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Mechanistic aspects of DnaA–RepA interaction as revealed by yeast forward and reverse two-hybrid analysis

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Using yeast forward and reverse two-hybrid analysis and biochemical techniques, we present novel and definitive *in vivo* and *in vitro* evidence that both the N-terminal domain I and C-terminal domain IV of the host-encoded DnaA initiator protein of *Escherichia coli* interact physically with plasmid-encoded RepA initiator of pSC101. The N-terminal, but not the C-terminal, region of RepA interacted with DnaA *in vitro*. These protein–protein interactions are critical for two very early steps of replication initiation, namely origin unwinding and helicase loading. Neither domain I nor IV of DnaA could individually collaborate with RepA to promote pSC101 replication. However, when the two domains are co-expressed within a common cell milieu and allowed to associate non-covalently with each other via a pair of leucine zippers, replication of the plasmid was supported *in vivo*. Thus, the result shows that physical tethering, either non-covalent or covalent, of domain I and IV of DnaA and interaction of both domains with RepA, are critical for replication initiation. The results also provide the molecular basis for a novel, potential, replication-based bacterial two-hybrid system.

Keywords: helicase loading/*Ori* unwinding/ protein–protein interaction/replication initiation/ yeast reverse two-hybrid

Introduction

DnaA is a ubiquitous, evolutionarily conserved protein that is necessary for the initiation of replication of bacterial chromosomes (Messer *et al*., 1999). Whereas bacterial replication requires the single initiator DnaA, replication of many plasmid chromosomes requires dual initiator proteins, namely DnaA and plasmid-encoded RepA-type initiators (Hasunuma and Sekiguchi, 1977; Gaylo *et al*., 1987; Lu *et al*., 1998). In this paper we have used the plasmid pSC101 as a model system to investigate the mechanistic roles of plasmid-encoded RepA and the host-encoded DnaA initiator proteins in two of the early steps of replication initiation, namely origin melting and helicase loading.

For over two decades, the plasmid pSC101 (Cohen and Chang, 1975) has been a popular model system in studies of replication and partition of plasmids (Vocke and Bastia, 1983a,b; Stenzel *et al*., 1987; Manen and Caro, 1991; Tucker *et al*., 1984; Miller and Cohen, 1993; Seitz *et al*., 2000). The origin of replication of pSC101 contains at least three distinct domains: (i) a *dnaA* box followed by (ii) an AT-rich region, which is naturally bent and is bent further upon binding of the integration host factor (IHF), and (iii) iterons that bind to the RepA protein (Vocke and Bastia, 1983b; Stenzel *et al*., 1987, 1991; see Figure 1). From footprinting studies of RepA and DnaA proteins with the normal and mutant forms of the *ori*, we had suggested that IHF-induced bending not only stabilizes the binding of DnaA to its cognate site located at the *ori*, but also promotes physical interaction between DnaA and RepA, which may be essential for initiation of replication (Stenzel *et al*., 1987, 1991). The dependence of the plasmid's replication on DnaA has previously been reported (Hasunuma and Sekiguchi, 1977). Despite the suggestions for possible occurrence of a DnaA–RepA interaction, critical and definitive evidence for this interaction was lacking. Furthermore, the mechanistic role of such an interaction in pSC101 replication was also not known. In this paper, we provide *in vivo* and *in vitro* evidence for direct physical interaction between the two proteins. We show further that the interaction is indispensable for two of the early steps of replication initiation, namely origin melting and helicase loading.

DnaA consists of four domains. Domain I is involved in oligomerization, II acts as a flexible linker, III is involved in ATP and phospholipid binding and IV is involved in binding to the *dnaA* box (Garner and Crooke, 1996; Sutton and Kaguni, 1997; Messer *et al*., 1999). The protein exists in an active, ATP-bound or an inactive, ADP-bound form with regard to bacterial replication. However, the ADP-bound form supports R6K and P1 replication (Lu *et al*., 1998; Skovgaard *et al*., 1998). Mutants of DnaA protein that support replication of pSC101, but not of *ori*C, have been described (Sutton and Kaguni, 1995; Seitz *et al*., 2000).

We have previously reported that RepA protein interacts physically with DnaB helicase, and non-interacting mutants located in either DnaB or in RepA fail to load the helicase onto the plasmid *ori in vitro*. Helicase loading requires purified RepA, DnaA, DnaC and IHF proteins (Datta *et al*., 1999). Since RepA–DnaB interaction results in helicase recruitment, one would like to know why DnaA is also needed in the process.

This work was initiated to determine whether DnaA and RepA interact *in vivo* and *in vitro* and to elucidate the possible role of this interaction in the early steps of initiation of plasmid replication. Here, we show that domains I and IV of DnaA interact physically with RepA and that this interaction is critical for *ori* melting and helicase loading. We show further that a non-covalent association of domains I and IV with each other and with RepA can elicit *in vivo* plasmid replication. The DNA

binding domain IV of DnaA could not be functionally replaced by a generic DNA binding domain. We present evidence that both domains I and IV interact with RepA and that this interaction is needed for *ori* unwinding.

Thus, the results show that domain IV is important not only for anchoring the protein to the *dnaA* box but also for critical protein–protein interactions with RepA initiator protein.

Results

Yeast forward two-hybrid analysis shows RepA–DnaA interaction in vivo

The protein–DNA interactions at the *ori* of pSC101 (Figure 1) have been published (Vocke and Bastia, 1983b; Stenzel *et al*., 1987, 1991). On the basis of our previous work, we had postulated that the IHF-induced bending of the *ori* DNA promoted direct physical interaction between DnaA and RepA proteins and that the interaction was critical for replication initiation (Figure 1B; Stenzel *et al*., 1991). However, direct evidence for this hypothesis was lacking. We wished to determine whether the two proteins interacted *in vivo* by using a yeast two-hybrid system (Fields and Song 1989; James *et al*., 1996). The system uses three separate reporters, driven by three separate yeast promoters to detect positive protein–protein interactions. The use of multiple promoters greatly reduced promoter-specific artifacts and false positives (James *et al*., 1996). We fused the reading frame of RepA with that of the transcriptional activation domain of yeast GAL4 protein in the vector pGAD424, and co-expressed the fusion protein in yeast cells that had a resident plasmid called pGBT9-DnaA. The latter plasmid contained the reading frame of DnaA fused with the DNA binding domain of GAL4 (Figure 2). Interaction of DnaA with RepA was indicated by transcriptional activation of Ade2, His3 and β-galactosidase reporters. Leaky expression of the His3 gene was suppressed by adding 2 mM 3-amino-triazol (AT) to the indicator plates lacking histidine. The known interaction between mammalian p53 and SV40 T antigen (Tag) was used as a positive control. The two vectors without any inserts, or pGAD424-RepA/pGBT9, pGAD424/pGBT9-DnaA were used pairwise as negative controls. Both p53-SV40 Tag and DnaA–RepA cloned in the appropriate vectors gave consistent positive signal. The growth on His^- plates (Figure 2B) and the positive signal on β-galactosidase indicator filters are shown (Figure 2C). The experiments using Ade^- plates gave results similar to that on His^- plates (not shown). The positive signals from all three reporters (along with the appropriate controls) provided preliminary evidence for the interaction between DnaA and RepA proteins in the yeast cell milieu.

