DnaA Box Sequences as the Site for Helicase Delivery during Plasmid RK2 Replication Initiation in Escherichia coli

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DnaA box sequences are a common motif present within the replication origin region of a diverse group of bacteria and prokaryotic extrachromosomal genetic elements. Although the origin opening caused by binding of the host DnaA protein has been shown to be critical for the loading of the DnaB helicase, to date there has been no direct evidence presented for the formation of the DnaB complex at the DnaA box site. For these studies, we used the replication origin of plasmid RK2 (oriV), containing a cluster of four DnaA boxes that bind DnaA proteins isolated from different bacterial species (Caspi, R., Helinski, D. R., Pacek, M., and Konieczny, I. (2000) J. Biol. Chem. 275, 18454–18461). Size exclusion chromatography, surface plasmon resonance, and electron microscopy experiments demonstrated that the DnaB helicase is delivered to the DnaA box region, which is localized ~200 base pairs upstream from the region of origin opening and a potential site for helicase entry. The DnaABC complex was formed on both double-stranded superhelical and linear RK2 templates. A strict DnaA box sequence requirement for stable formation of that nucleoprotein structure was confirmed. In addition, our experiments provide evidence for interaction between the plasmid initiation protein TrfA and the DnaABC prepriming complex, formed at DnaA box region. This interaction is facilitated via direct contact between TrfA and DnaB proteins.

The initiation of DNA replication of prokaryotic and eukaryotic replicons requires delivering and loading of a helicase at the replication origin (2, 3). In addition to unwinding double-stranded DNA, the helicase has to be positioned on the separated DNA strands. Although there is considerable information of helicase delivery to the origin and loading on ssDNA, the molecular mechanisms of these events are not fully understood.

DnaA box sequences are present within replication origins of many different bacterial species (4–9) and extrachromosomal prokaryotic genetic elements (10–20). Originally, the DnaA binding site consensus sequences was determined for the Escherichia coli DnaA protein (21). It has been shown that the consequence of DnaA protein binding to a DnaA box sequence can result in various types of functions, depending on the bacterial system. Binding of DnaA protein to the DnaA box consensus sequence, localized within a promoter region, can stimulate or inhibit transcriptional activity, hence, the regulation of gene expression and/or stimulation of the initiation of DNA replication (22). However, in most cases, the binding of DnaA protein to specific DnaA boxes, localized within bacterial replication origins, is essential for the initiation of DNA replication. The binding of DnaA to DnaA boxes at the E. coli chromosomal replication origin results in destabilization of duplex DNA at the A+T-rich region and an open complex formation (4, 23–26). The DnaB helicase (27), in the form of a DnaB/DnaC complex, is specifically loaded at the DnaA-induced open region of oriC (28–30). Physical interactions between the DnaA protein and the helicase are required for loading (4, 23, 31); however, the actual site of the initial formation of a DnaB complex within the replication origin is not known.

In the case of bacterial plasmids, the origin region of these extrachromosomal elements contains characteristic structural elements, i.e. multiple repeat sequences (iterons), DnaA box sequences, and an A+T-rich region (32) (Fig. 1). The one or more clusters of direct repeats are specific multiple binding sites for the plasmid replication initiation protein (Rep) (32). The DnaA box sequences are also often organized within clusters, and they act as binding sites for the host DnaA initiation protein (32). The positions and orientations of these sequences are equally critical for plasmid DNA replication (33–37). Unlike in the case for the E. coli replication, the origin binding of the host DnaA protein itself does not result in plasmid origin opening at the A+T-rich region (13, 38, 39). However, DnaA protein binding to DnaA box sequences does enhance unwinding of the origin region, initiated by the binding of the plasmid Rep protein to the iterons. The generated ssDNA lies within the 13-mer sequence at the A+T-rich region, and it is a potential site for bacterial helicase delivering and loading. Previous studies have demonstrated that interactions between the DnaA protein and the host DnaB helicase are critical for the recruitment of the helicase to the origin region (31, 43). In addition, the observed interactions of the replication initiation protein of plasmids R6K and pSC101 with the E. coli DnaB helicase are also likely to play a role in the positioning of the DnaB helicase at the origin of the narrow host range plasmid (40).

