NdeI</i> – <i>Bam</i> HI site	this work
pYBL111	K178A mutation in dnaA of pYBL110	this work
pYBL310 and pYBL320	wild-type and K178A of dnaA, respectively, cloned in a modified pET30a vector that has Cm ^R marker and the <i>ori</i> of pACYC184 (the <i>XbaI</i> – <i>BlnI</i> fragment was swapped from pYBL310 and 320 into the modified pET30a vector)	this work
pYBL220, 251, 252, 253 and 254	wild-type π and the m1, m2, m3 and m4 mutants, respectively, cloned in pET11b	this work
pYBL241 and 242	contain the N-terminal amino acids 1–116 and C-terminal 117–285, respectively, in pET11b vector	this work
pYBL261 and 262	wild-type and m3 π , respectively, expressed in a pET vector with Km ^R and Cm ^R markers, and pACYC <i>ori</i> , respectively	this work
pYBL750	<i>ori</i> γ ligated as <i>EcoRI</i> – <i>PstI</i> fragment to an Amp gene	this work

Interestingly, compared with its failure to support *oriC* replication *in vivo*, the purified mutant form of DnaA was as capable of supporting lagging-strand replication *in vitro* as the wild-type protein in an ABC primosome system (Masai *et al.*, 1990). The ABC primosome system measured replication initiated from a hairpin sequence containing a DnaA binding site present in a SSB-coated, single-stranded M13 template DNA. The replication required purified DnaA, DnaB, DnaC, DnaG, SSB, DNA pol III* and the β sliding clamp proteins (Figure 2B). Because the ABC primosome assay mimics the initiation and propagation of the lagging strand, we concluded that the ATP-bound form of DnaA was needed for initiation of the leading- but not that of the lagging strand.

We further investigated the ability of the mutant form of DnaA to unwind *oriC* as compared with that of the wild-type protein by chemical probing with KMnO₄. The procedure reveals regions of strand opening by KMnO₄-induced oxidation of the T residues exposed in the melted but not in the duplex DNA and subsequent detection of the oxidized residues by primer extension with an end-labeled primer (Mukhopadhyay *et al.*, 1993). Our results confirmed that wild-type DnaA, in combination with IHF, caused melting at *oriC* (Figure 3, lanes 5 and 6; see Sekimizu *et al.*, 1987) but the mutant form of the protein was completely inert in *oriC* unwinding (Figure 3, lanes 7 and 8). In contrast, results presented later will show that the same mutant form of DnaA was active in the unwinding of *ori* γ of R6K.

π protein specifically interacted with DnaA *in vitro*

The observation that replication from *ori* α and γ (but not *ori* β) required DnaA and π had raised the alternative possibilities that either DnaA and π controlled discrete and separate steps of initiation at *ori* α and γ , or that the two initiators functioned as a multiprotein complex and thus acted together to catalyze various substeps of initiation. The most direct way to distinguish between these two possibilities was by looking for specific, biologically relevant protein–protein interaction between the two proteins *in vitro*. DnaA has a tendency to form aggregates in solution, so we decided to present it in an immobilized form on an affinity matrix to ³⁵S-labeled π protein in solution. We prepared the affinity matrix by fusing the reading frame of DnaA in frame with a sequence encoding six tandem histidine moieties, purified the fusion protein and immobilized it on nickel-nitrilotriacetic acid agarose (Ni-agarose).

Table II. The ATP-bound form of DnaA is necessary for *oriC* but not for *ori* γ replication

Host strain	Resident plasmid	Number of colonies after putting in the second plasmid	
		<i>oriC</i> (Tc ^R)	<i>ori</i> γ (Amp ^R)
CM1793	none	2120	1867
	pYBL310 (wild-type DnaA, Cm ^R)	1757	1565
	pYBL320 (mutant DnaA, Cm ^R)	1671	1910
EH3827 (DnaA Δ)	none	0	2
	pYBL310 (wild-type DnaA, Cm ^R)	982	1620
	pYBL 320 (mutant DnaA, Cm ^R)	0	1059

We prepared ³⁵S-labeled full-length π , and the N- and C-terminal peptides by coupled *in vitro* transcription–translation. Similarly, we labeled the α subunit of DNA pol III and luciferase, and used these as negative controls (not shown). The labeled proteins were loaded onto DnaA-agarose and control NTA-agarose matrices, washed, the bound protein eluted, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Autoradiograms of the gels showed that full-length π (Figure 4A, lane 3), the N-terminal (Figure 4A, lane 9) but not the C-terminal peptide (Figure 4A, lane 6) bound to the DnaA matrix but not to the control matrix (Figure 4A, lanes 2, 5 and 8, respectively). Thus, DnaA appears to bind specifically to a region located in the the N-terminal peptide of π between amino acids 1 and 116.