Reverse two-hybrid analysis confirms the specificity of RepA–DnaA interaction

We wished not only to confirm the above-mentioned interaction, but also to look for the opportunity to isolate non-interacting mutants by performing reverse two-hybrid analysis. We performed random mutagenesis of the RepA open reading frame (ORF) by error-prone polymerase chain reaction (PCR) and introduced the mutagenized DNA pool contained in the pGAD424 vector into the appropriate yeast cells that had a resident pGBT9-DnaA plasmid, and we looked for those colonies that grew on Leu^- and Trp^- plates but failed to grow on His^- and Ade^- indicator plates. Several such clones were picked and the ORF of RepA present in each clone was sequenced. Four out of five clones had the mutation Y52S. One mutation was apparently located outside the RepA ORF (Figure 2D).

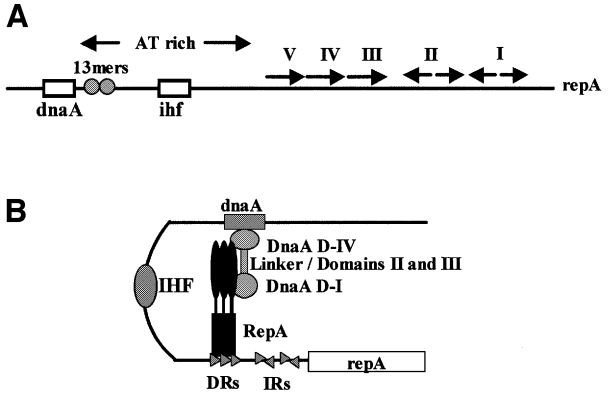

Fig. 1. (**A**) Diagram of the replication origin of pSC101. The *ori* consists of (left to right) the strong DnaA box (*dnaA*), two 13mers that may be the sequences that are initially melted, *ihf* site, three direct repeats (iterons V, IV and III) and two inverted repeats II and I that bind to the RepA protein. (**B**) The bending by IHF is believed not only to stabilize the *ori*–DnaA complex but also to promote contact between DnaA and RepA proteins.

ELISA and affinity chromatography confirmed RepA–DnaA interaction in vitro

We wished to confirm further the interaction between DnaA and RepA proteins observed *in vivo* by *in vitro* methods. We prepared DnaA protein that was tagged with six His residues at the N-terminus and immobilized it on nickel-nitrilo triacetic acid–agarose beads (Ni-NTA). We then allowed the affinity matrix and the control Ni-NTA matrix without DnaA to interact with [S^{35}]methionine-labeled wild-type (wt) RepA and the Y52S mutant form. An equal range of concentrations of the labeled wt and the mutant form of RepA were loaded onto the DnaA–His_6 and to the Ni-NTA control matrix The beads were washed extensively and the bound protein eluted with 1% SDS. The samples were precipitated and resolved by SDS–PAGE and autoradiographed. In some experiments equal aliquots of beads were boiled in SDS loading buffer and loaded directly onto an SDS–PAGE gel. A typical set of data, presented in Figure 3A, shows that whereas the wt RepA bound to the DnaA matrix, the binding of the mutant form of RepA was reduced at least by a factor of 4–5 in comparison with the wt RepA. Thus, the results obtained by *in vivo* reverse two-hybrid methods were confirmed by the affinity column method. We note that the reverse two-hybrid selection as performed in our work yielded mutants with substantial reduction in protein–protein interaction and that two rounds of random mutagenesis repeatedly yielded a single missense mutant, namely Y52S.

We wished to confirm further the *in vitro* binding data by ELISA and also to determine which domain of RepA was involved in the protein–protein interaction. We immobilized a constant saturating amount of the purified DnaA protein to wells of microtiter plates, blocked off the

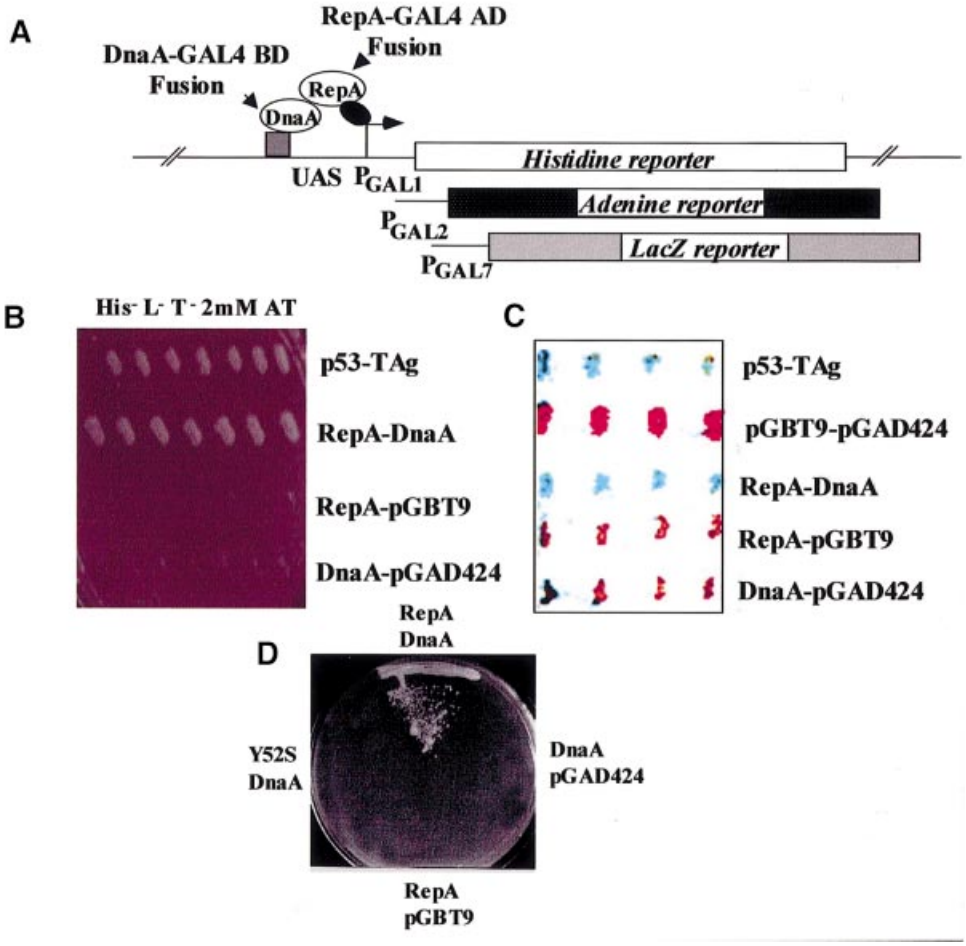

Fig. 2. (**A**) Schematic representation of the yeast two-hybrid system for *in vivo* demonstration of interaction between DnaA and RepA fused to DNA binding and activation domains of GAL4 protein, respectively. (**B** and **C**) The DnaA–RepA interaction activated transcription of all three reporter genes, namely histidine, adenine and β-galactosidase, as indicated by growth on His^- Leu^- Trp^- 2 mM AT and Ade^- His^- Leu^- Trp^- 2 mM AT (not shown) and blue color on β-galactosidase assay. (**D**) Non-interacting RepA mutant Y52S does not grow on Ade^- His^- Leu^- Trp^- 2 mM AT as shown by the reverse two-hybrid assay.

unbound, exposed plastic surface with bovine serum albumin (BSA) and challenged the immobilized protein with purified GST–RepA fusion proteins (both wt and Y52S mutant form) and also the N-terminal (residues 1–142) and the C-terminal (residues 143–309) peptides of wt RepA. The bound GST fusion proteins were detected with commercial enzyme-tagged anti-GST polyclonal antibodies. The data show (from three sets of measurements) that the N-terminal domain of RepA, but not the C-terminal domain, interacts with DnaA. The Y52S mutation significantly reduces the interaction between the two proteins (Figure 3B).

