

DnaA dependent replication of plasmid R1 occurs in the presence of point mutations that disrupt the dnaA box of *oriR*

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ABSTRACT

We have found that DnaA dependent replication of R1 still occurred when 5 of the 9 bases in the dnaA box present in *oriR* were changed by site directed mutagenesis although the replication efficiency decreased to 20% and 70% of the wild-type origin *in vitro* and *in vivo* respectively. Additional mutation of a second dnaA box, 28 bp upstream *oriR*, that differs in only one base from the consensus sequence, did not affect the level of replication whereas polyclonal antibodies against DnaA totally abolished *in vitro* replication in the absence of the dnaA box. Wild-type RepA as well as a RepA mutant, RepA2623, that binds to *oriR* but that is inactive in promoting *in vitro* replication of plasmid R1, induce efficient binding of DnaA to the dnaA box. However, specific binding of DnaA to *oriR* was not detected by DNase I protection experiments in the absence of the dnaA box. These results suggest that the entrance of the DnaA protein in *oriR* is promoted initially by interactions with a RepA-*oriR* pre-initiation complex and that, in the absence of the dnaA box, these interactions can support, with reduced efficiency, DnaA dependent replication of plasmid R1.

INTRODUCTION

Replication of plasmid R1 in *E. coli* extracts is dependent on, among other proteins, the plasmid RepA and the host DnaA proteins (1,2). The 9 bp recognition site for DnaA, or dnaA box (3) is present in *oriR*, the minimal origin of replication of R1 (2). RepA protein binds to *oriR* sequences that span nearly 100 bp downstream from the dnaA box and promotes binding of the DnaA protein to this box (2). It is not clear if this effect is modulated by DnaA-RepA interactions and/or by structural modifications of *oriR* induced by RepA.

The DnaA protein can act as a replisome organizer (4,5). It has been proposed, by analogy with the situation in *oriC*, that DnaA promotes assembly of a DnaB-DnaC pre-priming complex

at the 9 bp repeats present in the A+T-rich region of *oriR* that share homology with the 13-mers of *oriC* (6). Deletion of the A+T rich region of *oriR* does not prevent binding of the DnaA and RepA proteins, but results in complete loss of R1 replication (2). The potential role of DnaA in melting *oriR* at the A+T rich region is probably dispensable for R1 replication: the ADP form of DnaA, inactive in melting *oriC* sequences (6,7), seems to be sufficient for R1 replication (unpublished data mentioned in 8). In the absence of DnaA, RepA can promote replication of R1 *in vivo*, both in the autonomous and the integrated situations (9,10). This implies the ability of the RepA protein to open the helix and to promote the assembly of the replisome complex at the origin of replication. However, replication of R1 in the absence of DnaA and in the presence of normal levels of RepA protein is very inefficient (11) and this type of replication has not been detected *in vitro* (1). These data indicate that, although R1 can replicate in DnaA-dependent and DnaA-independent modes, under normal conditions the DnaA-dependent mode is predominant.

In this paper we further analyze interactions between RepA, DnaA and *oriR*, using wild-type and mutated RepA proteins and *oriR* sequences. The *repA* mutant used here codes for a RepA protein unable to support *in vitro* replication of R1 (12) but that still binds to *oriR* (Giraldo and Díaz, submitted). This mutant opens the possibility to investigate if the full replication capacity of the RepA protein is required to promote binding of DnaA to the dnaA box. The data we present are consistent with an initial entrance of the DnaA protein in *oriR* promoted by a RepA-*oriR* pre-initiation complex and indicate that although utilization of DnaA is favoured by the dnaA box present in *oriR*, this box is dispensable for the DnaA dependent replication of plasmid R1.

MATERIAL AND METHODS

Bacterial strains and plasmids

All bacterial strains were *E. coli* K12. TG1 (13) and BMH71-18 (14) were used for propagation of M13-based vectors or recombinants and in experiments using site-directed mutagenesis.

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JM83 (15) was used to rescue and identify pUC-type recombinants. C600 was used for the preparation of *in vitro* replication extracts and also as a host for mini-R1 plasmids.

Bacterial plasmids used in this work are indicated in Table 1.