The broad host range plasmid RK2 is able to replicate and maintain itself in a wide range of Gram-negative bacteria (41). The 393-base pair (bp) minimal origin, oriV, from the plasmid RK2, contains five 17-bp direct repeats (42), four DnaA boxes, and an A+T-rich repeat sequences (13-mers), which are the initial sites of helix destabilization (13) (Fig. 1). The DnaA binding sites localized within the RK2 replication origin consist of a cluster of two inverted pairs of DnaA box consensus sequences, separated by only a few bp (13). Footprinting and gel retarda-
tion experiments have shown that DnaA box 4 directs cooperative binding of the E. coli DnaA protein to the other boxes within the cluster (37). The nucleotide sequence and orientation of the essential DnaA box 4 are critical for stabilization of the nucleoprotein complex between E. coli DnaA protein and oriV in vitro (37). Binding of the DnaA protein at oriV stabilizes RK2 open complex formation, but it cannot on its own form an open complex (13). It has been demonstrated that the plasmid RK2-specific replication initiation protein, TrfA, binds to the iteron sequences and, in the presence of ATP and HU and/or DnaA, produces an opening of a set of 13-mers within the A+T-rich region (13). The DnaA protein also has an indispensable role in the delivery and loading of DnaB helicase at the RK2 origin (43). As for oriC (31), a specific interaction between the DnaA and DnaB proteins has been shown; however, both DnaA and TrfA proteins are required for helicase activity at RK2 DNA (43).

In this paper, we investigated the site of DnaB helicase interactions with the RK2 region of the replication origin by various techniques, including size exclusion chromatography, SPR, and EM. These results demonstrate that the DnaA box cluster, located within oriV, by itself serves as a site for the formation of the DnaABC complex. This formation does not require a superhelical DNA structure or open complex formation. This DnaA box cluster is localized ~200 bp upstream from the region of origin opening. Furthermore, the DnaABC complex is capable of interacting with the plasmid initiation protein TrfA, and this interaction is presumably initiated by the DnaB protein.

**MATERIALS AND METHODS**

**Proteins and Reagents**—Purified proteins that were used for various assays. DnaA (13), DnaC (44), and TrfA (35-kDa version of Trf8 protein) (42) proteins as well as histidine-tagged versions of DnaA (1), TrfA (His-tagged 33-kDa version of TrfA protein) (45), and the mutant TrfA254D/267L (His-tagged version of TrfA mutant protein, which contains two plasmid copy-up mutations and is fully functional in vivo and in vitro) (45), were purified as described previously. The construction and purification of the C-terminal His-tagged E. coli DnaB protein, which have been found active both in vitro and in vivo, will be described elsewhere. Polyclonal anti-DnaB antibody was provided by Dr. Jaroslaw Marszalek (University of Gdansk, Gdansk, Poland). Anti-TrfA antibody was as described previously (43). pTJS42 is a mini-replicon of the plasmid RK2 and contains the five iteron minimal oriV (46). Commercially available proteins and chemicals used in this study included: HU and SSB from Enzyco, Inc.; bovine serum albumin (fraction V), creatine phosphate, creatine kinase, Protein A (10 nm colloidal gold-labeled), reagents from Sigma; Paracoccus, Sepharose CL-4B from Amersham Pharmacia Biotech; nickel-nitrilotriacetic acid-agarose from Qiagen; and goat anti-rabbit IgG from Bio-Rad. Streptavidin matrix-coated Sensor Chip SA. (Pharmacia Biocore AB) was used for the SPR analysis.

**Isolation of the RK2 Prepriming Protein-DNA Complex by Gel Filtration**—Column gel filtration utilized to isolate the RK2 prepriming complex was performed as described previously (43). The reaction mixture (total volume, 100 μl; 40 mM Hepes/KOH, pH 8.0, 40 mM potassium glutamate, 10 mM magnesium acetate, 10 μM dithiothreitol, and 2 mM ATP), containing histidine-tagged versions of DnaA and DnaB proteins, DnaC, and pTJS42, which carries the RK2 minimal origin of replication, was incubated for 20 min at 32 °C. After incubation, proteins were removed by column filtration on Sepharose CL 4B. The void fractions were pooled together, and after cutting 1 cm across the column filtered sample and polyclonal antibodies against DnaB. Protein A-coated 10 nm gold particles were added cross-linked and diluted. Samples were adsorbed on mica sheets, stained with uranyl acetate, and platinum-carbon-coated. Binding of the DnaA protein to the oriV fragment was performed as described above; however, carbon staining instead of immunodetection was used. Samples were examined at 60 kV in a Philips CM100 electron microscope. Micrographs were digitalized for the position of the DNA-protein complex.