Y52S mutant form of RepA is not misfolded and binds normally to ori DNA and to DnaB

We wished to determine whether the Y52S mutation caused global misfolding of the protein or whether the effect was local. If the protein was extensively misfolded, it should also lose two other of its biochemical attributes, namely binding to DNA and to DnaB helicase (Datta *et al.*, 1999). We labeled a 686-bp-long *ori* fragment containing the iterons of pSC101 and another vector fragment of approximately equal length, and performed nitrocellulose filter binding experiments (Vocke and Bastia, 1983b). The data show that the wt and the mutant form of RepA were equally effective in binding to the *ori* fragment and not to the control fragment (Figure 4A).

We then proceeded to perform a comparative measurement of the binding of the wt and Y52S RepA to wt DnaB by ELISA. We immobilized equal amounts of the wt and mutant form of RepA to plastic wells and measured the interaction of the immobilized proteins with DnaB in solution using anti-DnaB polyclonal antibodies and secondary antibodies. Immobilized DnaA was used as a positive control. The data (average of three measurements) showed that the wt and the mutant form of RepA protein bound equally well to DnaB (Figure 4B). The background control was the signal generated by wells coated with BSA only, challenged by DnaB. From the results, we concluded that Y52S mutation did not cause global misfolding of RepA protein.

Y52S mutation abolishes pSC101 replication in vivo

At the present time, there is no available *in vitro* replication system for pSC101. We therefore decided to use an *in vivo* complementation system to determine the effect of

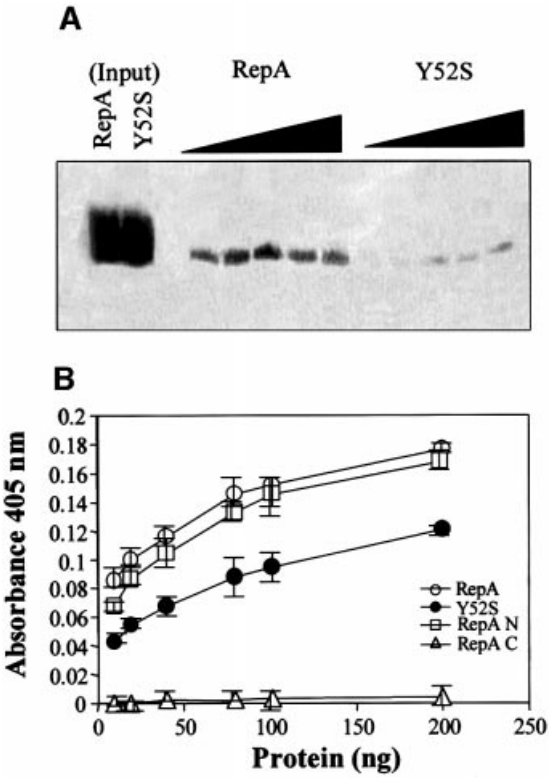

Fig. 3. (**A**) Physical interaction between DnaA and RepA as revealed by His_6–DnaA affinity chromatography. The retention of labeled Y52S RepA on His_6–DnaA column is reduced when compared with wt RepA. Input lanes contain 3 µl each of ^{35}S-labeled RepA and Y52S RepA followed by increasing amounts (0, 3, 6, 9, 12 and 15 µl) of labeled RepA and Y52S on His_6–DnaA beads. (**B**) ELISA showing that Y52S RepA (as compared with wt) has a reduced interaction with DnaA. The N-terminus of RepA (residues 1–142) and not the C-terminus of RepA interact with DnaA as seen by ELISA. Five hundred nanograms of DnaA were coated on the microtiter plate and overlayed with GST fusions of RepA, Y52S, N-terminal and C-terminal peptides of RepA. The bound proteins were detected by polyclonal anti-GST antibodies.

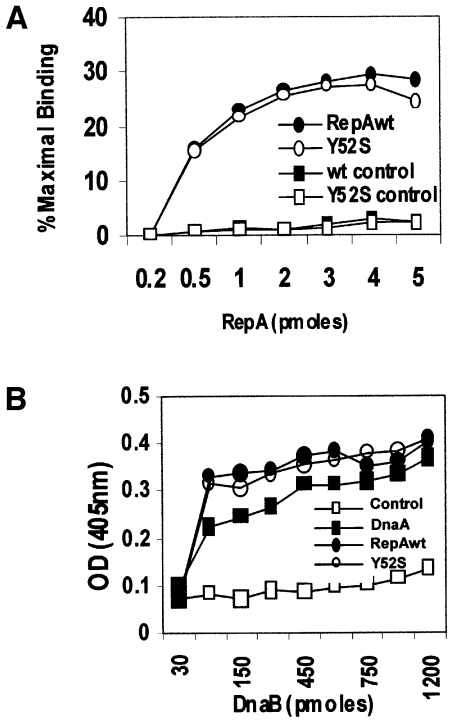

Fig. 4. Biochemical characterization of the Y52S form of RepA. (**A**) Nitrocellulose filter binding assay showing that the iteron DNA binding activities of wt and Y52S are similar, as seen by binding to [γ-^{32}P]ATP-labeled iteron and non-iteron control DNA. The labeled DNA was incubated with the same range of concentrations of wt and Y52S RepA, and passed through nitrocellulose filters. The protein–DNA complex trapped on the filters was measured. Note that the iteron DNA binds almost equally well to both forms of RepA. (**B**) ELISA showing that DnaB binds equally well to immobilized wt and Y52S forms of RepA, and to DnaA but not to immobilized BSA (control). Wild type, Y52S and DnaA (500 ng each) were coated on the plate and challenged with increasing amounts of DnaB. The binding was detected using polyclonal anti-DnaB antibodies.

the Y52S mutation of RepA on plasmid replication. We expressed wt and the mutant form of RepA from a ColE1 *ori*-based plasmid and checked to see whether the proteins could support the maintenance of a form of pSC101 that had a temperature-sensitive (ts) mutation in the repA gene (Hashimoto-Gotoh and Sekiguchi, 1977). Plasmids pET-RepA and pET-Y52S, expressing the wt and the Y52S mutant form, were introduced into *Escherichia coli* cells that contained a resident streptomycin-resistant pSC101 RepAts plasmid. The cells were selected for both the presence of the indicator ts plasmid and the RepA donor plasmid at 30°C on L-agar plates with appropriate antibiotics. The colonies were picked to a replica plate that had been grown at 42°C. The data in Table I show that the wt RepA donor, but not the Y52S donor plasmid, could rescue the pSC101-ts plasmid at 42°C, thus proving that the Y52S mutation abolished the ability of the mutant form of RepA to support pSC101 replication *in vivo*.

pSC101 replication does not require the ATP form of DnaA

We have described the isolation and biochemical characterization of a mutant form of DnaA that fails to bind ATP (Lu *et al*., 1998). We wanted to investigate whether the mutant form of DnaA could support pSC101 replication *in vivo*. We employed an *in vivo* complementation assay that tested the ability of a compatible plasmid expressing the wt and the mutant form of DnaA in a strain of *E.coli* that had suffered a deletion of the DnaA gene (Hansen and Yarmolinsky, 1986). The results showed that, whereas the mutant form of DnaA could not support the replication of the *ori*C plasmid, it readily supported the replication of pSC101 (Table II). While this work was in progress, Messer and colleagues showed that a mutant form of DnaA in which domains II and III had been replaced by a foreign linker was able to support pSC101 replication (Seitz *et al*., 2000). Thus, domains I and IV were necessary and sufficient for pSC101 replication although the efficiency of replication was lower in comparison with wt DnaA (Figure 7).

