A Critical DnaA Box Directs the Cooperative Binding of the Escherichia coli DnaA Protein to the Plasmid RK2 Replication Origin*

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Kelly S. Doran‡§, Donald R. Helinski‡¶, and Igor Konieczny‡**

From the ‡Department of Biology, Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92037-0232 and the ¶Department of Molecular and Cellular Biology, Faculty of Biotechnology, University of Gdańsk, 24 Klaków, PL-8022 Gdansk, Poland

The requirement of DnaA protein binding for plasmid RK2 replication initiation the Escherichia coli was investigated by constructing mutations in the plasmid replication origin that scrambled or deleted each of the four upstream DnaA boxes. Altered origins were analyzed for replication activity in vivo and in vitro and for binding to the E. coli DnaA protein using a gel mobility shift assay and Dnase I footprinting. Most strikingly, a mutation in one of the boxes, box 4, abolished replication activity and eliminated stable DnaA protein binding to all four boxes. Unlike DnaA binding to the E. coli origin, oriC, DnaA binding to two of the boxes (boxes 4 and 3) in the RK2 origin, oriV, is cooperative with box 4 acting as the “organizer” for the formation of the DnaA-oriV nucleoprotein complex. Interestingly, the inversion of box 4 also abolished replication activity, but did not result in a loss of binding to the other boxes. However, DnaA binding to this mutant origin was no longer cooperative. These results demonstrate that the sequence, position, and orientation of box 4 are crucial for cooperative DnaA binding and the formation of a nucleoprotein structure that is functional for the initiation of replication.

The DnaA protein is a key factor in the initiation of DNA replication in prokaryotes and is highly conserved among a diverse group of eubacterial species (for reviews, see Refs. 1–3). DnaA binds to conserved 9-base pair consensus sequences, DnaA boxes (5-9T(A/T)TNCACA), which have been identified both in prokaryotic chromosomal origins, including those of Escherichia coli (4), Pseudomonas putida (5), Pseudomonas aeruginosa (6), Bacillus subtilis (7) and Streptomyces lividans (8), and in plasmid origins of replication, including R6K (9), RK2 (10, 11), P3 (12), pSC101 (13), P1 (14), R1 (15), ColE1 (16), and pSP10 (17).

Whereas the interactions of DnaA at oriC have been studied in considerable detail (for a review, see Ref. 2), much less is known about the formation of DnaA-nucleoprotein structures at the replication origins of plasmid replicons. Unlike the more disperse arrangement of DnaA boxes at oriC and replication origins of other bacteria, the DnaA boxes at plasmid replication origins in general are closely associated forming one or more clusters. In addition, the DnaA protein must work in concert with a plasmid-encoded initiation protein (for reviews, see Refs. 18 and 19)). Finally, unlike the case with oriC, the ADP form of DnaA protein is sufficient for replication in vitro of plasmids R1 (15) and P1 (20) and for origin opening of F (21), RK2 (11), and R6K (22). These observations likely reflect fundamental differences in the structure of the nucleoprotein complexes formed by the DnaA protein at the replication origin of plasmids and the bacterial chromosome.

The broad host range plasmid RK2 exhibits the remarkable ability to replicate and maintain itself in a wide variety of Gram-negative bacteria (23) and, thus, is a particularly interesting system for studying fundamental questions about the interactions between plasmid- and host-encoded factors at a replication origin. The DnaA protein has been shown to be required for RK2 replication (24, 25), but its exact role has been less well studied. Only recently, it was demonstrated that there are two DnaA boxes in addition to two previously described boxes located in the leftward part of the RK2 origin, oriV, and that all four boxes bind the E. coli DnaA protein (11). The four DnaA boxes arranged as two pairs in an indirect orientation can potentially form a cruciform structure (see Fig. 1). Boxes 2, 3, and 4 contain one mismatch from the stringent DnaA box consensus sequence, whereas box 1 contains two mismatches.

Replication initiation of RK2 requires both DnaA and the plasmid-encoded initiation protein, TrfA. The TrfA protein binds to the iteron sequences in oriV (10, 26–28) and this binding results in the melting of all four 13-mers in the A+T-rich region in the presence of the DnaA and HU proteins (11). The DnaA protein also has the indispensable role of recruiting the DnaB-DnaC complex to the RK2 origin through a protein-protein (DnaA-DnaB) interaction (29). The loading and activation of the DnaB helicase at the open region requires both the DnaA and TrfA proteins (29) and the correct positioning of the DnaA boxes on the helical axis with respect to the rest of the origin (30). How the DnaA protein and the TrfA protein cooperate to achieve DnaB loading in not known.