Basic manipulations with DNA

Techniques used for the preparation of DNA, gel electrophoresis of DNA fragments and basic manipulations with enzymes that act on DNA, were as described (13). Purification of DNA fragments from agarose gels was done by elution using the GeneClean kit (BIO 101). Transformation of *E. coli* with DNA was done as described (16) with modification for M13 derivatives described in the 'M13 cloning and sequencing manual' edited by Amersham. DNA sequencing by the chain terminating method (17) was done using the M13 sequencing kit from Amersham according to the specifications of this supplier. Sequencing of linear fragments, A+G reactions, were done as described by Maxam and Gilbert (18). Oligonucleotide site-directed mutagenesis using M13 vectors was done using either the protocols described by Carter *et al.* (19) or the kit 'Oligonucleotide-directed *in vitro* mutagenesis system' (version 2) from AMERSHAM.

In vitro replication analysis

Cell-free extracts were prepared from cultures of C600. *In vitro* replication of the mini-R1 plasmids pRG10A and pRG10B were assayed in type I extracts, in which RepA is synthesized *de novo* during the assay (20). Replicative forms of M13-*oriR* recombinants were assayed in type II extracts supplemented with partially purified RepA protein, as described by Ortega *et al.* (1). Polyclonal antiserum against DnaA prepared from rabbit, was a kind gift of M.Kohiyama. This antiserum was added to the *in vitro* reaction mixtures at a dilution of 1:50 and these samples were incubated for 20 minutes at 0°C before starting the replication assay.

Copy number determinations

Relative copy number of the pKN177-type *copA* miniplasmids pRG10A and pRG10B were evaluated by determining the maximum level of resistance to ampicillin as described (21).

DNase I footprint analysis

Protocols were adapted by Giraldo and Díaz (Submitted for publication) from those described by Galas and Schmitz (22) and Masai and Arai (2). 12.5 ng (53.0 fmol, 25000 cpm) of the 359 bp *oriR* fragments of pRG22 and pRG220, 3'-end labelled at the *Xba*I site, 135 ng of RepA or RepA2623 proteins (4 pmol; molar ratios RepA/*oriR*=75.5) and 100–1000 ng of DnaA protein (1.8–18.0 pmol; molar ratios RepA/DnaA=2.2–0.27) were used. Incubation mixtures, 49 μ l in volume, were in 25 mM Hepes-KOH pH=8.0, 50 mM KCl, 10 mM Mg-acetate, 4 mM DTT, 2 mM ATP, 4% sucrose and 50 μ g/ml bovine serum albumine. The proteins and the *oriR* fragments were incubated for 15 min. at 0°C; then 1 μ l of calf thymus competitor DNA (0.5 mg/ml) was added to each sample and these were further incubated at 30°C for 15 min. Finally the samples were digested for 1 min. at room temperature with 2.5 or 5.0 ng (samples without or with proteins, respectively) DNase I. After the DNase I digestion, the samples were phenol-chloroform extracted, ethanol precipitated and loaded onto a 6% polyacrylamide-7M urea sequencing gel, in parallel with A+G chemical sequencing reactions of the same fragment (18).

Table 1. Plasmids

Plasmids	Relevant construction	dnaA boxes ¹		Marker	Source/Reference
		1	2		
M13mp9	cloning vector	–	–	Ap ^R	(25)
pSO3	M13mp9 \times BamHI + <i>oriR-Sau3A</i>	wt	wt	Ap ^R	(1)
pSO33	"	mut.	wt	Ap ^R	This work
pSO338	"	mut.	mut.	Ap ^R	This work
pUC18	cloning vector	–	–	Ap ^R	(15)
pRG22	pUC18 \times AccI + <i>oriR-HpaII</i>	wt	wt	Ap ^R	R.Giraldo
pRG220	"	mut.	wt	Ap ^R	This work
pRG10A ²	R1- <i>copA</i> -mini-plasmid	wt	wt	Ap ^R	This work
pRG10B ²	"	mut.	wt	Ap ^R	This work

¹dnaA box-1: 5'TTATCCACA3'(coordinates 1428–1436); dnaA box-1 mutant: 5'TGCGATACA3'. dnaA box-2: 5'TAATACAAA3' (coordinates 1392–1400). dnaA box-2 mutant: 5'TAATATCAA3'. (see Fig. 1A). ²The structure of pRG10A and pRG10B is the same of plasmid pKN177 (26,27), except for a 128 bp deletion at coordinates 1659–1787 (see Fig. 1A).