Direct interaction between RepA and DnaA is required for helicase loading

We investigated the effect of the Y52S mutation on the ability of RepA protein to promote loading of the DnaB helicase to the plasmid *ori* by gel filtration experiments. We tagged DnaB with a protein kinase recognition sequence and labeled the protein with [γ-^{32}P]ATP and

Table I. *In vivo* replication of pSC101

Host strain (Strep^R)	Plasmid (Amp^R)	Number of colonies (Strep^R and Amp^R)	
		30°C	42°C
pSC101 Repts	pET-RepA	3060	1224
pSC101 Repts	pET-Y52S	2987	0

Table II. pSC101 replication *in vivo* does not require the ATP form of DnaA

Host strain	Resident plasmid (Table I for description)	No. of colonies (Cm^R and Tet^R)	
		pYBL400 (*oriC*)	PSC101
CM1793 (wt)	none	2220	2391
CM1793	pYBL310 (wt DnaA)	1931	2183
CM1793	pYBL320 (mutant)	1983	2225
EH3827 (Δ*dnaA*)	none	0	5
EH3827	pYBL310	1895	2073
EH3827	pYBL320	0	1721

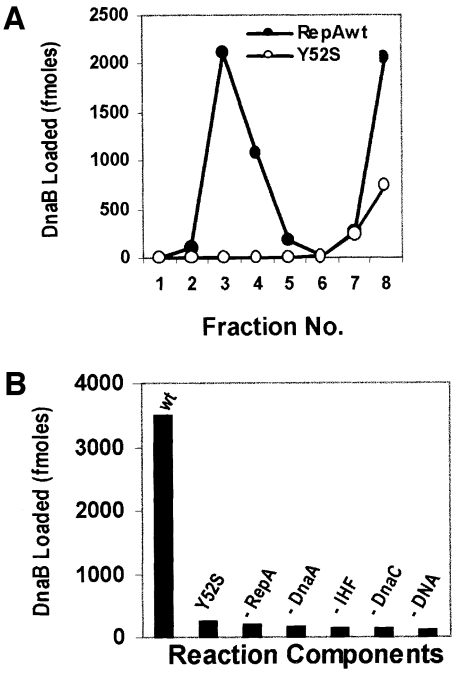

Fig. 5. Effect of Y52S mutation on recruitment of DnaB to the *ori* of pSC101. (**A**) DnaB protein was tagged with protein kinase recognition sequence and radiolabeled as described, incubated with pSC101 *ori* DNA along with purified IHF, DnaC, DnaA, SSB, and wt and Y52S RepA proteins, then passed through a gel filtration column. The profile of radioactivity in the excluded DNA–protein complex peak and in the included free protein fractions is shown. The loading of DnaB was markedly reduced when the Y52S protein was substituted for wt RepA. (**B**) Histograms showing the magnitude of DnaB loading when wt and Y52S RepA were used. Note the marked reduction in helicase loading, when one of the four proteins (DnaA, RepA, DnaC and IHF) was omitted one at a time from the reaction mixture.

muscle kinase as described (Datta *et al.*, 1999). We then incubated the labeled DnaB helicase with DnaA, IHF, wt and the mutant form of RepA, and supercoiled pSC101 DNA. The reaction mixture was passed through a gel filtration column (Datta *et al.*, 1999) and the radioactivity that was bound to DNA and present in the excluded peak was measured (Figure 5A and B). The data show that wt RepA promoted the loading of DnaB to the plasmid *ori*, whereas the Y52S form was totally defective in the process (Figure 5A and B). Exclusion of DNA, DnaA, IHF or DnaC almost completely abolished the loading of DnaB (Figure 5B). As shown later, DnaA–RepA interaction promotes DnaB loading by catalyzing the *ori* unwinding step.

Domain IV of DnaA could not be functionally replaced by a generic DNA binding domain

We considered the question as to whether the sole function of domain IV of DnaA was to anchor the protein to the *dnaA* box. Domain I of DnaA is thought to participate in other essential functions such as protein–protein interaction with RepA and other replication proteins.

If the sole function of domain IV of DnaA was to anchor the protein at the DnaA box, one should be able to functionally replace it with a different, generic DNA binding domain, provided that the *ori* sequence was suitably modified with a cognate DNA binding site to allow the binding of the hybrid DnaA in the correct stereochemical configuration. In order to test this hypothesis we constructed a modified plasmid *ori* in which the cognate DNA binding site TTATATACA of DnaA has been replaced by the 8mer, LexA binding half-operator site, CTGTATAT. The modified *ori* was tagged by a tetracycline-resistant marker and introduced into *E.coli* that had a resident plasmid encoding a chimeric *dnaA* gene. In the chimeric gene, domain IV in the 'Messer construction' (Figure 7B) was replaced by the DNA binding domain of LexA protein (Figure 7C). We observed that the hybrid protein failed to support the replication of the modified *ori*. Considering the possibility that the replacement of the *dnaA* box by the *lexA* operator half site might have inactivated the *ori* in a *cis* fashion (independent of the modified DnaA protein), we placed lexA operators at 250, 350 and 500 bp away from a fully functional minimal *ori*. We introduced the *ori*, thus modified, into the DnaAΔ strain that had a resident plasmid encoding the same LexA–hybrid DnaA protein mentioned above. None of the *lexA* operator-tagged modified *ori* replicated under these conditions (data not shown, but can be supplied on request).

Domains I and IV of DnaA both interact with RepA and the Y52S mutation reduces binding of RepA to domain I of DnaA

Does domain IV of DnaA, besides anchoring DnaA protein to the *dnaA* box, perform other functions necessary for initiation? In order to address this question we investigated the possible interaction between RepA and the separated domains I and IV of DnaA *in vitro*. We expressed domains I and IV as fusion proteins with GST, purified the fusion peptides and immobilized them on plastic surfaces of microtiter plates, and challenged the

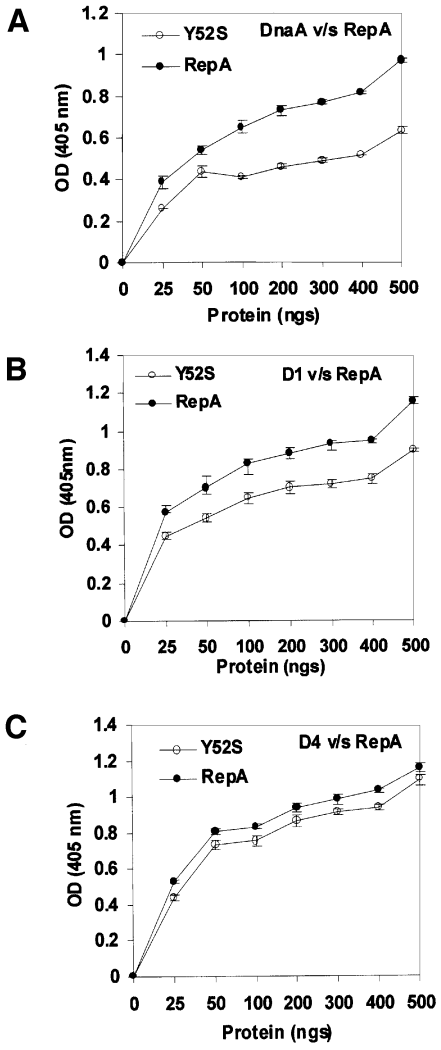

Fig. 6. ELISA showing that both the domains I (D1; residues 1–86) and IV (D4; residues 372–467) of DnaA interact with RepA. DnaA, D1 and D4 were immobilized on the plastic wells and reacted with GST fusions of wt and Y52S RepA proteins in solution. The bound RepA was detected with anti-GST antisera. When compared with wt the Y52S mutation knocks down the interaction with (**A** and **B**) full-length DnaA and (**C**) domain I. However, there is negligible effect of the Y52S mutation on the interaction with domain IV.