π –DnaA interaction was disrupted by the m3 mutation

In order to determine if the *in vitro* interaction between the N-terminal peptide of π and DnaA is biologically significant, we performed alanine scanning mutagenesis of the DNA encoding the N-terminal peptide of π and isolated four mutants called m1, m2, m3 and m4 (Figure 1C). The mutant forms were expressed and labeled *in vitro* with ³⁵S, and the relative binding affinities of the proteins for DnaA were measured by chromatography on the DnaA matrix. The wild-type, and the m1, m2 and m4 mutant forms bound to immobilized DnaA–His₆ with approximately equal avidity (Figure 4B, lanes 3, 4, 5 and 7). The m3 form of the protein however, showed greatly

Fig. 3. Autoradiogram of a DNA sequencing gel showing that the wild-type DnaA but not the mutant form can unwind oriC. Lanes G, A, T and C are the oriC sequencing ladders. Lane 1, no protein; lane 2, only IHF 15 ng; lane 3, only DnaA, 200 ng; lanes 4 and 5, IHF, 15 ng and wild-type DnaA protein, 100 and 200 ng, respectively; lanes 6, 7 and 8, 15 ng IHF and 100, 200 and 300 ng mutant DnaA protein, respectively.

diminished binding (Figure 4B, lane 6). We confirmed that the m3 form (R75A, K78A, E79A) of π was indeed defective in interaction with DnaA by titrating an equivalent increasing range of concentrations of wild-type and the m3 mutant form to a fixed amount of DnaA matrix. The bound protein samples were eluted from the DnaA beads and examined by SDS-PAGE followed by autoradiography. The data confirmed that the m3 form of π showed significantly reduced binding to DnaA in comparison with wild-type π (Figure 4C).

m3 π showed nearly normal iteron-specific DNA binding activity

We wished to determine whether the triple amino acid substitutions that comprised the m3 mutation (R75A, K78A, E79A) caused a gross misfolding and a general loss of function of the π protein, or if the effect was more localized. We tested the iteron-specific DNA binding activity (Germino and Bastia, 1983a,b) of m3 π in comparison with that of wild-type π using a nitrocellulose filter binding assay. A 470 bp DNA fragment containing ori γ and a control DNA fragment without the sequence were labeled with [α - 32 P]dNTPs, separately incubated with

Fig. 4. Physical interaction between DnaA and π as revealed by DnaA-His₆ affinity binding assay. (A) 35 S-labeled full-length, N-terminal peptide and C-terminal peptide of π were generated *in vitro* and applied to DnaA affinity beads. The proteins retained were resolved by SDS-PAGE. Lane 1, 3 μ l of input full-length π (F π); lane 2, binding of F π to the control beads; lane 3, binding of 3 μ l of F π to DnaA (5 μ g) beads; lane 4, input (4 μ l) of C-terminal π (C π); lane 5, binding of 4 μ l of C π to the control beads; lane 6, binding of 4 μ l of C π to the DnaA beads; lane 7, input (5 μ l) N-terminal π (N π); lane 8, binding of 5 μ l each of N π to the control beads, and lane 9 to the DnaA (10 μ g) beads, respectively. Note that the full-length and N-terminal peptide of π , but not the C-terminal peptide, bind to the DnaA beads. The N- and C-terminal peptides have been demarcated in Figure 1C. (B) Binding behavior of the wild-type and the m1-m4 mutant forms of π to DnaA. Lane 1, input (5 μ l) wild-type π (exactly equal numbers of CPMs of wild-type and the mutant forms of π were used in the binding experiments); lane 2, binding to control beads; lane 3, wild-type π binding to DnaA beads; lane 4, m1 binding to DnaA; lane 5, m2 binding to DnaA; lane 6, m3 binding to DnaA; and lane 7, m4 binding to DnaA. Note that the m3 form shows significantly reduced binding to DnaA. (C) Titration of increasing concentrations of wild-type and m3 π on a constant amount (10 μ g) of immobilized DnaA. Lane 1, input (3 μ l) wild-type π (same CPMs of m3 π was used in the binding). 10 μ g of His-tagged immobilized DnaA was used in each binding experiment; lanes 2-6, binding of 1, 2, 8, 16 and 32 μ l of input wild-type π to immobilized DnaA, respectively; lanes 7-11, binding of 1, 2, 8, 16 and 32 μ l of m3 π to immobilized DnaA.

wild-type and the m3 form of π , and passed through nitrocellulose filters to measure the labeled DNA retained on the filter as a function of the concentration of the wild-type and m3 form of π . The result showed that the m3 form of π was nearly as efficient in ori γ binding as wild-type π . The control experiment showed that the DNA fragment that did not contain an iteron did not bind to m3 π , and therefore binding was sequence specific (Figure 5). A further independent test of the overall physical integrity of m3 π was confirmed by results presented below.

The m3 mutant form of π supported ori β but not ori γ replication *in vivo*

We tested the relative abilities of the wild-type and m3 forms of π to support ori γ replication *in vivo* as follows. The wild-type and m3 form of π were separately expressed from a vector carrying the pACYC184 ori, and chloramphenicol and kanamycin resistance markers (pYBL 261 and 262, respectively; Table I) in *E. coli*, into which an ori γ replicon, tagged with a β -lactamase marker (pYBL750, Amp^R) was subsequently also introduced. The wild-type but not the m3 mutant form of π supported ori

Fig. 5. Filter-binding experiments showing that the wild-type and m3 π have comparable iteron DNA binding activities. 32 P-labeled iteron and non-iteron control DNA was incubated with the same range of concentrations of wild-type and m3 π , passed through nitrocellulose filters and the trapped labeled DNA was counted. Note that the iteron DNA binds almost equally well to both forms of π but the control shows that m3 π (and wild-type π) bind poorly to non-iteron DNA.

γ replication *in vivo* as shown by the yield of Amp^R and Cm^R colonies (Table III).