**Surface Plasmon Resonance (SPR)**—For the standard SPR analysis, a 64-μl 5'-biotinylated oligonucleotide (5'-AACGCCTGCATTATCCGCG-AAGTTTCCACATGATGATGACAAGCTGGGGATAAGTGCCCTGC-3') and non-biotinylated complementary oligonucleotide were annealed and immobilized on to the Sensor Chip SA surface as described previously (1). SPR analysis was performed on a BiaCore 1000, by injecting 10 μl of protein solutions in binding buffer (40 mM HEPES/KOH, pH 8.0, 40 mM potassium glutamate, 80 μg/ml BSA, 4% sucrose, 4 mM dithiothreitol, 10 mM magnesium acetate, and 2 mM ATP) for 2 min at room temperature. Protein DnaA, DnaC, and histidine-tagged proteins DnaB and TrfA254D/267L injections were followed by control DnaC injection (10 μl of HEPES buffer, pH 7.4, 150 mM NaCl, 10 μg/ml HU, 0.005% P20) for 3 min., and after subtraction of the background response signal (obtained in a control experiment), the results were plotted as sensograms. Following the completion of each protein binding analysis, the surface of the chip was regenrated by injection of 10 μl of 0.05% SDS, which releases all bound protein without affecting the binding capacity of the immobilized DNA. To analyse DnaA box requirement for helicase complex formation, the oligonucleotides A4 (AACGCCTGATTTCACGAGTTCGCCCTGTCG) and A1–3 (AACGCCTGATCGAGGATGCTAGGATTCTGGATGCTGGTGGTG) were used for detection TrfA-DnaB interactions. A histidine-tagged version of DnaB helicase (1000 ng) was incubated with nickel-nitrilotriacetic acid beads in buffer (40 mM Hepes, pH 8.0, 75 mM KCl, 10 mM MgCl2, 2 mM ATP) for 5 min at room temperature. After incubation, followed by three washes in incubation buffer, non-histidine-tagged TrfA protein (1 μg) was added to the reaction containing resin bound DnaB helicase. The protein complexes were formed for 60 min at room temperature, and then unbound protein was removed by three washes in incubation buffer, containing 20 μM imidazole. DnaB-TrfA complexes were analysed by SDS-gel electrophoresis, followed by Western blot analysis with anti-TrfA polyclonal antibodies.

**Enzyme-linked Immunosorbent Assay (ELISA)**—A modified ELISA assay with glutaraldehyde cross-linking reagent (31) and polyclonal anti-DnaB antibodies was used for detection TrfA-DnaB interactions. DnaC protein, BSA, and histidine-tagged versions of DnaA, DnaB, and TrfA proteins were used.

**RESULTS**

**Protein Requirements for the Formation of the DnaB Helicase Complex at oriV**—Requirements for DnaB helicase activity during replication initiation at the plasmid RK2 origin has characterized previously (43). It has been shown that a specific interaction between DnaB and TrfA is critical for the helicase activity (43). Moreover, helicase activation strictly depends on TrfA binding at the RK2 origin and formation of an open complex (43). In this work, we analyzed the minimal requirements for formation of the helicase prepriming complex at oriV by performing size exclusion chromatography with a Sepharose CL-4B column. Superoiled plasmid DNA (pTJS42), containing the minimal RK2 origin (Fig. 1), was incubated with purified proteins and then analyzed for the presence of bound DnaB helicase. The results show clearly that...
Replication proceeds toward the G + C-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region. The TrfA protein binds to iterons located between the DnaA boxes and the A + T-rich region.

both DnaA and DnaC proteins are required for the complexing of the DnaB helicase with the oriV template DNA (Fig. 2A). The DnaB helicase did not form a stable complex with the template DNA if the incubations were carried out without the DnaA or DnaC proteins (Fig. 2, B and C). Furthermore, addition of formaldehyde in order to stabilize DnaB-DNA interaction did not result with isolation of DnaB complexes with oriV when DnaA and DnaC were not present in the reaction mixture (data not shown).