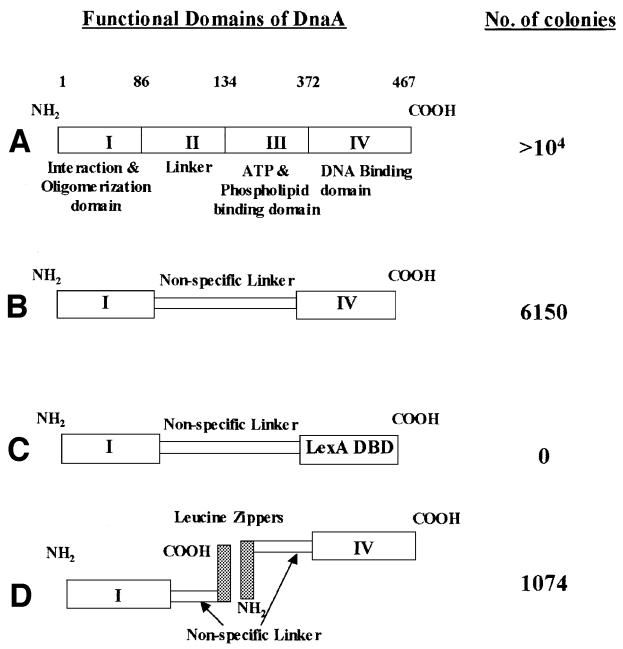

Fig. 7. (**A**) Schematic representation of functional domains of DnaA. (**B**) Domain I (D1); amino acids 1–86 and domain IV (D4); amino acids 372–467 linked by a non-specific linker is able to support pSC101 replication (this work and Seitz *et al.*, 2000). (**C**) Domain IV can not be replaced by a generic DNA binding domain such as the DNA binding domain of LexA. To test this hypothesis, a series of replicons was constructed where the *dnaA* box consensus sequence was replaced with either an 8 bp *lexA* half site or 16 bp *lexA* full site. In another case the *lexA* half site or full site was inserted at ~250, 350 and 500 bp, in a mini pSC101 *ori*, upstream of the *dnaA* box, leaving the latter intact. None of these replicons was supported by hybrid DnaA molecules, where domain IV of DnaA was replaced with the DNA binding domain of LexA protein. A similar set of experiments was conducted where the Pi initiator protein of R6K and the cognate 22 bp iteron were used in place of LexA, half site or full site combination. It was observed that domain IV of DnaA could not be replaced in either case (data not shown). (**D**) Domains I and IV can non-covalently associate via LZs to support plasmid replication. The numbers on the right indicate the number of transformants containing the pSC101 tester replicon.

immobilized peptides with wt RepA and Y52S proteins in solution. The data from three separate ELISA measurements show that (i) RepA interacted with both domains I and IV of DnaA (Figure 6B and C), and (ii) the Y52S mutations reduced the interaction not only between wt DnaA and RepA but also between domain I of DnaA and RepA (Figure 6A and B). However, the mutation caused only a small reduction in the interaction between domain IV and RepA (Figure 6C). From the data, we suggest that interaction of RepA with both domains I and IV of DnaA is necessary for pSC101 replication.

Domains I and IV can non-covalently associate via leucine zippers to support plasmid replication

Is the physical continuity (covalent joining) between domains I and IV necessary for replication initiation? We constructed vectors expressing domains I and IV individually as fusion proteins with leucine zippers (LZs) separated by a linker, and expressed from the plasmids that had compatible replication origins and different antibiotic resistance markers (plasmids pTacD1BLZ, Amp^R and pTacLZBD4-29, Cm^R; see Table III). We transformed the plasmids into the DnaAΔ strain of *E.coli*. The reporter pSC101 plasmid (Tet^R) was then introduced into the cells expressing the two domains fused in-frame with LZs. We observed that the two domains apparently associated via LZs and supported pSC101 replication (Figure 7C), although less efficiently than the covalently joined domains (Figure 7B). When one or more of the LZs were left out, there was no replication of pSC101 (not shown). The various domains of DnaA are shown schematically in Figure 7A.

Interaction between RepA and DnaA is needed for ori unwinding in vitro

Our earlier work has shown that although direct interaction between RepA and DnaB is needed to load the helicase to the plasmid *ori*, the process also needs DnaA, RepA, DnaC and IHF (Datta *et al.*, 1999). What then is the role of DnaA

Table III. Plasmid constructs

Plasmid	Description	Reference
pYBL310, pYBL320	wt and K178A DnaA (ATP non binding), with Cm^R and pACYC184 *ori*	Lu *et al.* (1998)
pYBL 400	*ori*C with Tet^R	Lu *et al*. (1998)
pMOT, pLHT and pLFT, pLHTX1, pLHTX2 and pLHTX3, pLFTX1, pLFTX2 and pLFTX3,	test replicons with Tet^R based on minimal origin of replication of pSC101 (Materials and methods)	this work
pTacD1BLZ, pTacD1BlexA and pTacLZBD4	Amp^R, ColE1 origin of replication to provide fusion proteins for transformation experiments under the control of Tac promoter	this work
pTacLZBD4-29	Cm^R and RSF1030 origin of replication	this work
pGADRepA and pGBTDnaA	Amp^R GAL4 activation domain and DNA binding domain fusion for yeast two hybrid analysis	this work
pGEX2TK-D1, pGEX4T1-D4, pGEX2TK-RepA, pGEX4T1-Y52S, pGEX2TK-RepAN and pGEX2TK-RepAC	Amp^R for production of GST fusion proteins of fragments of DnaA and RepA (see Materials and methods)	this work and Datta *et al.* (1999)
pET-RepA and pET-Y52S	Amp^R, for production of RepA and Y52S RepA under the control of T7 promoter	this work and Datta *et al.* (1999)
pET15b-DnaA	Amp^R, for production of His_6–DnaA under the control of T7 promoter	Datta *et al.* (1999)

in the recruitment of DnaB? Since DnaB does not bind to double-stranded DNA, we had suspected that RepA–DnaA interaction might be needed to melt the *ori*, thus making it competent to receive the helicase. We wished to investigate the validity of the hypothesis as follows.

We incubated supercoiled pSC101 DNA with purified wt RepA, DnaA and IHF and treated the DNA–protein complex with $KMnO_4$ solution, which oxidizes any T residues that are present in the single-stranded state. The oxidation 'fixes' the localized melted regions, which were identified by linear PCR with the end-labeled primers MELTOP and MELTBOT (see Materials and methods). A DNA sequencing ladder generated by the same primer–template combination was used to map the melted regions. An autoradiogram showing the melted ($KMnO_4$ reactive) DNA (top strand of the *ori*) is shown in Figure 8A. *Ori* melting required wt RepA, DnaA and IHF. The rows of five Ts followed by seven Ts were melted (Figure 8A, lane 1). Substitution of wt RepA by the Y52S protein greatly reduced the melting signal (Figure 8A, lane 2). The wt RepA and the mutant form in the presence of IHF, but without DnaA, elicited no detectable melting (Figure 8A, lanes 3 and 4). DnaA and IHF without added wt RepA elicited a low level of melting of the five Ts (Figure 8A, lane 5). All of the melted regions of the top strand and the bottom (not shown) were confined to the region between the *dnaA* box and the IHF binding site (Figure 8A).