Do the requirements for DnaA protein binding and function in plasmid systems differ from that of bacterial chromosomes, specifically oriC? In this paper, we present the molecular and biochemical characterization of the structural requirements for functional binding of DnaA protein during plasmid replication initiation, specifically plasmid RK2. Our results demonstrate that one of the four DnaA boxes, box 4, directs the cooperative binding of DnaA to the other DnaA boxes, essentially acting as the “organizer” in the formation of a functional DnaA-oriV nucleoprotein structure. These features of DnaA protein bind-
ing to a plasmid replication origin may be important in understanding the differences between plasmid and chromosomal replication initiation and, in the case of RK2, its broad host range properties.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Proteins—**Plasmid pKD19L1, a dual RK2-R6K mini-replicon, was used for constructing all mutations using the QuickChange polymerase chain reaction-based site-directed mutagenesis kit (Stratagene, Inc.) as described previously (30). All oligonucleotide primers used for polymerase chain reaction were synthesized by OPERON, Inc. The E. coli strains used in this study were XLI-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (‘F’ proAB lacZDMD15 Tn10)) from Stratagene, Inc., C600 (thr leu thi lacY supE44 tonA), CC118 (araD139 Dara, leu7697 DlacX476 phoA20 gaIE thir psps rpoB argE3 recA1), and CC118Apr (CC118 (Aprir), (31); the latter two strains were kindly provided by Dr. Ken Timmis. Analysis of in vivo and in vitro replication activity of mutated plasmid constructs was performed as described previously (30). The E. coli DnaA protein was provided by Dr. Alessandra Blasina. The TrfA protein, His6-TrfA G254D/S267L, was purified as described previously (32). In the in vitro experiments performed here and described previously (30, 33) the his-tagged version of the largely monomeric (34) 33-kDa TrfA mutant (His6-TrfA G254D/S267L) which exhibits elevated DNA binding (32) and is fully functional in vivo and in vitro for replication was used. Commercially available proteins were as follows: HU from Enzyco; bovine serum albumin (fraction V) from Sigma; Pf1 DNA polymerase and DpnI restriction endonuclease from Stratagene, Inc.; and other restriction endonucleases and T4 DNA ligase from Promega.

**DNase I Footprinting—**DNA probes were prepared as described (11) with [α-32P]dATP (6000 Ci/mmol) and the HindIII fragment of E. coli DNA polymerase (NEB). Unincorporated ATP was removed using nucleotide removal spin columns (Qiagen). Binding of DnaA protein to origin DNA containing mutated DnaA box sequences was performed essentially as described previously (11), except that DnaA protein was incubated with DNA at 37 °C for 15 min and DNase I (NEN Life Science Products), 0.67 units/reaction, at 37 °C for 30 s.

For footprinting of specific nucleoprotein complexes, reactions were assembled as for gel mobility shift assays described below except that the amount of probe used was increased. After incubating the reaction containing labeled DNA template and DnaA protein for 15 min at 37 °C, DNaI was added (6.7 units/reaction) for 2 min at the same temperature. Reactions were then run on a 5% nondenaturing polyacrylamide gel. After electrophoresis, specific complexes were identified and isolated from the gel and eluted overnight at 30 °C in 1 μL ICN 20 mM Tris-HCl, pH 7.6, 0.2% SDS, 0.2 mM EDTA. Eluant was collected over a spin column (Bio-Rad), extracted with phenol-chloroform, ethanol-precipitated, and resuspended in loading buffer.

**Gel Mobility Shift Assay—**Complexes between oriV DNA and DnaA protein were formed and analyzed essentially as described previously (35) except that the reaction mixture, based on standard conditions used for RK2 in vitro replication (29), contained 40 mM Hepes/KOH, pH 8.0, 25 mM Tris/HCl, pH 7.4, 80 μg/ml bovine serum albumin, 4% sucrose, 4 mM dithiothreitol, 11 mM magnesium acetate. Reactions also contained 25 μg/ml poly(dI-dC) (Boehringer Mannheim) and 0.1% Nonidet P-40. Incubation was at 34–37 °C for 20 min.