The RepA protein used in the DNase I protection experiments was obtained first in an insoluble form (1) and then solubilized by treatment with 3M guanidium hydrochloride, followed by addition of the zwitterionic detergent CHAPS to 1.0% and dialysis against 25 mM Hepes-KOH pH=8.0, 0.5 M KCl, 2 mM DTT, 0.1 mM EDTA and 10% ethylene glycol (Giraldo and Díaz, submitted). The RepA wt and RepA2623 preparations used contained respectively 1550 μ g/ml and 1260 μ g/ml of total protein and were 15–20% pure. The DnaA preparation (23) was a kind gift of M.Kohiyama. This preparation contained 2300 μ g/ml of total protein and was 40% pure.

RESULTS

Plasmid R1 replicates *in vitro* in a DnaA dependent mode in the absence of the dnaA box present in *oriR*

The strict requirement of DnaA for *in vitro* replication of plasmid R1 (1), made it interesting to test if the dnaA box present in *oriR* is an absolute requirement for this replication. For this purpose we isolated, by site-directed mutagenesis, a M13-*oriR* mutant, pSO33, in which the dnaA box at coordinates 1428–1436, 5'TTATCCACA3', was mutated in 5 positions (Fig. 1A). Data in Fig. 1B indicate that replicative forms of this *oriR* mutant can be replicated in type II extracts supplemented with partially purified RepA protein. However, the level of replication of pSO33 was only 20% of the pSO3 control, which indicates that although the dnaA box is not strictly required, its presence determines an increased efficiency of replication. The occurrence, 28 bp upstream from the minimal origin, of a 9 bp sequence, 5'TAATACAAA3', differing in only one base (in bold) from the consensus dnaA box, made it interesting to test if the replication of pSO33 involved the utilization of this box. For this purpose we constructed pSO338, a mutant of pSO33 mutated in both sequences sharing homology to the consensus dnaA box (Fig. 1A), and tested replication of this mutant *in vitro*. It was found that pSO338 and pSO33 replicate with similar efficiencies (Fig. 1B). This result indicates that the RepA-dependent replication of pSO33 which lacks the dnaA box present in *oriR* is not due to the utilization of the second and imperfect dnaA box present 28 bp upstream from *oriR*.

According to these results it should be possible to isolate R1 plasmids containing the mutated dnaA box present in pSO33.