We proceeded to investigate the relative contributions of each of the domains I and IV to melting. Domains I and IV of DnaA added to a reaction mixture also containing IHF and wt RepA showed no unwinding (Figure 8B, lanes 2 and 3). DnaA and IHF induced significant unwinding without RepA, but at a different location in comparison with that induced when all three wt proteins were present (Figure 8B, compare lanes 4 and 7). Domain IV with IHF, but without added RepA, generated a melting pattern similar to that of wt DnaA plus IHF (Figure 8B, compare lanes 7 and 9). Addition of wt RepA almost completely suppressed the melting generated by domain IV and IHF (Figure 8B, compare lanes 3 and 9). Similar results were obtained with melting at three Ts located on the bottom strand, just before the *ihf* box (Figure 8C). Here also, addition of wt RepA suppressed melting generated by domain IV and IHF (Figure 8C). Adding purified domain I to that shown in lane 9 (Figure 8B) did not change the pattern, and melting was also suppressed by the addition of wt RepA (not shown). Thus, the results clearly showed that domains I and IV have to be attached to each other to cooperate with RepA (in the presence of IHF) to melt the *ori* at the row of 5Ts and the 7Ts of the top strand. The data confirm the requirement for both domains I and IV of DnaA, along with IHF and RepA, for proper *ori* melting.

Discussion

This work presents several significant results that shed light, not only on the mechanism of early stages of initiation of plasmid replication, but also on the functions of domains I and IV of DnaA protein, which is an important initiator required for both host and plasmid replication. Interestingly, when DnaA-dependent plasmid replicons integrate into the bacterial chromosome and drive replication of the chromosome from the plasmid *ori*, for reasons that are still obscure, the need for DnaA protein is completely bypassed (a phenomenon called integrative suppression; Hansen and Yarmolinsky, 1986; Bernander *et al*., 1991). We have shown that *ori* α and γ of plasmid R6K require both π (plasmid-encoded initiator) and DnaA, whereas initiation from *ori* β is independent of DnaA (Kelley *et al*., 1992; Miron *et al.*, 1992). While the mechanism that causes replicons to be independent of DnaA is not understood, we have endeavored to find the biochemical role of DnaA in those replicons where such a need can not easily be dispensed with (Hasunuma and Sekiguchi, 1977; Lu *et al*., 1998).

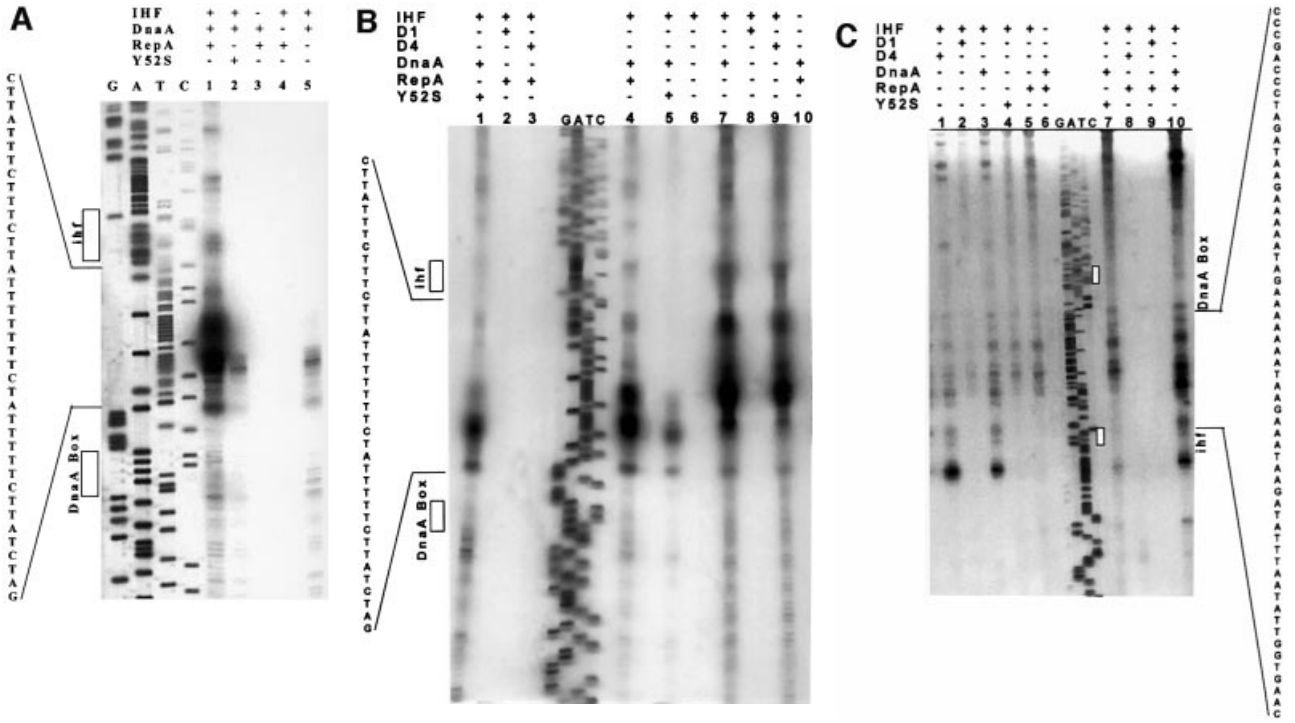

Fig. 8. pSC101 *ori* unwinding activity of purified initiator proteins. (**A**) Autoradiogram of a sequencing gel showing that RepA, IHF and DnaA are all required for *ori* unwinding, and that the Y52S RepA has reduced unwinding activity. The boxes on the left margin mark the protein binding sites (i.e. *dnaA* box and *ihf* site). The sequence of unwound region is indicated on left. (**B**) Domains I and IV alone are not able to unwind pSC101 *ori*. (**C**) Unwound region on the bottom strand. Lanes are marked on the top and the experimental conditions are described in Materials and methods.

This paper provides the first definitive evidence that plasmid-encoded RepA interacts with host-encoded DnaA and that this interaction is essential for *ori* unwinding and helicase loading. Keeping in mind that three proteins, namely DnaA, RepA and IHF, are all needed for correct and efficient origin unwinding, it is worthwhile considering what role each of the three proteins play in the unwinding reaction.

From our previous work, the role of IHF appears to be at least two-fold: (i) promoting and stabilizing efficient binding of DnaA to the single consensus *dnaA* box by bending the DNA and bringing the single strong *dnaA* site into contact with several weak DnaA binding sites located in the iterons, a phenomenon called cooperativity at a distance (Stenzel *et al*., 1991); and (ii) promoting the physical interaction between RepA and DnaA, which are bound to physically non-contiguous sites in the *ori* region (Stenzel *et al*., 1987, 1991 and this work).

What is the role of RepA in *ori* melting and helicase loading? The result presented here shows that RepA by itself, or when present together with IHF, did not melt DNA, but acted as a specificity factor that caused, in the presence of DnaA, melting to occur at the correct place. The data presented here show that intact DnaA by itself or domain IV of DnaA can elicit unwinding but not at the correct location. In fact, RepA suppressed spurious unwinding induced by domain IV of DnaA. However, when present together with DnaA and IHF, the unwinding was greatly stimulated at the right location of the AT-rich region. It is also clear that RepA by itself could not promote any detectable unwinding without direct physical contact with DnaA and the iterons. An additional function of RepA is, of course, recruitment of DnaB by direct protein–protein interaction. Our previous work shows that mutational disruption of DnaB–RepA interaction by a mutation in DnaB did not impair loading of the mutant form of DnaB to *ori*C but sharply reduced helicase loading to the plasmid *ori* (Datta *et al.*, 1999). This result shows that DnaA–DnaB interaction can not load the helicase to the plasmid *ori*. Although DnaA also interacts with DnaB (Marszalek and Kaguni, 1994), we believe that this interaction may only be needed for helicase loading at the host *ori*C. However, direct proof that DnaA–DnaB interaction is not needed for plasmid replication awaits isolation of mutant forms of DnaA that can not interact with DnaB (or the converse) but can still support plasmid replication.