We wished to ensure the m3 form of π had not suffered a gross defect in initiation in the following manner. ori β (in contrast to ori α and ori γ) of R6K does not require DnaA for replication (Kelley *et al.*, 1992). If the m3 π had retained its ability as an initiator other than its loss of binding to DnaA, we would have expected the mutant form to support ori β replication. We introduced the m3 mutation into the π open reading frame (ORF) of a β replicon (pAM838; Miron *et al.*, 1992) that lacked the DnaA box but included the seven iterons at γ and the half iteron at β (Figure 1). The plasmid was transformed into the same *E.coli* strain except that it did not carry a π donor plasmid. The results clearly showed that both the wild-type and the m3 form of π supported ori β replication (Table III). We sequenced a sample of the recovered ori β replicon to make sure that each of these retained all seven iterons at γ and had not suffered any structural alterations, and to confirm the presence of the m3 mutation (data not shown). Thus, we concluded from the data that the m3 mutation selectively destroyed initiation at the DnaA-dependent ori γ but not at the DnaA-independent ori β replicon.

π -DnaA interaction was necessary for ori γ unwinding *in vitro* and the m3 mutant form of the π protein was defective in unwinding of ori γ

We wished to investigate whether the known requirement for π , DnaA and IHF for initiation from ori γ *in vivo* paralleled that required for ori γ unwinding *in vitro*. We incubated supercoiled ori γ DNA with purified IHF, wild-type DnaA and wild-type π proteins. Incubations were also performed in the presence of the individual proteins and in combinations of two: π -IHF, DnaA-IHF and π -DnaA. Following the incubations, the reaction mixtures were treated with KMnO₄, the plasmid DNA extracted, hybridized to an appropriate 5'-end labeled primer and extended by polymerase chain reaction (PCR). The extension products were resolved in DNA sequencing gels with an appropriate sequencing ladder used as markers. The results showed that all three proteins, namely π , DnaA and IHF were needed for ori γ unwinding (Figure 6A).

Table III. The m3 mutant of π that showed reduced binding to DnaA failed to support ori γ but not ori β replication

Plasmid	Host cells	
	DH5 α (wild-type π)	DH5 α (m3 π)
pUC19 (amp ^R)	2479	2082
ori γ (pYBL750, amp ^R)	1465	3
host DH5 α		
ori β (m3 π)		
pAM838 (m3, amp ^R)	721	
ori β (wild-type π)		
pAM838 (wild-type, amp ^R)	640	

The unwound region extended from the dnaA box up to, but not beyond, the ihf site (Figure 6B). The wild-type as well as the GAT mutant form of DnaA were almost equally capable of ori γ unwinding *in vitro* (Figure 6A, lanes 8 and 9). It is worth recalling that in contrast to this result, the same mutant form of DnaA was totally incapable of unwinding oriC (Figure 3, lanes 6–8).

We also wished to examine whether a specific DnaA- π interaction was needed for ori γ unwinding. KMnO₄ probing revealed that the m3 mutant form of π , as compared with the wild-type protein, showed significant quantitative and qualitative differences in its ability to unwind ori γ (Figure 6C, compare lanes 1, 2 and 3 with lanes 4, 5 and 6, respectively). The unwinding footprint of the wild-type protein was not only more intense in comparison with the ones generated by the m3 form but the banding pattern was also somewhat different (Figure 6C).

IHF enhanced the binding of DnaA to its cognate binding site

We wished to investigate the possible role of IHF in initiation from ori γ by performing binding experiments. The location of an ihf site between the dnaA box and the π -binding iterons had suggested the possibility that the bending of DNA at the ihf site might bring into contact DnaA and π that are bound to physically noncontiguous binding sites, and in addition stabilize the binding of DnaA by sandwiching the DnaA within a bent DNA so that DnaA makes contact with additional sites at or near the iterons. We made use of the observation that IHF by itself binds poorly to nitrocellulose filters (Stenzel *et al.*, 1991). Filter binding experiments confirmed that either DnaA or IHF bound weakly to the ori DNA, and the binding of DnaA was greatly enhanced in the presence of IHF (Figure 7, graph). Our interpretation of the basis of this enhancement is shown in the lower part of Figure 7. This interpretation is based on lack of observable binding between DnaA or π with IHF *in vitro* (unpublished) and the promotion of binding of DnaA to weak sites in a similar system (Stenzel *et al.*, 1991).

Discussion

The results presented in this paper are significant because they illuminate for the first time an interesting and important problem, namely the nature of the biochemical con-

Fig. 6. Ori γ unwinding activity of purified initiator proteins. **(A)** Autoradiogram of a sequencing gel showing that π , IHF and DnaA are all required for ori γ unwinding, and that the wild-type and the mutant form of DnaA that fails to bind ATP are equally proficient in collaborating in the unwinding reaction. The bracket on the left margin marks the protein binding sites (i.e. dnaA box, ihf site and the iterons. Lanes 1–3 are +IHF (15 ng), +DnaA (100 ng) and + π (200 ng), respectively (single protein). Lanes 4–6 contain IHF +DnaA, IHF +DnaA and DnaA + π , respectively (amounts of each of the two proteins were as shown in lanes 1–3). Lanes 8 and 9 contain IHF (15 ng) + DnaA mutant form (100 ng) + wild-type π (200 ng) and IHF (15 ng) + wild-type DnaA (100 ng) + wild-type π (200 ng), respectively. **(B)** Nucleotide sequence of ori γ showing the region that is unwound as determined by reactivity to KMnO_4 . The dotted lines on top of the sequence mark the unwound region; note that the AT-rich region between the DnaA box and the ihf site has been unwound, and the AT-rich region downstream from the ihf site, and the first iteron shows no reactivity to KMnO_4 . The iterons are underlined and numbered and the χ sequence located next to the 7th iteron is an essential part of the minimal ori sequence but does not show any reactivity to KMnO_4 . **(C)** Autoradiogram of a DNA sequencing gel showing that the m3 mutant form of π has less ori γ unwinding activity in comparison with wild-type π . Lanes 1, 2 and 3 show unwinding in the presence of 100, 150 and 200 ng of wild-type, π . Lanes 4, 5 and 6 show unwinding due to 100, 150 and 200 ng of m3 π . The amount of IHF and DnaA was kept constant at 15 and 100 ng, respectively, in each of lanes 1–6.

tributions of the component initiator proteins in a replicon that is driven by multiple initiators, i.e. DnaA and π , and the role of IHF in promoting this interaction.