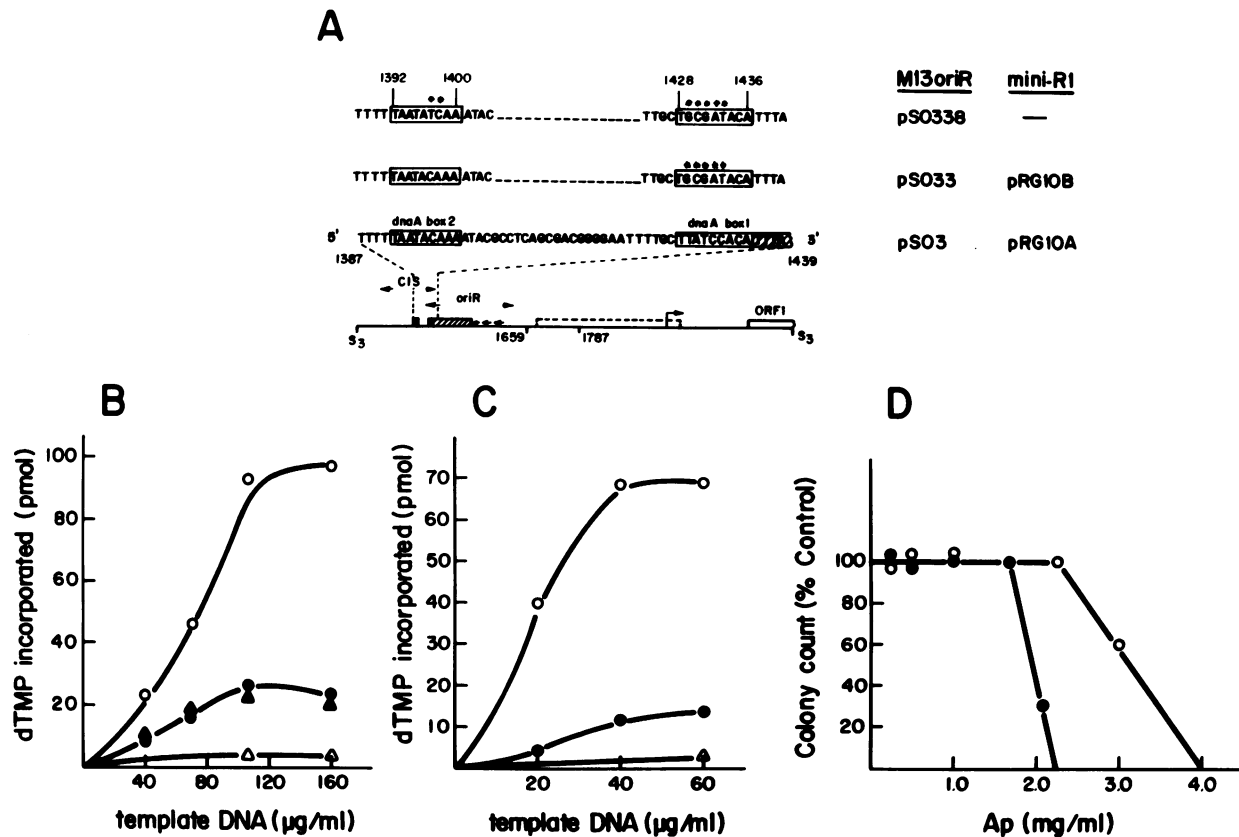


Figure 1. A. Relevant information on the *Sau3A* fragment of R1 that includes *oriR*. ■ *dnaA* boxes, ▨ RepA binding region, ↔ 9-mers present in the A+T rich region, □ origin region as defined by electron microscopy, → initiation of leading strand synthesis. Enlarged: region that contains two sequences homologous to the consensus *dnaA* box of *E. coli*. * indicates base changes in these sequences introduced by site directed mutagenesis. The relevant sequences corresponding to the M13-*oriR* and mini-R1 plasmids used, are indicated. Coordinates are as described by Ryder *et al.* (28). B. *In vitro* replication of M13-*oriR* recombinants pSO3 (○), pSO33 (●) and pSO338 (▲) in type II extracts supplemented with partially purified RepA protein. The background line (△) corresponds to samples of pSO3 treated with novobiocin (25 µg/ml). C. *In vitro* replication analysis of R1 miniplasmids in type I extracts: (○) replication of pRG10A, (●) replication of pRG10B, (△) replication of pRG10B in the presence of polyclonal antibodies against DnaA protein. D. Ampicillin resistance levels determined by plasmids pRG10A (○) and pRG10B (●).

As predicted, a *copA* mini-R1 derivative containing the mutated *dnaA* box, pRG10B, was easily isolated (Table 1). Replication of this mutant and the parental plasmid, pRG10A, were tested *in vitro* using type I extracts. Again, the mutation in the *dnaA* box reduced the level of replication to 20% of that observed with the parental miniplasmid (Fig. 1C). Replication of the mutants lacking the *dnaA* box sequences should be DnaA-dependent because *in vitro* replication of plasmid R1 requires DnaA (1). This prediction was evaluated directly by measuring the effect of polyclonal antibodies against DnaA on the *in vitro* replication of pRG10B. Addition of DnaA antiserum to the replication assays reduced replication of both pRG10A and pRG10B to the background level, and the same amount of preimmune serum inhibited less than 15% replication of these miniplasmids. From these results it can be concluded that the *dnaA* box is not absolutely required for the action of the DnaA protein in initiation of R1 replication *in vitro*, although its presence improves the efficiency of the process.