What might be the role of DnaA in unwinding of the plasmid *ori* and loading of the helicase? Clearly, the ATP form of DNA, which is essential for *ori*C replication, is not needed for pSC101 or R6K replication (Lu *et al*., 1998). As shown by Messer and co-workers (Seitz *et al*., 2000) and confirmed here, one can completely dispense with the linker-like domain II and domain III, which has the phospholipid and ATP binding sites (Garner and Crooke, 1996; Messer *et al*., 1999), but still elicit pSC101 replication. It is also clear from the data presented that specific unwinding requires cooperation between domain I and IV of DnaA and RepA proteins. There is evidence from *ori*C–DnaA interaction that the ATP form and not the ADP form of DnaA contacts the melted 13mers located in the AT-rich region of *ori*C (Speck and Messer, 2001). Further work will be needed to determine whether DnaA or RepA or a RepA–DnaA complex makes direct contact

with the 13mer sequences of pSC101 *ori*. It is worth keeping in mind in this context that in the broad host range plasmid RK2, as in *ori*C, interaction between DnaA and DnaB appears to be needed for loading of the helicase to the plasmid *ori* (Konieczny and Helinski, 1997; Konieczny *et al*., 1997).

The data presented in this paper show that the DNA binding domain IV of DnaA could not be replaced with a generic DNA binding domain. Our data tend to help one distinguish between two alternative interpretations of this observation. First, the interaction of C-terminal domain IV of intact, wt DnaA with the *dnaA* box could induce an allosteric change in the protein, which is transmitted through the body of the protein, and activate the N-terminal domain I. The activated domain I could then cooperate with RepA to promote *ori* unwinding. This type of DNA-induced allosteric signal transduction has been observed in the repressor protein of phage 434 (Ciubotaru *et al*., 1999). The second, alternative hypothesis was that domain IV performs other essential function(s) besides just anchoring DnaA to the *dnaA* box. Our results show that domain IV (and domain I) interacts with RepA. The observation that non-covalent association of domains I and IV is capable of promoting pSC101 replication also tends to argue against an allosteric signal transduction hypothesis.

The loading of DnaB helicase to the *ori* of pSC101 required IHF, RepA, DnaA and DnaC proteins. Our previous work showed that direct physical interaction between RepA and DnaB was essential for helicase loading and that mutations either in RepA or DnaB, which caused a reduction in protein–protein interaction, caused marked reduction in helicase loading (Datta *et al*., 1999). What then is the role of DnaC in helicase recruitment and loading? McMacken and co-workers (Learn *et al*., 1997) have shown that DnaC has cryptic single-stranded DNA binding activity, which is needed to load DnaB onto DNA. After loading the helicase, the DnaC protein falls off the complex (Fang *et al*., 1999). Further work is needed to fully elucidate the role of DnaC in the helicase recruitment and loading mechanism.

Finally, the observation that domains I and IV of DnaA, fused to LZs, can associate *in vivo* to support pSC101 replication provides us with a novel way to perform bacterial two-hybrid analysis. In principle, domain I could be fused to a gene X and domain IV to a gene Y, and if X and Y interact, the associated domains could conceivably interact with RepA and promote replication of an antibiotic marker-tagged pSC101 *ori*. Work is currently in progress to work out a potential bacterial two-hybrid system based on activation of replication of plasmid DNA.

Materials and methods

Bacterial strains, plasmids and oligonucleotides

Escherichia coli strain DH5α [F′ *sup* E44 *lac*U169 (ϕ80 *lacZ* ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1] was used for cloning. Strain BL21{DE3}[F$^-$ *omp*T *hsd*S (rB$^-$ mB$^-$) *gal*], containing the plasmid pLysS, was used for expressing proteins in pET vectors (Novagen). Plasmid pSC101 Rept$_s$ (Hashimoto-Gotoh and Sekiguchi, 1977) was used for *in vivo* replication assay, and EH3827(*dnaA*Δ) (CM1793, *dnaA* mad-1 Km^R; Hansen and Yarmolinsky, 1986) for DnaA complementation experiments. The yeast two-hybrid kit (Clontech, USA) and the *Saccharomyces cerevisiae* strain PJ69-4A [*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ*] (James *et al*., 1996) were used for two-hybrid assays. The expression plasmid pMalC2X (New England Biolabs) and GST fusion vectors pGEX2TK and pGEX4T1 (Pharmacia, USA) were also purchased. A brief description of the plasmids constructed and used in this work is given in Table III. The oligonucleotides were synthesized commercially (Life Technologies, USA) and the sequences of all the oligonucleotides will be supplied upon request.

Yeast two-hybrid assay and isolation of DnaA non-interacting mutations in RepA

Yeast two-hybrid assays were carried out following the supplier's (Clontech) instructions using the yeast strain PJ69-4A (James *et al*., 1996). RepA and DnaA were PCR amplified using the primer pairs RepAFP/RepARP and DnaA2HFP/DnaA2HRP and cloned in the pGAD424 and pGBT9 plasmids (Clontech), respectively. Positive interactions were identified by growth on (i) His$^-$ Leu$^-$ Trp$^-$ 2 mM AT and (ii) His$^-$ Leu$^-$ Trp$^-$ Ade$^-$ plates and (iii) with the β-galactoside assay.

RepA fragment was mutagenized by error-prone PCR using Amplitaq (Perkin Elmer, USA) in the presence of 1 mM $MnCl_2$, digested with *Eco*RI–*Pst*I and ligated to *Eco*RI–*Pst*I cut pGAD424 plasmid. This mix was transformed into *E.coli* and plasmid DNA was extracted from the pool of transformants. The DNA pool was then transformed into PJ69-4A yeast cells containing the pGBT-DnaA and transformants were selected on Leu$^-$ Trp$^-$ plates. Several transformants were replica plated on (i) Leu$^-$ Trp$^-$, (ii) His$^-$ Leu$^-$ Trp$^-$ 2 mM AT and (iii) His$^-$ Leu$^-$ Trp$^-$ Ade$^-$ plates. DNA extracted from yeast colonies auxotrophic for histidine and adenine were sequenced to look for mutants in RepA that no longer interact with DnaA.

Plasmid construction, site-directed mutagenesis and transformation experiments

Plasmid pET-RepA was mutagenized with the oligonucleotides RepAMUTFP and RepAMUTRP to generate pET-Y52S using Quick Change™ site-directed mutagenesis kit (Stratagene, USA) following the supplier's instructions.