The first significant finding reported here is that unlike the host oriC replicon that requires an ATP-activated form of DnaA for ori unwinding and initiation (Sekimizu *et al.*, 1987), the ori γ of R6K (and in pSC101; H.J.Datta and D.Bastia, in preparation) did not exhibit such a requirement. The data would strongly suggest that DnaA either makes different contributions to host and plasmid initiation mechanisms, or its contribution to plasmid replication is only a subset of that necessary for host replication. The results also show that a mutation in the canonical GKT motif to a GAT uncouples the catalytic role of DnaA in the replication of the lagging strand from that of the leading strand. The implications of the observation for control of plasmid replication are discussed below.

The ATPase activity of DnaA has been proposed to have a negative regulatory effect on *oriC* replication, presumably by converting the active ATP-bound DnaA to an inactive ADP-bound form. Consistent with this notion, an ATPase defective form of DnaA caused chromosome over-replication when expressed in the cell (Mizushima

et al., 1997). Since the ATP-activated form of DnaA is not needed for replication of R6K and possibly the activity of many other plasmids, it is unlikely to play a controlling role in this and perhaps other replicons.

The second significant finding reported here is the biologically relevant, direct physical interaction between DnaA and π and the fact that this specific contact was essential for proper ori unwinding. In contrast with the need for DnaA, IHF and π for ori γ unwinding (Kelley and Bastia, 1991; MacAllister *et al.*, 1991; Miron *et al.*, 1992), a recent investigation of replication of the broad host-range plasmid RK2 showed that a combination of either DnaA and the plasmid-encoded trfA initiators, or HU and trfA were sufficient for ori unwinding *in vitro* (Konieczny *et al.*, 1997). It appears, at least *in vitro*, that DnaA can take the place of HU which is a DNA-binding protein of little or no sequence specificity. Thus the requirement for ori unwinding appears to be different in R6K and RK2.

In sharp and interesting contrast with the failure to support ori γ replication, the mutant form of π showed no detectable loss of the ability to drive ori β replication. This result is consistent with our earlier observation that

Fig. 7. Enhancement of DnaA binding to ori γ in the presence of IHF. (Upper) Nitrocellulose filter binding showing that IHF by itself does not trap labeled γ DNA, and DnaA by itself shows low levels of binding. However the presence of a constant amount of 50 ng of IHF in the reaction mixture binding of DnaA to ori γ is significantly enhanced. (Lower) A model to explain the enhancement of DnaA binding to ori γ by IHF. The model posits that the bending of the DNA causes DnaA that is bound to the strong site (dnaA_S) to interact with weaker sites present around or in the iterons. The bent complex probably lowers the dissociation constant of the bound DnaA.

ori β , unlike ori γ , can replicate in a DnaA Δ strain of *E. coli* (Kelley and Bastia, 1992; Kelley *et al.*, 1992). The initiation at ori β requires π -mediated DNA looping between the γ iterons and the half iteron at β (Mukherjee *et al.*, 1988a,b; Kelley and Bastia, 1992; Miron *et al.*, 1992). Analysis of the mechanism of ori opening and helicase loading at ori β should be interesting and might provide some clues as to how the requirement for DnaA is dispensed with for initiation at ori β . Such work is now in progress.

The third significant result reported here is that IHF significantly enhances the binding of DnaA to the ori *in vitro*. We have previously reported observations supporting co-operation between IHF and the RepA initiator of pSC101 that were consistent with this general model (Stenzel *et al.*, 1991). Co-operation between π and DnaA, but not between IHF and π , was previously noted (Kelley and Bastia, 1991).

The primary mechanism for regulating plasmid copy number is believed to be the interaction between the plasmid-encoded initiator and the iterons. In fact, most of the mutations that affect plasmid copy number are located either in the ORF that encodes the initiator protein or in the ori itself (Nordstrom, 1991; Inuzuka and Wada, 1995; Chatteraj and Schneider, 1997). An attractive model for replication control postulates that after duplication of the plasmid chromosomes, the initiator protein couples or 'handcuffs' the two daughter molecules and prevents reinitiation (Pal and Chatteraj, 1988; McEachern *et al.*, 1989; Ables and Austin, 1991; Kittel and Helinski, 1991; Blasina *et al.*, 1996).

Despite evidence that vests the principal control of plasmid replication in the initiator–iteron interaction, there is also evidence that some host-encoded genes can control plasmid copy number (Macrina *et al.*, 1974). In fact, as described below, an increasing number of host-encoded

replisomal proteins that interact with the plasmid-encoded initiator have recently come to light. Besides the DnaA– π interaction reported here, we have previously reported interaction between DnaB and π (Ratnakar *et al.*, 1996). We have recently discovered that both π and RepA (of pSC101) specifically interact with DnaG primase and the τ subunit of pol III (H.J.Datta and D.Bastia, unpublished results). These observations raise intriguing possibilities regarding a role of initiator proteins not only in ori recognition and helicase loading at the ori, but possibly also in DNA chain elongation and primer synthesis. Thus, we believe that the mechanistic investigations of protein–protein interactions between replication initiator proteins and other replisomal proteins should continue to be a rewarding endeavor.

