Role of TrfA and DnaA Proteins in Origin Opening during Initiation of DNA Replication of the Broad Host Range Plasmid RK2*

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The Escherichia coli protein DnaA and the plasmid RK2-encoded TrfA protein are required for initiation of replication of the broad host range plasmid RK2. The TrfA protein has been shown to bind to five 17-base pair repeat sequences, referred to as iterons, at the minimal replication origin (oriV). Using DNase I footprinting and a gel mobility shift assay, purified DnaA protein was found to bind to four DnaA consensus binding sequences immediately upstream of the five iterons at the RK2 origin of replication. Binding of the TrfA protein to the iterons results in localized strand opening within the A+T-rich region of the replication origin as determined by reactivity of the top and bottom strands to potassium permanganate (KMnO₄). The presence of either the E. coli DnaA or HU protein is required for the TrfA-mediated strand opening. Although the DnaA protein itself did not produce an RK2 open complex, it did enhance and/or stabilize the TrfA-induced strand opening.

Many plasmids found in Gram-negative bacteria contain one or more clusters of direct repeats (iterons) within the replication origin (ori), one or more essential binding sites for the DnaA protein (DnaA box), and an A+T-rich region (1). For these plasmids, replication initiation involves binding of a plasmid-encoded replication initiation protein to the iterons which, in the presence of bound DnaA protein, results in the formation of a specific nucleoprotein structure and structural changes within the origin sequence (1–3). Initiation of replication at the origin of Escherichia coli (oriC) has been studied extensively, and models have been proposed which include the formation of several distinct nucleoprotein complexes (4). The DnaA protein binds to DnaA boxes within the E. coli origin resulting in initial complex formation (5, 6). Detailed studies have determined that a specific DNA consensus sequence is required for DnaA protein binding (5–9). In the presence of HU protein and ATP, a compact, ellipsoid structure is formed which is partially unwound (open complex) at the E. coli origin (10–12). This opening, which occurs at the A+T-rich 13-mer repeat sequences in the left part of oriC, has been demonstrated in vitro with P1 nuclease and both in vivo and in vitro by reactivity to potassium permanganate (KMnO₄) (10, 13, 14). The role of the DnaA protein in plasmid P1 replication has also been investigated (15, 16). In this system, the DnaA protein alone induces open complex formation. The P1-encoded replication initiation protein, RepP, cannot form an open complex by itself, but it enhances DnaA protein-induced reactivity of the P1 origin to KMnO₄ (16). In the case of the initiation of bacteriophage λ DNA replication, the initiation protein (O) induces helix destabilization at the λ origin (17). More recently it has been shown that the F plasmid-encoded RepE protein, in the presence of the HU protein, produces localized melting within an F replication origin, and this open complex is extended by DnaA (18).

The mechanism of DNA replication initiation of the broad host range plasmid RK2 is largely unknown. RK2 is 60 kilobases and replicates at a copy number of 5–8/chromosome in E. coli (19). This antibiotic resistance plasmid is of particular interest because of its ability to replicate and to be stably maintained in a wide range of Gram-negative bacteria (19). DNA replication of RK2 requires a single plasmid origin (oriV) and a plasmid-encoded initiation protein (TrfA) (20–24) which binds as a monomer to the 17-bp direct repeats in oriV (25, 26). As in the case of plasmids R1, P1, pSC101, F, and R6K (3, 16, 27–29), it has been shown that the DnaA protein binds to DnaA consensus sequences within the RK2 origin (30, 31).

In the present work, we examined in vitro binding of the E. coli DnaA protein to the RK2 origin of replication and the role of the TrfA and DnaA proteins in forming an open complex. Four DnaA binding sites, arranged in two pairs in an inverted orientation with respect to each other, were found within the RK2 minimal origin. DNase I protection assays showed that the binding pattern of the DnaA and TrfA proteins to the origin region is essentially the summation of binding by either protein alone. Using an in vitro KMnO₄ reactivity assay, we have found that the RK2 initiation protein, TrfA, in the presence of HU can produce localized opening at the A+T-rich region of oriV. The DnaA protein stimulates and/or stabilizes this RK2 open complex formation, but cannot, on its own, form an open complex.