For transformation experiments domain I (residues 1–86) and domain IV (residues 372–467) of DnaA were fused to LZs and the DNA binding domain of LexA repressor of *E.coli*. Sequence encoding for D1-B-D4 [DnaA (1–86)-biotin linker-(372–467)] was PCR amplified from the plasmid DNA from WM2869 (Seitz *et al*., 2000) using the primer pair DnaAFPNde/DnaARPXba. The LZs were PCR amplified from pT25-Zip (Karimova *et al*., 1998) using two different primer pairs, namely FRLZFP/FRLZRP and BKLZFP/BKLZRP. The former (FRLZ) and the latter (BKLZ) PCR products were digested with *Nde*I–*Bam*HI and *Xho*I–*Xba*I, respectively. The *Nde*I–*Xho*I digested D1BD4 PCR product was ligated in a three-piece ligation reaction to BKLZ and pMALC2X, respectively digested with *Xho*I–*Xba*I and *Nde*I–*Xba*I (New England Biolabs, USA) to give pTacD1BLZ. The DNA binding domain (Oertel-Buchheit *et al*., 1993) of LexA repressor (residues 1–87) was PCR amplified using the primers LexAFP/LexARP, digested with *Xho*I–*Xba*I and used to replace the LZ fragment of pTacD1BLZ to generate pTacD1BLexA. For pTacLZBD4, the *Bam*HI–*Xba*I digested D1BD4 PCR product was ligated to FRLZ PCR product and pMalC2X, respectively digested with *Nde*I–*Bam*HI and *Nde*I–*Xba*I. The Tac-promoter-LZBD4 cassette from pTacLZBD4 digested as a *Msc*I–*Hin*dIII fragment was cloned in *Sma*I–*Hin*dIII cut pDHC29 (Phillips *et al*., 2000) to generate pTacLZBD4-29 providing RSF1030 origin of replication and cloramphenicol resistance. Various hybrid replicons were constructed for transformation experiments. Minimal *ori* of pSC101 from DnaA box to end of RepA was PCR amplified using OriFP and RepARP primers. The PCR product digested with *Eco*RI–*Bam*HI was ligated to *Eco*RI–*Bgl*II cut tetracycline resistance gene to generate pMOT. The DnaA box in pMOT was substituted with *lexA* half site (CTGTATAT) and full site (CTGTATATATATACAG) (Oertel-Buchheit *et al*., 1993). The half and full sites were engineered by PCR using primer pairs HalfFP/OriRP and FullFP/OriRP, respectively, and ligated to the tetracycline gene as in pMOT, producing the constructs pLHT and pLFT. The ligation mixes were transformed in *E.coli* EH3827 containing pTacD1BlexA and plated on LB agar containing 25 μg of kanamycin, 50 μg of ampicillin, 5 μg of tetracycline and 40 μM isopropyl β-D-thiogalactopyranoside (IPTG). In order to eliminate the possibility of disrupting the *ori* in a *cis*-dependent fashion, the half and full sites of *lexA* were cloned 236, 336 and 484 bp upstream of the DNA box leaving the minimal *ori* intact. For this, non-specific DNA fragments of respective lengths, digested with *Pst*I from other plasmid vectors, were cloned at the *Pst*I site of pMOT upstream of the DnaA box. The half and full sites of LexA were inserted as *Eco*RI cassettes (using self-annealing oligonucleo-

tide pairs HalfTop/HalfBot and FullTop/FullBot) at the *Eco*RI site upstream of *Pst*I to develop constructs pLHTX1, pLHTX 2, pLHTX 3, pLFTX1, LFTX2 and LFTX2.

For expression as GST fusion proteins, domains I and IV were PCR amplified using D1BamFP/D1RP and D4FP/DnaASalRP primers, digested with *Bam*HI–*Mfe*I and *Bam*HI–*Sal*I, and cloned at compatible sites in pGEX2TK and pGEX4T1 (Pharmacia) to give pGEX2TK-D1 and pGEX4T1-D4, respectively. The 0.9 kb wt and Y52S RepA gene were amplified using RepAFP/RepARP primers and cloned in pGEX4T1 for expression as GST fusions.

In vivo replication assay

The plasmids pET-RepA and pET-Y52S were transformed into pSC101 $Rept_s$ strain using electroporation following the manufacturer's instructions (Biorad, USA). Transformants were selected at 30°C on medium containing streptomycin (10 μg/ml) and ampicillin (100 μg/ml). The colonies were replica plated and grown at 42°C to check for complementation at non-permissive temperature.

Purification of proteins

Purification of DnaA, His_6–DnaA, RepA (wt and Y52S), RepA-N (N-terminal RepA, residues 1–142), RepA-C (C-terminal RepA, residues 143–309), IHF, DnaB and DnaC has been described earlier (Datta *et al.*, 1999). Single-stranded DNA binding protein was purchased from USB. The D-I (domain I of DnaA, residues 1–86), D-IV (domain IV of DnaA, residues 372–467), RepA and Y52S RepA proteins expressed as GST fusion proteins were purified on glutathione–Sepharose 4B beads following the supplier's instructions (Pharmacia, USA).

Protein–protein interaction studies by ELISA

ELISA was carried out essentially as reported earlier (Datta *et al.*, 1999). For RepA–DnaA interactions, DnaA, D-I or D-IV was coated on microtiter plate wells and challenged with GST fusions of RepA, RepA-N, RepA-C or RepA-Y52S in solution. The bound proteins were detected by enzyme-conjugated anti-GST antibody (1:30 000 dilution) following the supplier's instructions (Pharmacia).

Protein–protein interaction studies by protein affinity column chromatography

Protein affinity column chromatography was performed as described (Lu *et al*., 1998). Purified His_6–DnaA or GST-fused domain I and IV was immobilized on Ni-NTA or glutathione–Sepharose beads, respectively. ^{35}S-labeled RepA and Y52S proteins were obtained using the TNT T7 Quick Coupled Transcription/Translation System (Promega) as per the supplier's instructions. Binding of various amounts of ^{35}S-labeled peptides was checked by SDS–PAGE and autoradiography.

Filter binding assays

Filter binding assays were carried out as reported (Datta *et al.*, 1999). Binding of the wt and Y52S mutant forms of RepA protein was checked with a 686 bp DNA fragment, labeled with [γ-^{32}P]ATP containing the *ori* of pSC101 [*Hin*fI b fragment of pCV2 (Vocke and Bastia, 1983b)] and to a control fragment without the *ori* sequence [a *Hin*fI-digested 616 bp fragment from Litmus38 (New England Biolabs, USA)].

Loading of DnaB helicase

Helicase loading was carried out as described (Datta *et al.*, 1999) whereby different purified proteins, namely single-stranded DNA binding protein, IHF, DnaC, DnaA, wt or Y52S RepA, and [γ-^{32}P]ATP-labeled DnaB were incubated with supercoiled pSC101 DNA substrates. The reaction mixture was gel-filtered and the radioactivity in the excluded and the included fractions was measured.

In vitro unwinding of the ori and $KMnO_4$ footprinting

The unwinding reactions were carried out as described (Konieczny *et al*., 1997) using 300 ng of supercoiled pSC101 DNA, 50 ng of IHF, 100 ng of DnaA/D1/D4 and 500 ng of wt or Y52S RepA as the purified components.

Primer extensions, carried out using PCR with [γ-^{32}P]ATP-labeled MELTOP and MELTBOT primers for top and bottom strands, respectively, were analyzed by electrophoresis on an 8 M urea–6% polyacrylamide gel and autoradiogaphy. The position of the area modified by $KMnO_4$ was determined by comparison to a sequencing ladder run in parallel in the same gel.

Leucine zipper transformation experiments

Escherichia coli EH3827 was sequentially transformed with pTacD1BLZ-29 and pTacLZBD4, and transformants were selected on LB agar plates containing 25 μg/ml kanamycin, 50 μg/ml ampicillin and 17 μg/ml chloremphenicol at 37°C. The competent cells made from these transformants were transformed with pSC101 and plated on LB agar plates containing 5 μg/ml tetracycline and 40 μM IPTG in addition to the antibiotics above. For control, pGEX4T1-D4 was used in place of pTacLZBD4. The ability to support pSC101 replication in terms of number of transformants obtained with the LZ DnaA constructs was compared with WM2869 (Seitz *et al*., 2000) and full-length DnaA.

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