**RESULTS**

**Requirement of DnaA Boxes for oriV Function—**To address the importance of each DnaA box present in the RK2 origin (Fig. 1), individual box sequences were replaced with the scramble sequence 5′-GATATCCTG) in place of the wild-type DnaA box sequence (Fig. 2) (29), containing 40 mM Hepes/KOH, pH 8.0, 25 mM Tris/HCl, pH 7.4, 80 μg/ml bovine serum albumin, 4% sucrose, 4 mM dithiothreitol, 11 mM magnesium acetate. Reactions also contained 25 μg/ml poly(dI-dC) (Boehringer Mannheim) and 0.1% Nonidet P-40. Incubation was at 34–37 °C for 20 min.

**Box 4 is Essential for DnaA Binding to Other Boxes at the Replication Origin—**We analyzed the binding of DnaA protein to oriV fragments containing the DnaA box scramble mutations using DNase I footprinting and a gel mobility shift assay. The DNase I pattern of DnaA protection for wild-type oriV (Fig. 3) was as has been previously shown for both linear initiator protein. In the parental strain, CC118, only the RK2 origin may be utilized. Thus, the ratio of transformation frequencies obtained in CC118 versus CC118Apr was used to quantify oriV function in vivo. Ratios obtained for altered origin constructs were normalized to those obtained for pKD19L1 (wild-type oriV) (Table I). Origins that contained a scrambled sequence in DnaA box 1 (oriV A1) or box 2 (oriV A2) or a deletion of both boxes 1 and 2 (oriV A1-2) did not negatively effect oriV function and actually resulted in a somewhat higher level of plasmid transformation frequencies. However, a scrambled sequence in DnaA box 3 (oriV A3) or box 4 (oriV A4) reduced or completely abolished plasmid transformation, respectively. Similarly, an origin containing a deletion of all four DnaA boxes (oriV A1-4) rendered the plasmid inactive. An origin containing only DnaA box 4 (oriV A1-3) was only 74% active compared with wild-type (Table I). The oriV A1-3 mutant is highly variable; in another set of experiments, replication activity was as low as 32% of the activity of a wild-type origin (data not shown). Additionally, the transformants that were obtained with the oriV A1-3 mutant were lost fairly rapidly compared with the wild-type plasmid in the absence of antibiotic selection. Thus, whereas both DnaA boxes 3 and 4 are required for replication activity in vivo, the requirement for DnaA box 4 is the more stringent.

The mutated origins were also analyzed for replication activity in vitro using an in vitro replication assay for RK2 that has been described previously (33). This assay utilizes a 40% (NH₄)₂SO₄ fraction from a soluble E. coli extract to replicate supercoiled DNA containing an RK2 origin in the presence of the initiator protein, TrfA. Fig. 2 shows DNA synthesis for wild-type and altered origin templates. In vitro replication activity with the scrambled DnaA box origin templates was relatively similar to the activity that was observed in vivo (Fig. 2A). Scramble mutations in DnaA boxes 1 and 2 had no inhibitory effect on DNA synthesis, whereas mutations in DnaA boxes 3 and 4 resulted in a lower level of replication activity, with mutant oriV A4 displaying only 50% of wild-type levels. Replication activity of origin templates with deletion mutations (Fig. 2B) again showed that DnaA boxes 1and 2 can be deleted without affecting replication levels, whereas deleting all four DnaA boxes abolished DNA synthesis. Replication of an origin template with only DnaA box 4 (oriV A1-3) was slightly less than wild-type except at the highest amount of TrfA protein.