MATERIALS AND METHODS

Bacterial Strains, Proteins, and Reagents—Highly purified proteins were used for the various assays. The mutant His6 TrfA254D/267L protein was purified as described previously (32). This protein, containing two plasmid copy up mutations, initiates replication with kinetics similar to those of the wild-type protein but is present largely in the form of active monomers. The E. coli DnaA protein used was either a generous gift from Dr. D. Bramhill or was purified following published procedures (33) using the overproducer strain WM1202 pDNA107A, kindly provided by Dr. W. Messer. Basic cloning methods were performed according to Sambrook et al. (34). pTJS42 and pSP6 are mini-

1 The abbreviations used are: KMnO₄, potassium permanganate; bp, base pair(s); ATPγS, adenosine 5'-O-(thiotriphosphate).
2 A. Toukdarian, unpublished observations.
replicons of plasmid RK2 and contain the five-iteron minimal orfV (25, 35). Commercially available proteins and chemicals were used. HU and SSB were purchased from Enzyco, Inc. Bovine serum albumin (fraction V), creatine phosphate, creatine kinase, nNTPs, and KMoO₄ were from Sigma. Restriction endonucleases, T₄ polynucleotide kinase, the Kle- nove complementation of E. coli DNA polymerase, and DNase I were from New England Biolabs, Life Technologies, Inc., Boehringer Mannheim, Strat- agene, or Promega and were used according to the manufacturer’s recommendations. The Ultra Clean DNA purification kit was from MoBio Laboratories. AmpliTaq polymerase was from Perkin-Elmer. 30-mer pBR322 primer was from New England Biolabs. A 25-mer orfV1 primer (5'-GAATTCTACTCCGACATTGGTGAAGGT-3’) was from OPERON.

Preparation of DNA Probes—Plasmid DNA, pSP6, used as a source of DNA probes, was purified by two rounds of ethidium bromide-CsCl gradient centrifugation. The procedure for labeling the 400-bp probe containing the linear RK2 origin of replication has been described previously (25). Oligonucleotide primers used for primer extension were end labeled (30 pmol of DNA) with [γ-³²P]ATP (6,000 Ci/mmol, NEN Life Science Products) in the presence of seven units of T₄ polynucle- otide kinase. The labeled oligonucleotide primers were separated from unincorporated ATP using the NuTrap push column (Stratagene) or by gel filtration through a 1-ml Sephadex G-50 column equilibrated with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) in the presence of 50 mM NaCl.

Gel Mobility Shift and DNase I Footprints—These experiments were performed as described previously (25, 36, 37) with the exception that all of the binding reactions were carried out in the presence of 5 mM ATP. The reaction volume for the gel mobility shift experiments was 20 μl. Poly(dI-dC) was present at 25 μg/ml for both the gel mobility shift and DNase I protection experiments. In all cases, His6 TrfA254D/267L and DnaA proteins were incubated together or separately with DNA at 27 °C for 20 min. The amount of DNase I used was 12.5 ng/reaction. The position of the protected area was determined by comparison with a sequencing ladder run in parallel on the same gel.

Permanganate Footprinting and Primer Extension—Permanganate footprinting was carried out as described (13) with modifications. The standard reaction mixture (25 μl), based on the RK2 in vitro replication system reconstituted with purified components, 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; 2 mM MgCl₂; 500 μM (each) CTP, GTP, and UTP; 8 mM creatine phosphate; 20 μg/ml creatine kinase; 8 ng of HU; 100 ng of DnaA; 300 ng of orfV DNA (pTJS42); and 400 ng of His6 TrfA254D/267L. After incuba-
tion for 10 min at 37 °C, KMoO₄ was added to a final concentration of 10 mM. After a 5-min incubation at 37 °C, the reactions were stopped by the addition of 3 μl of 13.4 mM β-mercaptoethanol and EDTA to a final concentration of 15 mM. Each sample was purified further by using the Ultra Clean DNA purification kit.

Primer extension reactions were carried out using a polymerase chain reaction with radiolabeled primer and AmpliTaq polymerase. Two synthetic 32P-labeled primers were used. For top strand analysis, the 30-mer pBR322 1238 primer (New England Biolabs) was used. For the bottom strand the 25-mer synthesized by Stratagene or by gel filtration through a 1-ml Sephadex G-50 column equilibrated with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) in the presence of 50 mM NaCl.