Helicase Is Delivered to the DnaA Box Region at oriV—Previous studies demonstrated that direct interactions between E. coli DnaA and DnaB proteins are essential for the plasmid RK2 replication activity (43). In addition, it has been shown that the position of the DnaA boxes at oriV is critical for origin activity (35). If the correct helical phasing is disrupted by the insertion of less or greater than one helical turn, DnaB cannot be efficiently loaded at the open region and replication activity is substantially reduced (35). Size exclusion experiments, described in Fig. 2, show that both E. coli DnaA and DnaC proteins are required to form the helicase complex at RK2 DNA. To investigate possibility that the DnaA box region is the site for DnaBC complex delivery to the RK2 replication origin, we performed EM and SPR studies. After incubation of DnaA, DnaB, and DnaC with the supercoiled oriV template, proteins and DNA were cross-linked with glutaraldehyde, digested with EcoRI and HindIII restriction enzymes, and prepared for EM analysis. Utilization of antibodies against DnaB and protein A-colloidal gold particles allowed us to determine the position of the DnaB helicase on the plasmid DNA fragment. The results from this procedure demonstrated that the DnaB helicase particles were located within the minimal RK2 replication origin. Moreover, statistical analysis indicates that the majority of DnaB complexes were detected at a site that corresponds to the position of the DnaA box region at oriV (Fig. 3). We further examined the requirements and real time kinetics of helicase complex formation at the oriV DnaA box region by utilizing the SPR technique. A 64-bp linear double-stranded DNA fragment, containing four DnaA box sequences, was linked to the sensor chip. The DnaA, DnaB, and DnaC proteins were injected over the sensor matrix and tested for binding. Sensograms, which indicate change of mass at the attached DNA fragment, clearly show signals from DnaA protein binding to the DnaA box fragment (Fig. 4). In addition, a DnaBC complex was detected at the linear DnaA box DNA, following the formation of a complex between the DnaA protein and DNA (Fig. 4A). The association of DnaB with the DnaA nucleoprotein complex at the DnaA box fragment requires DnaC (Fig. 4B).

Specific DnaA Boxes Are Required for the Formation of the Helicase Complex at oriV—We have recently shown that specific DnaA box sequences at oriV are critical for the cooperative binding of the E. coli DnaA protein (37). In this work, DnaA box mutants were constructed and tested for activity in vivo and in vitro. The scrambled DnaA box sequence GATACTGTC, which was shown to be defective in binding to the E. coli DnaA protein (37, 47), was introduced into the A4 and A1–3 positions of the 64-bp fragment of oriV, respectively. The mutant DNA fragments were immobilized on to the streptavidin matrix-coated sensor chip. The two altered DnaA box fragments, designated A4 (DnaA box 4 scrambled and wild type sequences of DnaA boxes 1, 2, and 3) and A1–3 (DnaA boxes 1, 2, and 3 scrambled...
and wild type sequence of DnaA box 4), were analyzed for binding of the DnaB/DnaC complex in the presence of DnaA protein. Only a slight amount of the helicase complex at DnaA box fragment A4 was detected (Fig. 5). The magnitude of the response signal was not substantially increased following the addition of either the DnaA protein or the DnaBC complex. Similar results were obtained with the A1–3 mutant DNA fragment, attached to the sensor chip matrix (Fig. 5). Thus, a single wild-type A4 box structure was not sufficient for the formation of a stable DnaA/oriV complex or a stable complex of oriV and DnaB helicase. In contrast to these results, the 64-bp oriV fragment, containing the wild-type DnaA box sequence, reproducibly formed a stable ABC complex. These results are consistent with previously published observations, which showed that the DnaA box A4 is critical for cooperative binding and stable association of E. coli DnaA protein with oriV (37).