The *dnaA* box of *oriR* is required for maximal efficiency of mini-R1 replication *in vivo*

The effect of the mutation in the *dnaA* box on the efficiency of R1 replication was also evaluated *in vivo*. For this purpose we

determined the relative copy number of pRG10A and pRG10B comparing the maximum levels of resistance to ampicillin conferred by these plasmids. Data shown in Fig. 1D indicates that 1.75 mg/ml and 2.5 mg/ml are the maximum ampicillin concentrations to which 100% of the cells bearing pRG10B and pRG10A, respectively, are resistant. This indicated that the mutation in the *dnaA* box reduces to 70% the efficiency of replication of R1 miniplasmids *in vivo*. Although pKN177 can replicate *in vivo* in the absence of DnaA, this replication is only 7% of the control (data not shown) which indicates that replication of R1 in the absence of the *dnaA* box is mainly DnaA dependent. It can be concluded that, both *in vivo* and *in vitro*, the *dnaA* box present in *oriR* is dispensable for the action of the DnaA protein in R1 replication, but it is required for maximal efficiency of replication.

Binding of DnaA to *oriR* can be promoted by a RepA mutant protein that binds to *oriR* but fails to support replication of R1, and requires the sequences of the *dnaA* box

The capacity of RepA, probably as part of a RepA-*oriR* complex, to promote binding of DnaA to the *dnaA* box of the minimal origin, lead us to question if the full replication capacity of RepA is required to promote this binding. To answer this question we

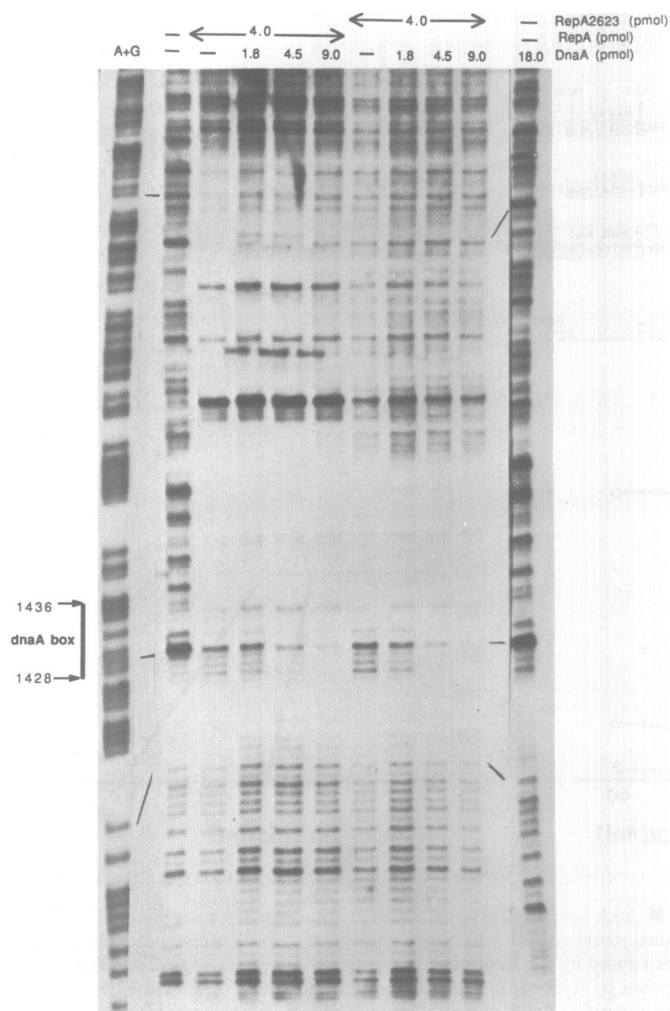


Figure 2. DNase I protection of wild-type *oriR* sequences by RepA, RepA2623 and DnaA proteins. 53 fmol of wild-type 3'-³²P-*oriR* 359 bp fragment labelled in *Xba*I, was treated with DNase I in the presence of proteins RepA wild-type or mutant and/or DnaA as indicated. pmol of proteins used are indicated above the tracks. The combination of proteins are indicated. A + G: Maxam and Gilbert reactions for these bases on the *oriR* fragment. The coordinates corresponding to the *dnaA* box are indicated on the left side of the figure.