Materials and methods

Bacterial strains and plasmids

All plasmids used in this work are described in Table I. The *E. coli* strain DH5 α [F' *supE44*, *lacU169* (ϕ 80 *lacZ* Δ M15), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*] was used for all subcloning and for *in vivo* assay of R6K ori γ and ori β replication. Wild-type DnaA strain CM1793 [*asnB32*, *relA1*, *spoT1*, *thi-1*, *fuc-1*, *lysA*, *ilv-192*, *zla::pKN500* (miniR1), *Km^R*] and dnaA Δ strain EH3827 (CM1793, dnaA *mad-1*, *Km^R*; Hansen and Yarmolinsky, 1986) were used in *in vivo* studies on the role of ATP-bound form of dnaA protein in R6K replication. BL21 [DE3] (F[−], *ompT*, *hsdS*, [*rK[−]*, *MK[−]*] *gal*) containing the plasmid pLysS was used for protein expression. The IHF⁺ strain HN458 [LE392: F[−], *supE44*, *supF58*, *hsdR514*, *LacY1* or (*LacIZY6*), *galK2*, *galT22*, *metB1*, *trpR55*], and IHF[−] strain HN840 [E582: HN458, *hima* (Δ 82::Tn10)*hip*(Δ 3:CAM)] were used in the *in vivo* replication assay.

The protein coding sequences were expressed in the T7 promoter-based pET vectors (Novagen) or the *tac* promoter based pGEX vectors (Pharmacia). Plasmids pYBL110 and pYBL111 are wild-type dnaA and mutant dnaA (K178A) subcloned into pET15b as a *NdeI*–*BamHI* fragment. Wild-type and mutant, his-tagged DnaA protein were produced using these two expression plasmids. Plasmids pYBL310 and pYBL320 are wild-type DnaA and mutant DnaA (K178A) subcloned by swapping (as *XbaI*–*BlnI*) from pYBL110 and pYBL111, respectively, into a modified pET30a vector, pYBL300, which contains Cm^R and Km^R genes, and the ori of pACYC184. Plasmid pYBL400 was constructed by inserting a *BstBI* fragment containing the tetracycline resistance gene (Tc^R) into the *BstBI* site of the the original oriC plasmid, pCM960 (von Meyenburg *et al.*, 1979). Plasmids pCM960 and pAM738 α 0 (the mini-ori- γ plasmid) (Miron *et al.*, 1992) were used in the origin unwinding-footprinting experiments. Plasmids pYBL220, pYBL251, pYBL252, pYBL253 and pYBL254 are wild-type π , m1, m2, m3 and m4 mutants of π (m1 = K11A, K12A, K14A; m2 = K46A, E47A, E50A, R51A; m3 = R75A, K78A, E79A; m4 = K110A, S112A, E114A, E115A), respectively, subcloned into pET11b as *NdeI*–*BamHI*. The plasmids pYBL261 and pYBL262 encoded wild-type and m3 π , respectively, and were expressed in a version of pET vector that had Cm^R, pACYC 184 ori and a T7 promoter. Plasmids pYBL241 and pYBL242 were constructed by inserting the N-terminal (amino acids 1–116) and C-terminal of π (amino acids 117–285) into pET11b as *NdeI*–*BglII* and *BglII*–*BamHI* fragments, respectively. Plasmid pYBL750 (ori γ -amp^r) was constructed by fusion of the minimal ori- γ sequence (in *EcoRI*–*PstI*) with an *EcoRI*–*PstI* fragment containing the amp^r gene. The wild-type ori- β plasmid pAM838 (Miron *et al.*, 1992) and the mutant form containing the m3 mutation in the π ORF were used for transformation experiments shown in Table II.

Protein purification

Wild-type and mutant DnaA proteins were expressed from an overproducer clone, pTS102, in a T7 promoter vector (Stenzel *et al.*, 1987) and purified as previously described (Sekimizu *et al.*, 1987). Briefly, the overproducer carrying pYBL110 (DnaA in pET15b) was harvested 3 h after IPTG (0.4 mM) induction, lysed and centrifuged. The supernatant (Fraction I) was incubated with charged Ni-beads with mixing for 3 h. The beads were then spun down and washed with washing buffer [Buffer C (50 mM HEPES, 0.2 M KCl, 0.1% β -mercaptoethanol) plus 50 mM

imidazole]. The protein was then eluted from the beads with buffer C containing 1 M imidazole, 3 M guanidine HCl, 10 mM magnesium acetate, and 0.6 M ammonium sulfate. The protein was further purified by gel filtration through a Superose-12 column (Pharmacia FPLC HR16/50) equilibrated with buffer D (50 mM HEPES-KOH pH 7.6, 1 mM EDTA, 20% sucrose, 0.2 M $(\text{NH}_4)_2\text{SO}_4$, 0.1% β -mercaptoethanol) at a flow rate of 1 ml/min. Active fractions were pooled and the DnaA activity was assayed by *in vitro* oriC replication and the ABC primosome assay. Wild-type mutant (m3) π proteins were purified as previously described (Miron *et al.*, 1992). IHF was purified from *E. coli* as previously described (Nash *et al.*, 1987).