RESULTS

The DnaA Protein Binds to Four DnaA Boxes Located within the RK2 Minimal Origin—Replication of the broad host range plasmid RK2 in E. coli is dependent on the plasmid origin of replication, the plasmid-encoded initiation protein TrfA, and host replication factors. E. coli DnaA, DnaB, DnaC, G primase, DNA gyrase, polymerase III holoenzyme, and SSB proteins are required for RK2 replication in crude extracts (38, 39) and in an in vitro replication system reconstituted with purified components. The binding of the TrfA protein to the ite- rons at the minimal RK2 origin of replication has been characterized in detail (25, 26, 37, 40); however, little is known about the nature of binding of the DnaA protein to orfV. We examined this binding using both the gel mobility shift assay and DNase I footprinting. The probe used in the gel mobility experiments is a DNA restriction fragment containing the minimal RK2 origin required for replication in E. coli and for replication in vitro using either an E. coli crude extract (39) or a system reconstituted with purified components. Gel retardation analysis demonstrated the ability of the DnaA protein to bind specifically to orfV. At a low concentration of DnaA protein only two retarded bands were observed; however, four distinct DNA-protein complexes were resolved by gel electrophoresis at higher DnaA concentrations (Fig. 1). The omission of ATP had no effect on the formation of the four retarded bands (data not shown). The presence of two DnaA protein binding sites upstream of the iters within the RK2 minimal origin has been reported previously based on RK2 DNA sequence analysis (22, 41), replication activity in E. coli of RK2 mini-replicon deletion mutants (31), and in vitro immunoprecipitation experiments using restriction fragments containing orfV (30). The finding of four retarded bands in our gel mobility shift experiments led us to examine in greater detail the location and number of DnaA protein binding sites in orfV.

DNase I footprinting was carried out to localize the DnaA binding sites within the RK2 origin. Using a 32P-labeled EcoRI-XhoI orfV restriction fragment, we analyzed the extent of DnaA protection upstream of the A+T-rich site. Fig. 2 shows the protection obtained with either the DnaA and the TrfA protein alone or in combination. In this and subsequent experiments, we used a mutant protein, His6 TrfA254D/267L, which has been shown to be largely in the active monomer form (32). In all of the experiments that follow, the mutant protein is referred to as TrfA. As shown before with the wild-type TrfA protein (25, 26), the His6 TrfA254D/267L mutant protein completely protects the cluster of five iters against DNase I digestion as well as producing DNase I-hypersensitive sites (Fig. 2, A and B). The DnaA protein alone, or in the presence of TrfA, protected four distinct regions localized upstream of the iters (Fig. 2, A and B). A part of the protected area corre-
both of which correspond to the DnaA binding consensus sequence (9) (Fig. 2A; see also Fig. 6A). The same protection pattern was observed when a supercoiled DNA template containing oriV was used instead of the linear oriV fragment (data not shown). The DNase I protection pattern obtained in the presence of both proteins was essentially the summation of that obtained for either protein alone.

The presence of a DnaA box has also been reported downstream of the iterons in the A1T-rich region (30, 41). To test binding of the DnaA protein within the A1T-rich region, a 32P-labeled KpnI-BamHI restriction fragment containing oriV was used. A clearly defined region of DNase I protection with the DnaA protein alone or in the presence of TrfA was not observed within the A1T-rich region under the same conditions that showed protection of the DnaA boxes localized upstream from the iterons (Fig. 2B). However, we did observe that increasing amounts of the DnaA protein gave a slightly different DNase I pattern downstream from the iterons in the region containing the previously reported DnaA box.

Opening of the RK2 Replication Origin by the TrfA Protein—As shown above, both the host-encoded DnaA protein and the plasmid-encoded TrfA protein bind to the RK2 origin. To determine the role of these two proteins in the formation of an open complex, KMnO4 was used as a reagent to probe any structural changes at the RK2 origin as a result of binding by one or both proteins. KMnO4 reacts preferentially with pyrimidines in single-stranded DNA, modifying primarily T and to a less extent C residues. Specific modification of plasmid supercoiled DNA can be detected by termination of the primer extension reaction from a radiolabeled primer at modified residues. In our experiments, supercoiled DNA containing the RK2 minimal oriV region (plasmid pTJS42) and two specific primers, one for each of the two strands, was used. Fig. 3 shows the pattern of KMnO4 modifications of the top and bottom strands of the RK2 origin. Some of the bands visible on the autoradiogram correspond to terminations that occur during primer extension even in the absence of KMnO4 treatment. Also, certain base positions were found to be hypersensitive to oxidation even in the absence of the TrfA or DnaA protein (Fig. 3). The addition of TrfA alone or both TrfA and DnaA proteins, however, clearly induced a modification of a 46-bp region with coordinates of 500–545 in the top strand (Fig. 3). Similarly, a 13-bp region of the bottom strand with coordinates 519–531 was found to be sensitive to oxidation by KMnO4 in the presence of TrfA or both TrfA and DnaA (Fig. 3). The modified regions on the top and bottom strands contain a total of four 13-mers that correspond in sequence to the 13-mer consensus sequence within E. coli oriC (10). DnaA itself did not induce KMnO4 reactivity; however, the observed pattern of modified residues after the addition of both TrfA and DnaA proteins is different with respect to the intensity of the bands compared with incubation with TrfA alone. When both proteins are pres-
ent, the position and number of bands are the same as with TrfA alone; however, the intensity of certain individual bands is greater (Fig. 3).