TrfA Protein Interacts with the ABC Prepriming Complex at the DnaA Box Region—A specific DnaA-DnaB interaction is required for the formation of a helicase complex at oriV; however, both DnaA and TrfA proteins are necessary for DnaB helicase loading and activation (43). The TrfA protein binds to iteron sequences at the origin, and these interactions result in the unwinding of the A+T-rich oriV region (13). The mechanisms for helicase loading on to the ssDNA in the open region and helicase activation for DNA helix unwinding are not yet known. In this paper, we have demonstrated the formation of a specific DnaABC complex at the DnaA boxes of oriV linear and supercoiled DNA templates. We proceeded further to test whether the TrfA protein could interact with such a DnaABC complex, formed at the DnaA box region. Once again, a double-stranded linear fragment, containing wild-type sequences of the four DnaA boxes, was attached to the sensor chip. The DnaABC complex was formed by injection of DnaA, followed by injection of DnaBC and, finally, injection of the TrfA protein. A strong response signal was observed after addition of the TrfA protein (Fig. 6A), despite the absence of DNA binding sites for the TrfA protein, indicating that the observed response is due to TrfA interactions with the DnaABC complex at the DnaA boxes. In a control experiment, the DnaBC complex was not injected prior to the injection of the TrfA protein, which resulted in no detectable response signals (Fig. 6B). Interestingly, after formation of the DnaA box/DnaABC/TrfA complex, a very rapid dissociation from the sensor chip was observed (Fig. 6A).

TrfA Protein Interacts with the DnaB Helicase—SPR studies demonstrated that TrfA protein interacts with the DnaABC prepriming complex, formed at the DnaA box oriV region. We further determined whether or not these interactions are due to physical contacts between the TrfA protein and bacterial helicase. Affinity chromatography and ELISA studies verified that TrfA interacts with DnaB (Fig. 7, A and B). Following cross-linking with glutaraldehyde, a DnaB-TrfA complex was
For details, see “Materials and Methods.” Assay plate, and then increasing amounts of DnaB protein was added. Tagged versions of DnaA or TrfA proteins were bound to plastic wells of immunodetection by ELISA, and the magnitude of the observed signal was very similar to that obtained for the DnaB-DnaA complex. In control experiments, DnaB protein formed a strong complex with DnaC, but it did not interact with BSA (Fig. 7B).

**Fig. 6.** TrfA protein interacts with DnaABC, the prepriming complex formed on the DnaA boxes region. The experiment was carried out as described under “Materials and Methods” and in the legend to Fig. 5, except for the addition of histidine-tagged RK2 replication initiation protein, TrxA254D/267L. The sequences of injections were as follows. A. TrfA protein was injected after the formation of the ABC complex; B. TrfA protein was injected after complex formation between the DnaA protein and the four DnaA box sequences, present on the 64-bp DNA fragment.

**Fig. 7.** TrfA protein interacts with DnaB helicase. Immunochromatography and ELISA tests were used for detection DnaB-TrfA interactions. A. Histidine-tagged version of DnaB helicase was bound to nickel-nitrilotriacetic acid beads, and then TrfA protein was added to the reaction. DnaB-TrfA complexes were analyzed by SDS-gel electrophoresis, followed by Western blot with anti-TrfA polyclonal antibodies. Arrow indicates position of TrfA protein. B. DnaC, BSA, and histidine-tagged versions of DnaA or TrfA proteins were bound to plastic wells of assay plate, and then increasing amounts of DnaB protein was added. For details, see “Materials and Methods.”

Our results demonstrate for the first time the primary formation of an E. coli DnaB helicase complex at the DnaA box region of a plasmid replication origin. SPR experiments showed that this structure depends on the binding of DnaA protein to the DnaA boxes and the DnaB helicase in the form of a complex with its accessory protein DnaC (Figs. 4 and 5). In general, these results are consistent with the original studies on DNA replication initiation at the E. coli replication origin oriC (2, 48). Similar to the bacterial system, a Dnabc prepriming complex is formed at the plasmid RK2 origin (Fig. 2) (43). In addition, specific DnaA-DnaB interactions along with the presence of DnaA box sequences are critical for the formation of prepriming complexes on both oriC (31) and oriV (43). Despite these similarities, bacterial and plasmid RK2 helicase delivery strategies differ in some respects. After the Dnabc complex is established at oriC, the binding of DnaA protein itself in the presence of ATP results in unwinding of the origin at the A+T-rich region, which then allows helicase loading on to the ssDNA (49). Similarly, the E. coli DnaB helicase is positioned on ssDNA in the DNA/RNA heteroduplex region of plasmid pBR322 after RNA polymerase moves through the replication origin destabilizing helical structure (50). In contrast to these observations, helicase is delivered to the plasmid RK2 replication origin without TrfA-dependent helix destabilization within the A+T-rich region (Fig. 4) (13, 43). Moreover, our experiments clearly show that the DnaB helicase forms a complex of ~200 bp, upstream from the 13-mer structures, that is the site of ssDNA formation (Fig. 3). In addition, our work shows that the stable association of helicase with oriV depends on the presence of the DnaA boxes (Fig. 5). Mutations in these sequences, which inactivate oriV, also prevent DnaB complex formation. Interestingly, helicase formation within the DnaA box region requires the presence of DnaC. It has been shown that DnaC protein possesses a cryptic ssDNA binding activity that is probably involved in DnaB helicase loading on ssDNA (51). Our experiments demonstrate that DnaC is required for the formation of a helicase complex on dsDNA. Probably, the helicase loading is a two-step reaction. The first step is Dnabc complex formation on dsDNA. In the second step, a cryptic ssDNA binding activity of DnaC ATPase is involved in helicase loading on to ssDNA.