***In vitro* coupled transcription-translation**

DNAs encoding full-length π (wild-type or mutant) and N- and C-terminals of π were cloned downstream from a T7 promoter (in pET11b), and transcribed and translated *in vitro* with [^{35}S]methionine (10 mCi/ml), amino acid mixture (1 mM, lacking methionine), T7 RNA polymerase (4 U), RNase inhibitor (RNasin, 40 U) in a commercial rabbit reticulocyte lysate system (TNT, Promega) at 30°C for 2 h. The labeled products were analyzed by SDS-PAGE and autoradiography.

Filter binding assay

Filter binding assay was used to determine the ATP-binding activity of wild-type or mutant DnaA as described (Sekimizu *et al.*, 1987). Filter-binding assay was also used to show the initiator proteins (IHF, wild-type DnaA, and wild-type and mutant π) to the 470 bp ori- γ DNA fragment. The proteins of various amount were incubated with the [γ - ^{32}P]ATP-labeled 470 bp ori- γ DNA at 30°C for 10 min in 50 μl of buffer containing 40 mM HEPES-KOH pH 8.0, 40 mM potassium glutamate, 10 mM magnesium, 1 mM ATP, 10 μM NAD, 4 μM cAMP and 6% PEG 6000. Samples were passed through nitrocellulose membranes (Millipore HA 0.45 μm). The radioactivity remaining on the filters was counted in a liquid passed through nitrocellulose membranes (Millipore HA 0.45 μm). The radioactivity remaining on the filters was counted using a liquid scintillation counter.

His-tag protein affinity column chromatography

Five micrograms of His-tag DnaA protein was immobilized onto 20 μl Ni-beads and incubated with 1 $\times$ TBS buffer (50 mM HEPES-KOH pH 7.4, 10 mM MgCl_2 , 0.1% β -mercaptoethanol) on ice for 20 min. The beads were blocked with 100 μl of 5% bovine serum albumin (BSA in TBS-NP-40, 0.1%) for 1 h at 0°C. The beads were then allowed to pellet, and various amounts of ^{35}S -labeled π , or its N- or C-terminal peptides were allowed to bind to the immobilized DnaA on the beads for 30 min on ice. The beads were washed vigorously with TBS-NP-40, 0.3%. The beads were extracted with 0.1% SDS, 10 mM β -mercaptoethanol, 10 mM Tris pH 7.4 and analyzed by a SDS-PAGE (15%). The ^{35}S -labeled peptides were visualized by autoradiography.

ABC primosome assay

The reaction mixture (25 μl) contained the following: HEPES-KOH pH 8.0 at 1 mM and 40 mM; potassium glutamate, 40 mM; sucrose, 4%; dithiothreitol, 4 mM; BSA, 100 $\mu\text{g}/\text{ml}$; ATP, 2 mM; CTP, GTP, and UTP, 250 μM each; dNTPs, 20 μM each with [^3H]TTP at 200–400 c.p.m./pmol of total deoxyribonucleotide; rifampicin, 20 $\mu\text{g}/\text{ml}$; template single-stranded DNA, 0.1 μg ; single strand DNA binding protein (SSB), 800 ng; DnaA protein in various concentrations; DnaB protein, 100 ng; DnaC protein, 50 ng; primase, 50 ng; DNA poly III (core polymerase 50 ng, γ complex 50 ng, and β subunit 75 ng). Incubation was for 10 min at 30°C. Total nucleotide incorporation (pmol) was measured by liquid scintillation counting after precipitation of nucleic acid with 10% trichloroacetic acid and filtration onto Whatman GF/C glass-fiber filters.

Permanganate footprinting and primer extension

oriC unwinding was probed with KMnO_4 in a reaction mixture (25 μl) that contained 5 mM MgSO_4 , 3 mM EDTA, 7.5% glycerol, 0.2 mg/ml BSA, 0.1 mg/ml creatine kinase, 40 mM creatine phosphate pH 7.5, 5 mM ATP, 300 ng supercoiled oriC DNA, 0.005% Triton X-100, 200 ng DnaA protein and 15 ng IHF protein. After preincubation for 15 min at 37°C, KMnO_4 was added to a final concentration of 10 mM. After a 3 min incubation at 37°C, the reaction was stopped by the addition of 2 μl of 14.3 M β -mercaptoethanol and EDTA to a final concentration of 15 mM. Each DNA sample was purified by phenol, phenol-chloroform, chloroform extractions and passage through a 1 ml Sephadex G-25 spin column. For R6K ori γ unwinding, in addition to these components, 5 mM ATP, 500 μM each of CTP, UTP and GTP, 200 ng of π protein

and 300 ng of supercoiled ori γ DNA were added to the reaction mixture (Borowicz *et al.*, 1987).

Primer extension reactions were carried out using PCR and vent DNA polymerase. Oligonucleotide primers used for primer extension were end-labeled (100 pmol of DNA) with [γ - ^{32}P]ATP (7000 Ci/mmol, ICN biochemical) in the presence of 7 U of T4 polynucleotide kinase. The labeled oligonucleotide primers were separated from unincorporated ATP using 1 ml Sephadex G-25 spin column equilibrated with TE (0.01 M Tris-HCl pH 8.0, 0.001 M EDTA). PCR was stopped by the addition of formamide buffer followed by electrophoresis on an 8 M urea, 6% polyacrylamide gel and autoradiography. The position of the area modified by KMnO_4 was determined by comparison with a sequencing ladder run in parallel on the same gel.