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and supercoiled oriV templates (11). When a DnaA box contained the scrambled sequence, full Dnase I protection was not observed in that box. Thus, the replacement of the wild-type sequence with the scramble sequence had altered DnaA binding to the respective box. Interestingly, the sequence in DnaA box 4 (oriV A4) greatly reduced the amount of protection observed for box 3 (Fig. 3, lanes 12–15). We further analyzed DnaA binding to mutants oriV A3 and oriV A4 using a gel mobility shift assay (Fig. 4). As expected for wild-type oriV, increasing DnaA concentration led to four specific nucleoprotein complexes (Fig. 4, lanes 1–5); however, on the basis of the relative intensity of the various retarded bands, their formation did not proceed sequentially. Even with smaller amounts of DnaA protein, the appearance of complex I never occurred in the absence of complex II, and usually there was much less of complex I (data not shown and Ref. 11). Thus, binding of DnaA protein to linear oriV DNA appears to be more stable when in the form of complex II, suggesting the possibility of cooperative binding by DnaA. DnaA binding to scramble mutants oriV A3 and A4 support this idea. When DnaA box 3 was scrambled, only complex II and, to a lesser extent, complex I was seen (Fig. 4, lanes 6–10). When DnaA box 4 was scrambled, no nucleoprotein complexes were observed (Fig. 4, lanes 11–15). This is consistent with the lack of replication activity observed for mutant oriV A4 in vitro (Table I) and the substantially reduced activity in vitro (Fig. 2A), suggesting that the function of DnaA box 4 is to initiate DnaA binding to oriV.

**DnaA Binds Initially to Boxes 4 and 3**—We further analyzed DnaA binding by carrying out footprint analysis on specific DnaA-oriV nucleoprotein complexes. DnaA was incubated with wild-type oriV probe, treated with DnaseI, and run on a native gel in order to isolate specific complexes. DnaA protein concentrations were adjusted in order to obtain only complexes I and II (see Fig. 4). DnaA-oriV complexes I and II were isolated, gel purified, and run on a denaturing gel in order to visualize

### Table I

| Origina | CC118 | CC118pir | CC118/CC118pirb | Relative % |
|---------|-------|----------|-----------------|------------|
| oriV wt | $1.6 \times 10^5$ | $2.1 \times 10^5$ | $100$ |
| oriV A1 | $2.9 \times 10^5$ | $3.5 \times 10^5$ | $109$ |
| oriV A2 | $2 \times 10^5$ | $1.9 \times 10^5$ | $138$ |
| oriV A3 | $2.8 \times 10^6$ | $2.6 \times 10^6$ | $14$ |
| oriV A4 | $0$ | $2.1 \times 10^5$ | $0$ |
| oriV A Δ1–2 | $1.4 \times 10^5$ | $1.4 \times 10^5$ | $134$ |
| oriV A Δ1–3 | $9.7 \times 10^4$ | $2.8 \times 10^5$ | $74$ |
| oriV A Δ1–4 | $9.6 \times 10^6$ | $2.3 \times 10^5$ | $0$ |

a Both wild-type and mutant origins are contained on plasmid pKD19L1.
b Ratio of transformants obtained in CC118 (pir–) and CC118pir (pir+) was normalized to that obtained for wild-type.
c Transformants were detected only after 2 nights of incubation.

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**FIG. 2.** *In vitro replication of oriV templates containing DnaA box mutations.* In *in vitro* replication was performed using an *E. coli* C600 extract (fraction II) active for RK2 replication described under “Experimental Procedures.” Reactions contained 0.3 μg of supercoiled wild-type and mutant pKD19L1 template and the indicated amount of TrfA protein. A, the DnaA box scramble mutants: filled circle, wild-type oriV; open circle, oriV A1; open square, oriV A2; filled square, oriV A3; ×, oriV A4. B, the DnaA box deletion mutants: filled circle, wild-type oriV; open circle, oriV A Δ1–2; open square, oriV A Δ1–3; filled square, oriV A Δ1–4.

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**FIG. 3.** DNase I footprint of DnaA box scramble mutations. Restriction fragments (0.6 ng) containing the oriV region with wild-type or mutated DnaA boxes were incubated with DnaA protein for 15 min at 37 °C followed by DNase I treatment for 30 s. The left lane in each template set (−) is the reaction without protein followed by reactions incubated with 625 and 2500 ng of DnaA protein (+ and ++, respectively). Protection from DNase I corresponding to each DnaA box is indicated by brackets. Mutant templates did not display full DNase I protection in the region of the box that contains the scrambled sequence.