used a mutated RepA protein, RepA2623, that is unable to support *in vitro* replication of plasmid R1 (12). Recent studies indicate that purified RepA2623 protein can bind to *oriR* sequences but that this mutant is affected in cooperative interactions that led to the formation of high order RepA-*oriR* complexes (Giraldo and Díaz, submitted). The ability of RepA2623 protein to promote binding of DnaA to wild-type *oriR* sequences was evaluated comparatively with that of the RepA wild-type protein by DNase I protection experiments as indicated in Material and Methods. Saturating amounts of the mutant and wild-type replication proteins (4.0 pmol) and increasing amounts of the DnaA protein (1.8–18.0 pmol) were used. Protection of *oriR* from DNase I digestion by RepA, RepA2623 or DnaA proteins alone, was also performed. Data in Fig. 2 indicate that i) RepA is required, as described, for binding of DnaA to the *dnaA* box, ii) RepA2623 induces, with similar efficiency to wild-type RepA protein, binding of DnaA to the *dnaA* box. Note that RepA2623 binds to *oriR* but interacts less efficiently than the RepA protein with

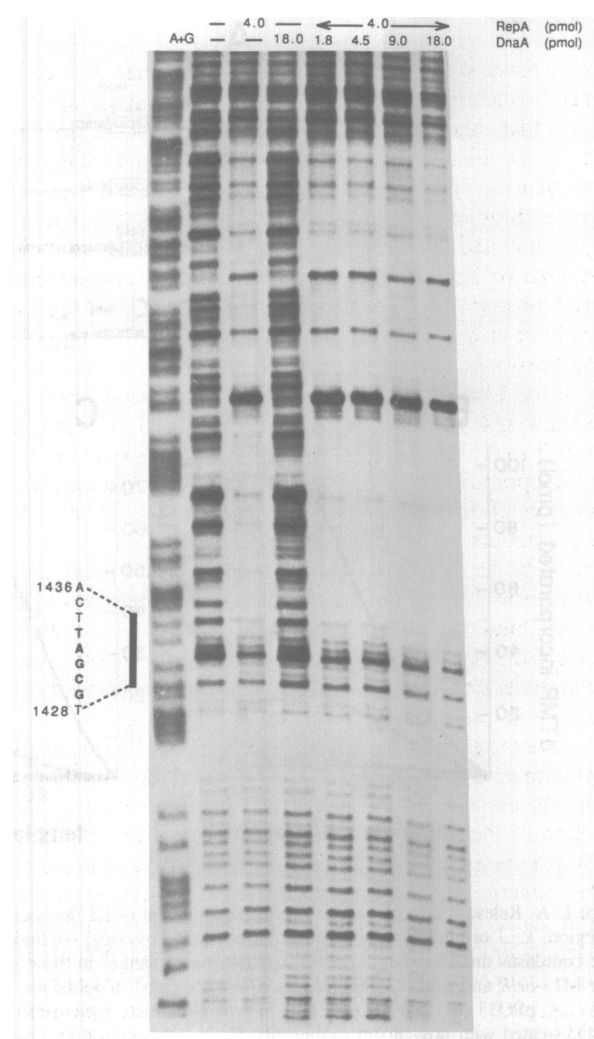


Figure 3. DNase I protection by RepA and/or DnaA of *oriR* sequences mutated in the *dnaA* box-1. 53 fmol of 3'-³²P-*oriR* 359 bp mutated fragment labelled in *Xba*I, were digested with DNase I in the presence of proteins RepA and/or DnaA as indicated. pmol of proteins used are indicated above the tracks. A + G indicate Maxam and Gilbert reactions. Bases altered by mutagenesis are in bold character. The coordinates corresponding to the *dnaA* box are indicated on the left side of the figure.

the region of *oriR* distal to the *dnaA* box. These results are consistent with a role of a RepA-*oriR* complex in promoting binding of DnaA to the *dnaA* box. In addition, they indicate that the full replication activity of RepA is not required for this binding.