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References

- Ables,A.L. and Austin,S.J. (1991) Antiparallel plasmid-plasmid pairing may control P1 plasmid replication. *Proc. Natl Acad. Sci. USA*, **88**, 9011–9015.
- Bell,S.P. and Stillman,B. (1992) ATP-dependent recognition of eukaryotic origin of replication by a multiprotein complex. *Nature*, **357**, 128–134.
- Bernander,R., Krabbe,M. and Nordström,K. (1992) Mapping of the *in vivo* start site for leading strand DNA synthesis in plasmid R1. *EMBO J.*, **11**, 4481–4487.
- Blasina,A., Kittel,B., Toukdarian,A.E. and Helinski,D.R. (1996) Copy-up mutants of plasmid RK2 replication initiation protein are defective in coupling RK2 replication origins. *Proc. Natl Acad. Sci. USA*, **93**, 3559–3564.
- Borowicz,J.A., Zhang,L., Sasse-Dwight,S. and Gralla,J.D. (1987) DNA-supercoiling promotes formation of a bent repression loop in lac DNA. *J. Mol. Biol.*, **196**, 101–111.
- Chattoraj,D.K. and Schneider,T.D. (1997) Replication control of plasmid P1 and its host chromosome: the common ground. *Prog. Nucleic Acids Res. Mol. Biol.*, **57**, 145–186.
- Crosa,J.H. (1980) Three origins of DNA replication are active *in vivo* in *Escherichia coli*. *J. Biol. Chem.*, **255**, 11075–11077.
- Crosa,J.H., Luttrop,L.K. and Falkow,S. (1976) Mode of replication of the conjugative R-plasmid R6K in *Escherichia coli*. *J. Bacteriol.*, **126**, 454–456.
- Crosa,J.H., Luttrop,L. and Falkow,S. (1978) Molecular cloning of replication and incompatibility regions of the R plasmid R6K. *J. Mol. Biol.*, **124**, 443–468.
- Filutowicz,M. and Appelt,K. (1988) The integration host factor of *E. coli* binds to multiple sites at plasmid R6K γ origin and is essential for replication. *Nucleic Acids Res.*, **16**, 3829–3841.
- Filutowicz,M., Uhlenhopp,E. and Helinski,D.R. (1985) Binding of purified π initiator protein to a replication origin region of R6K. *J. Mol. Biol.*, **187**, 225–239.
- Gaylo,P.J., Turjman,N. and Bastia,D. (1987) DnaA protein is required for replication of the minimal replicon of the broad-host-range plasmid RK2 in *Escherichia coli*. *J. Bacteriol.*, **169**, 4703–4709.
- Germino,J. and Bastia,D. (1983a) Interaction of the plasmid R6K-encoded replication initiator protein with its binding sites on DNA. *Cell*, **34**, 125–134.
- Germino,J. and Bastia,D. (1983b) The replication initiator protein of plasmid R6K tagged with beta-galactosidase shows sequence-specific DNA-binding. *Cell*, **32**, 131–140.
- Gille,H. and Messer,W. (1991) Localized DNA melting and structural perturbations in the origin of replication, oriC, of *Escherichia coli* *in vitro* and *in vivo*. *EMBO J.*, **10**, 1579–1584.
- Hansen,E.B. and Yarmolinsky,M.B. (1986) Host participation in plasmid maintenance: dependence upon dnaA of replicons derived from P1 and F. *Proc. Natl Acad. Sci. USA*, **83**, 4423–4427.
- Hasunuma,K. and Sekiguchi,M. (1977) Replication of plasmid pSC101 in *Escherichia coli* K12: requirement for dnaA function. *Mol. Gen. Genet.*, **154**, 225–230.
- Hwang,D.S. and Kornberg,A. (1992) Opening of the replication origin of *Escherichia coli* by DnaA protein with protein HU or IHF. *J. Biol. Chem.*, **267**, 23083–23086.