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**FIG. 4.** DnaA binding to oriV fragments using gel mobility shift assay. Restriction fragments (0.15 ng) containing wild-type oriV (A), a scramble mutation in box 3 (B), and a scramble mutation in box 4 (C) were incubated with increasing amounts of DnaA protein for 20 min at 34 °C. The first lane in each template set (−) is without protein, followed by 31, 62, 124, and 248 ng of DnaA protein (lanes from left to right). The positions of the four complexes (I–IV) previously described (11) are indicated. The shaded arrows indicate that a strong or weak band is present, and the open arrows indicate that no band is present at the expected position.
construct in the box 3 position (oriV 3-4, see Table II and Fig. 6B). If box 4 was flipped (oriV 4-Flip) so that it faced away from the other boxes, the effect was even more dramatic in that complexes were not formed at all with DnaA protein (Fig. 6C), and the origin was completely inactive in vivo (Table II). Because the sequences surrounding DnaA boxes in oriC have been shown to effect DnaA binding (36), it was possible that DnaA could not bind to box 4 in the oriV 4-Flip mutant because the adjoining sequences were not also placed in the flipped position. Therefore, a mutant was constructed that flipped box 4 and the adjoining sequences (3 base pairs on either side of the box, oriV 4-FlipX). This construct was also inactive in vivo (Table II), but nucleoprotein complexes were observed with DnaA protein (Fig. 6D), suggesting that now DnaA could bind to box 4 as well as the other boxes. However, the binding of DnaA to the boxes in this mutant template proceeded in a stepwise, noncooperative fashion, which is not what is observed for a wild-type oriV template (see Fig. 4, lanes 1–5). Thus, the orientation of DnaA box 4 and its adjoining sequences, relative to box 3, is critical for cooperative binding and it is this binding that is required for replication activity of oriV.

**DISCUSSION**

We have investigated the binding of the DnaA protein to the RK2 replication origin and the DnaA box requirement for plasmid RK2 replication. Our results clearly showed that in *E. coli*, boxes 3 and 4 are essential for optimal replication activity, whereas boxes 1 and 2 are dispensable. The DnaA-oriV interaction is dependent upon one critical box, box 4, as mutations in box 4 abolished stable DnaA binding to all four boxes. DNaseI footprinting of specific DnaA-oriV complexes demonstrated that the first boxes to be filled are boxes 4 and 3. These results, along with the nonlinear progression of DnaA-oriV complex formation observed in the gel mobility shift assay, indicate that DnaA binds cooperatively to the boxes at the RK2 origin. This is in contrast to what has been observed at oriC, where several approaches failed to detect cooperative DnaA binding (37, 38).

DnaA was shown to bind preferentially to box R4, but the binding preference of DnaA to the other sites was not altered when box R4 was mutated (38).

The DNase I footprinting experiments performed here do not reveal which box in oriV, 4 or 3, actually binds DnaA first. We cannot exclude the possibility that DnaA binds initially to either box 4 or box 3 without a preference. This could account for the observed footprint by DnaA in complex I (Fig. 5, middle lane), which indicates a mixture of both boxes. However, we propose that because a mutation in box 4 abolishes replication activity and the stable binding of DnaA to the other boxes, DnaA probably binds first to box 4 and then cooperatively, possibly via a conformational change in the DNA, to box 3. What is clear is that a fully functional origin requires the formation of a complex II that contains DnaA protein bound to both boxes 4 and 3.