Our results are consistent with previously published studies on mutant DnaA boxes (36, 37). DnaA box 4 is critical for cooperative binding of DnaA protein to all four DnaA boxes, located within the RK2 origin. SPR analysis also demonstrated that these mutations in DnaA box 4 destabilized Dnabc complex formation at oriV (Fig. 5). The finding that DnaB complex formation occurs within a 64-bp segment of a functional RK2 minimal origin of replication, as well as the failure of DnaB complex formation within this mutant forms of this segment, together indicate that the DnaA box region within the RK2 replication origin is the site of Dnabc complex formation prior to loading on the ssDNA in the A+T-rich region.

It should also be noted that the type of DnaA box required for plasmid RK2 replication is host-dependent (36, 52–54). Plasmids with mutations in DnaA box 4 or a deletion of all four DnaA boxes are not stably maintained in E. coli and P. putida; however, these mutants displayed some replication activity in P. aeruginosa (36). Interestingly, the effects of deleting all four boxes can be overcome in vitro by increasing the concentrations of the DnaA protein. Possibly, low affinity binding of DnaA protein elsewhere in oriV would explain DnaA-dependent replication initiation in the absence of the four DnaA boxes (36). At very high DnaA protein levels, diffuse protection of the 13-mer sequences and the surrounding region was observed by the
Dnase I footprinting technique (13). For oriC, DnaA has been shown to bind to the 13-mer by the methods of filter binding, oligonucleotide displacement (55), and, more recently, SPR studies. Although the deletion of the entire oriV DnaA box region renders the origin non-functional in vivo in E. coli (36), high concentration of DnaA protein in vitro possibly could result in the association of the E. coli DnaABC with the A+T rich region of the RK2 origin. Another possible explanation for the activity DnaA box deletion mutants of oriV in vitro at high DnaA concentrations and in vivo in P. aeruginosa may be that these activities are brought about or enhanced by DnaA-TrfA and/or DnaB-TrfA interactions. Our SPR experiments demonstrated interactions between the TrfA protein and the DnaABC complex, formed at the DnaA box sequences (Fig. 6). Specific interactions between the TrfA protein and DnaB helicase have been shown by the ELISA technique and affinity chromatography. Further investigations are in progress to examine the specificity of TrfA interactions with DnaB by utilizing DnaB proteins isolated from different bacterial species. Mutational analysis and binding assays have shown that the plasmid pSC101 (40) and R6K (56) encoded Rep proteins specifically interact with E. coli DnaB helicase. These interactions are critical for pSC101 replication. In addition, it has been shown that RepA-DnaB interaction is required for DnaB complex formation at the pSC101 origin. Our results demonstrate that, although the replication initiation protein TrfA interacts with DnaB helicase (Figs. 6 and 7), this interaction is not required for DnaB helicase complex formation at the DnaA boxes. The formation of a DnaB complex at the replication origin in the absence of TrfA protein may be an important feature of RK2 replication initiation, which contributes to the broad host range replication activity of this plasmid. In this scenario, a DnaB helicase complex is formed at the RK2 origin, and it is activated by the TrfA-dependent opening of the 13-mer region and TrfA interaction with the DnaB helicase.

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