- Inuzuka,M. and Wada,Y. (1995) Single amino acid alteration in the initiator protein is responsible for the DNA overproduction phenotype of copy number mutants of plasmid R6K. *EMBO J.*, **4**, 2301–2307.
- Jacob,F., Brenner,S. and Cuzin,F. (1964) On the regulation of DNA replication in bacteria. *Cold Spring Harb. Symp. Quant. Biol.*, **28**, 329–348.
- Kelley,W.L. and Bastia,D. (1992) Activation *in vivo* of the minimal replication origin β of plasmid R6K requires a small target sequence essential for DNA looping. *New Biol.*, **4**, 569–580.
- Kelley,W.L. and Bastia,D. (1991) Conformational changes induced by integration host factor at origin γ of R6K and copy number control. *J. Biol. Chem.*, **266**, 15924–15937.
- Kelley,W.L., Patel,I. and Bastia,D. (1992) Structural and functional analysis of a replication enhancer: separation of enhancer activity from origin function by mutational dissection of the replication origin γ of plasmid R6K. *Proc. Natl Acad. Sci. USA*, **89**, 5078–5082.
- Kittel,B.L. and Helinski,D.R. (1991) Iteron inhibition of plasmid RK2 *in vitro*: evidence for intermolecular coupling of replication origins as a mechanism of RK2 replication control. *Proc. Natl Acad. Sci. USA*, **88**, 1389–1393.
- Kolter,R. and Helinski,D.R. (1978) Construction of plasmid R6K derivatives *in vitro*: characterization of the R6K replication region. *Plasmid*, **1**, 571–580.
- Kolter,R., Stalker,D.M., Thomas,C.M. and Helinski,D.R. (1982) Plasmid R6K DNA replication II: direct nucleotide sequence repeats are required for an active γ origin. *J. Mol. Biol.*, **161**, 45–56.
- Konieczny,I., Doran,K.S., Helinski,D.R. and Blasina,A. (1997) Role of TrfA and DnaA proteins in origin opening during initiation of DNA replication the broad host range plasmid RK2. *J. Biol. Chem.*, **272**, 20173–20178.
- Lovett,M., Sparks,R.A. and Helinski,D.R. (1975) Bidirectional replication of plasmid R6K DNA in *E.coli*: Correspondence between origin of replication and a single strand break in relaxed complexes. *Proc. Natl Acad. Sci. USA*, **72**, 2905–2909.
- Macrina,F., Weatherly,G.G. and Curtis,R. (1974) R6K plasmid replication: influence of chromosomal genotypes in mini cell producing strains of *E.coli*. *J. Bacteriol.*, **120**, 1387–1400.
- MacAllister,T., Kelley,W., Miron,A., Stenzel,T. and Bastia,D. (1991) Replication of plasmid R6K γ origin *in vitro*: dependence on dual initiator proteins and inhibition by transcription. *J. Biol. Chem.*, **266**, 16056–16062.
- Masai,H., Nomura,N. and Arai,K.-I. (1990) The ABC primosome system. *J. Biol. Chem.*, **265**, 15134–15144.
- McEachern,M., Bott,M.A., Tooker,P.A. and Helinski,D.R. (1989) Negative control of plasmid R6K replication: possible role of intermolecular coupling of replication origins. *Proc. Natl Acad. Sci. USA*, **86**, 7942–7946.
- Miron,A., Mukherjee,S. and Bastia,D. (1992) Activation of distant replication origins *in vivo* by DNA looping as revealed by a novel mutant form of an initiator protein defective in cooperativity at a distance. *EMBO J.*, **11**, 1205–1211.
- Mizushima,T., Nishida,S., Kurokawa,K., Katayama,T., Miki,T. and Sekimizu,K. (1997) Negative control of DNA replication by hydrolysis of ATP-bound DnaA protein, the initiation of chromosomal DNA replication of *Escherichia coli*. *EMBO J.*, **16**, 3724–3730.
- Mukherjee,S., Erickson,H. and Bastia,D. (1988a) Detection of DNA looping due to simultaneous interaction of a DNA-binding protein with two spatially separated binding sites on DNA. *Proc. Natl Acad. Sci. USA*, **85**, 6287–6291.
- Mukherjee,S., Erickson,H. and Bastia,D. (1988b) Enhancer–origin interaction in plasmid R6K involves a DNA loop mediated by initiator protein. *Cell*, **52**, 375–383.
- Mukhopadhyay,G., Carr,K.M., Kaguni,J.M. and Chatteraj,D.K. (1993) Open-complex formation by the host initiator, DnaA, at the origin of P1 plasmid replication. *EMBO J.*, **12**, 4547–4554.
- Nash,H., Robertson,C.A., Flamm,E., Weisberg,R.A. and Miller,H.I. (1987) Overproduction of *Escherichia coli* integration host factor, a protein with nonidentical subunits. *J. Bacteriol.*, **169**, 4124–4127.
- Nordstrom,K. (1991) Control of plasmid replication—how do DNA iterons set the replication frequency. *Cell*, **63**, 1121–1124.
- Pal,S.K. and Chatteraj,D.K. (1988) P1 plasmid replication initiator sequestration is inadequate to explain control by initiator binding sites. *J. Bacteriol.*, **170**, 3554–3560.
- Ratnakar,P.V.A.L., Mohanty,B.K., Lobert,M. and Bastia,D. (1986) The replicator initiator protein II of plasmid R6K physically interacts with the replicative helicase DnaB. *Proc. Natl Acad. Sci. USA*, **93**, 5522–5526.
- Sekimizu,K., Bramhill,D., Kornberg,A., Baker,T.A., Funnell,B.E. and Kornberg,A. (1987) ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the *E.coli* chromosome. *Cell*, **50**, 259–265.
- Shon,M., Germino,J. and Bastia,D. (1982) The nucleotide sequence of the replication origin β of the plasmid R6K. *J. Biol. Chem.*, **257**, 13823–13827.
- Stalker,D.M., Kolter,R., Tolun,A. and Helinski,D.R. (1982a) Plasmid R6K DNA replication. I. Complete nucleotide sequence of an autonomously replicating segment: Separation of the minimal replication region of the F plasmid into a replication origin segment and a *trans*-acting segment. *J. Mol. Biol.*, **161**, 33–43.
- Stalker,D.M., Thomas,C.M. and Helinski,D.R. (1982b) Plasmid R6K DNA replication. II. Direct nucleotide sequence repeats are required for an active γ -origin. *J. Mol. Biol.*, **161**, 45–56.
- Stenzel,T.T., MacAllister,T. and Bastia,D. (1991) Cooperativity at a distance promoted by the combined action of two replication initiator proteins and a DNA bending protein at the replication origin of pSC101. *Genes Dev.*, **5**, 1453–1463.
- von Meyenburg,K., Hansen,F.G., Riise,E., Bergman,H.E.N., Meijer,M. and Messer,W. (1979) Origin of replication oriC of *Escherichia coli* K12: genetic mapping and minichromosome replication. *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 121–128.

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