Further analysis of DnaA box 4 argues that there are specific requirements for both its sequence and position for oriV functionality. If box 4 is replaced with the sequence of box 1 (mutant oriV 4-1) the origin is inactive (Table II) and DnaA binding is greatly reduced as judged by gel retardation analysis (Fig. 6A). The sequence of box 1 has less homology to the DnaA box consensus than box 4, and in footprinting experiments more DnaA protein is required to see protection in box 1 than in box 4 (Fig. 3 and data not shown). Thus, the data suggest that box 4 has a higher affinity for DnaA or forms a more stable complex with DnaA than the other DnaA boxes and, in this capacity, acts to recruit DnaA protein to oriV. However, the critical role of box 4 is also dependent upon its relative position within the origin. In mutant oriV 3-4, the box 4 sequence replaces DnaA...
It has been shown for oriC that the preference for DnaA box R4 depended on it being in the correct orientation (38). We found for oriV that if box 4 was inverted (mutant oriV 4-Flip), DnaA protein did not form stable complexes with any of the DnaA boxes unless adjoining sequences were also inverted (mutant oriV 4-FlipX) (Fig. 6). Thus, with the oriV 4-FlipX mutant, DnaA did bind to all four boxes, but this binding no longer exhibited cooperativity. Possibly, in this case the binding of DnaA to the high affinity but inverted box 4 increased the local concentration of DnaA protein and, thus, enhanced the probability of other DnaA-DnaA box contacts. Interestingly, the oriV 4-FlipX mutant was not active in vivo. Presumably when DnaA is bound to the inverted box 4, it is now on the opposite face of the DNA helix with respect to box 3, thus disrupting the structure and/or critical protein contacts required for cooperative binding. These results demonstrate that the correct orientation of box 4 is crucial for directing not only the cooperative binding of DnaA but the formation of a nucleoprotein structure that is required for a functional initiation complex. More than likely, this specific structure is critical for the loading of DnaB helicase. We have previously demonstrated that when all four DnaA boxes are on the opposite side of the DNA helix with respect to the rest of the origin, DnaB helicase activity at oriV is reduced even though the origin is fully open (30). Unlike replication initiation at oriC, where origin opening and helicase loading occur as the result of a single initiator protein (DnaA), which is responsible for both steps, initiation at a plasmid origin usually requires two initiator proteins (DnaA and a plasmid-encoded replication initiator protein) and, therefore, may involve a fundamentally different nucleoprotein structure for facilitating each step of replication initiation.

Most plasmid replication origins contain more than one DnaA box or cluster of DnaA boxes. In the case of the narrow host range plasmids P1 (39) and R6K (40), one box is sufficient for a functional origin. Evidence for cooperative DnaA binding to the DnaA boxes at the origin of pSC101 has been demonstrated to occur at a distance (DNA looping) with the combined action of the RepA initiator protein and IHF (41). However, for RK2, the cooperative binding exhibited by the DnaA protein occurs at boxes that are separated by only three to five base pairs and is not dependent on the binding of additional proteins, including the initiator protein, TrfA. Recently it was shown for plasmid R6K that the DnaA protein physically interacts with the R6K plasmid specific initiator protein and that this interaction is required for origin opening (22). There is no evidence for an interaction between DnaA and the RK2 initiator protein, TrfA. Preliminary results using ELISA to detect protein-protein interactions suggest that if an interaction does occur, it is a relatively weak interaction.2 An interaction between the DnaA and TrfA proteins clearly is not required for binding of the two proteins to their respective sites, DnaA boxes or iteron sequences (11), or for the cooperative DnaA-oriV binding interaction presented here. It is possible that these differences in molecular interactions at the replication origin observed between the narrow host range plasmids, P1 and R6K, and RK2 at least partially account for their different host range.

It was surprising to us that DnaA boxes 1 and 2 can be deleted without affecting the efficiency of transformation of E. coli (Table I) or in vitro replication activity (Fig. 2), especially

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2 K. S. Doran and D. R. Helinski, unpublished observations.
because the four boxes potentially can form a cruciform structure (11). However, in view of the broad host range replicating activity of RK2, the experiments performed here with E. coli do not rule out the possibility that a cruciform structure and/or all four DnaA boxes are required in bacterial hosts other than E. coli for optimal replication. Prior to the discovery of all four DnaA boxes, mutagenesis studies of the RK2 origin region demonstrated a differential requirement of DnaA boxes between E. coli and P. aeruginosa (42–44). Thus, it is of interest to test the DnaA box mutants constructed in this study in other bacterial hosts, as different DnaA and other host replication proteins may act differently at the RK2 origin.

The finding that boxes 4 and 3 are sufficient for oriV activity indicates that the DnaA-oriV complex is a much more compact structure than is found for oriC, where the nucleoprotein complex extends over approximately 200 base pairs. Do the structural differences reflect the differences in DnaA function for the formation of a prepriming complex. This may represent a novel type of DnaA cooperative binding at oriC, where the nucleoprotein complex forms an open complex. Our results have further demonstrated through mutational analysis and protein binding experiments that one box, box 4, effectively acts as the organizer for DnaA cooperative binding at oriV. This binding then initiates a nucleoprotein structure that is functional for the formation of a prepriming complex. This may represent a novel type of DnaA binding at a prokaryotic origin. It will be of interest to determine whether this type of DnaA interaction is found for other plasmid systems and whether it is a critical factor for the broad host range properties of the RK2 plasmid.

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