The strict requirement of the RepA protein for the interaction of DnaA with the *dnaA* box and the DnaA dependent replication of R1 observed in the *dnaA* box mutant, made it interesting to test if this box is required for the interaction of the DnaA protein with *oriR*. For this purpose we evaluated, by DNase I protection experiments, binding of DnaA to *oriR* carrying the 5 bp changes in the *dnaA* box. The results obtained show that DnaA, alone or in combination with saturating amounts of RepA, fails to protect sequences in the mutated *dnaA* box (Fig. 3). This result indicates that the footprint of DnaA in the *dnaA* box is the consequence of specific interactions of DnaA with these sequences.

DISCUSSION

In this work we show that mutants having the *dnaA* box of *oriR* disrupted by mutation replicate *in vitro* at a reduced but significant level and that this replication, that is dependent on the RepA protein, is specifically inhibited by DnaA antibodies. These data indicate that the specific sequences of the *dnaA* box present in *oriR* are not required for the DnaA-dependent initiation of plasmid R1 replication and are consistent with previous data that indicate that *in vitro* replication of plasmid R1 is strictly dependent on the DnaA protein (1). The *dnaA* box mutant pRG10B replicates efficiently *in vivo* in a *dnaA*⁺ background. This result confirms that the *dnaA* box present in *oriR* is not strictly required for the action of the DnaA protein in initiation of R1 replication. It is interesting to note that the ratio of pRG10B to pRG10A replication is almost 3 fold higher *in vivo* than *in vitro*. This could indicate either an optimum level of DnaA protein *in vivo* and/or the contribution of factor(s) which are not present or present in limiting amounts in the *in vitro* replication assay.

Because of the dispensability of the *dnaA* box for the DnaA-dependent replication of plasmid R1 and the requirement of RepA for specific binding of DnaA to *oriR*, it was of interest to analyze interactions of the DnaA and RepA proteins with the *oriR* mutant deficient in the *dnaA* box. It can be imagined that RepA protein bound to *oriR* can exert a strong positional effect for entry of DnaA and that, even in the absence of the *dnaA* box, DnaA can interact with *oriR* in the region adjacent to the RepA binding site. The results presented here indicate that efficient and specific interaction of the DnaA protein with *oriR* requires, in addition to the RepA protein, the sequences of the *dnaA* box. It is proposed that the induction of this binding by RepA protein is probably due to an initial interaction of the DnaA protein with RepA protein bound at the *oriR* region. Subsequent specific interaction of DnaA with the sequences of the *dnaA* box will optimize this entry, but in the absence of the *dnaA* box the initial protein-protein interaction is probably sufficient for incorporation of DnaA to the initiation complex.

The situation in R1 has similarities and differences with the situation in pSC101. It has been proposed, recently, that the replication protein of pSC101, Rep, enhances, by protein-protein interactions, binding of the DnaA protein to a *dnaA* box that is an essential part of the origin of replication (24). Significant binding of the DnaA protein to the *dnaA* box, and also to weak affinity sites for DnaA that overlap with the RepA binding site, were found in the absence of the Rep protein of pSC101. In this plasmid, deletion of the high-affinity binding-site for DnaA leads to inactivation of plasmid replication (26). Although in R1 destruction of the *dnaA* box present in *oriR* by punctual mutations is compatible with replication, it is not known if deletion of this box leads to the inactivation of R1 replication. This could well be the case if this box was placed in an spacer or if the overlap between the high-affinity binding-site for RepA and the *dnaA* box (Giraldo and Díaz, submitted) were relevant for the action of RepA in *oriR*. On the other hand, the possibility that point mutations in the *dnaA* box of pSC101 were compatible with replication has not been explored.

Finally, our results underline the flexibility of the mechanism of initiation of replication catalyzed by RepA: this protein promotes initiation of R1 replication that is maximally efficient in the presence of DnaA and the *dnaA* box but that can also occur, with decreased efficiency, in the absence of the *dnaA* box and even in the absence of the DnaA protein. Although RepA is able

to catalyze opening of the helix and assembly of the replisome complex at the origin of replication in the absence of DnaA, the *dnaA* box and the DnaA protein are important to optimize the initiation frequency in order to allow the copy number control system to couple replication of the plasmid to the growth of the host cell efficiently.

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