**DnaA or HU Protein Enhances and/or Stabilizes oriV Opening**—The HU and IHF proteins have been shown to stimulate oriC replication in vivo and in vitro (43, 44). Using sensitivity to P1 nuclease activity as a probe, it has been shown that HU or IHF is required for oriC opening mediated by the DnaA protein (12). For the experiments described above, HU was present in the standard reaction mix. To test if RK2 oriV open complex formation actually requires the presence of HU protein, we examined TrfA oriV opening activity in the presence and absence of HU protein. The results showed that TrfA mediated oriV opening is strictly dependent on HU in the absence of the DnaA protein. In contrast, HU is dispensable when both TrfA and DnaA proteins are present. Also, at any HU concentration tested, the DnaA protein alone could not open the RK2 origin (Fig. 4). Interestingly, even at high concentrations of HU the TrfA-induced KMnO₄ modification pattern was not as intense in the downstream portion of the open region compared with the presence of both the TrfA and DnaA proteins in the reaction mixture (Fig. 4). This observation indicates that although both the DnaA and the HU proteins can stimulate and/or stabilize TrfA-mediated origin melting, the two proteins show differences in their ability to enhance the formation of the open complex.

**Requirement of ATP for Open Complex Formation**—Duplex opening by DnaA protein at oriC is ATP-dependent (10, 12). In contrast, during the initiation of bacteriophage λ DNA replication, the λO initiation protein can generate a local helix destabilization in the absence of nucleotides (17). We tested the nucleotide requirement for the opening of the RK2 origin. Reactions were performed in the presence of TrfA and HU or TrfA, DnaA, and HU. A strict ATP dependence was not observed for KMnO₄ reactivity. However, the extent of the open region was greater in the presence of ATP. This effect is not the result of ATP hydrolysis because the addition of ATPγS gave a pattern similar to that observed for ATP. Unlike the duplex opening experiments described above (Figs. 3 and 4) where GTP, CTP, and UTP are present in these reactions, these results obtained in the absence of these ribonucleotides (Fig. 5) also indicate that ribonucleotides other than ATP are not required for the opening at the RK2 origin.

**DISCUSSION**

**Binding of the DnaA Protein at the RK2 Minimal Origin**—RK2 is a broad host range plasmid that requires host initiation factors for DNA replication including the DnaA protein which is indispensable, in vivo and in vitro (30, 38, 39). Our results show the specific binding of E. coli DnaA protein to four DnaA
The sequences of the two newly identified, as well as the two previously described, DnaA binding sites match well (a single mismatch in each case) to the DnaA binding relaxed consensus sequence proposed by Schaefer and Messer (9) (Fig. 6A). Binding of the DnaA protein to oriV at the A+T-rich region has been reported previously (30); however, in the present study we could not detect a clearly defined segment of DNaI protection in this region. This discrepancy may be because of the use of different methods for the determination of DnaA protein binding. For the four upstream boxes, DNaI footprinting (Fig. 2A) showed similar binding of the DnaA protein in the absence and presence of TrfA. Although the pattern of DNaI footprinting in the presence of both proteins was the sum of each protein alone, we cannot exclude that under different set of conditions there is some cooperativity in binding by these two initiation proteins.

It has been shown that the DnaA protein binds to five DnaA boxes at oriC, and this binding results in bending of the DNA. It was also observed that nucleotide sequences flanking the DnaA boxes influenced DnaA binding affinity (8). It has been estimated that the complex of DnaA proteins and its binding site consists of one DnaA monomer/DnaA box, but multimeric higher order complexes are formed on larger DNA fragments (8). In our gel mobility shift experiments we observed four retardation bands that presumably correspond to the four DnaA binding sites determined by DNaI footprinting are shown. L, M1, M2, and R correspond to 13-mer sequences localized within the A+T-rich sequence of oriV (panel B). Internal stability of the nucleotide sequences within the RK2 minimal origin was determined using OLIGO software. Nucleotide coordinates are as described (22).

Additional work is required to determine the specific DNA structural requirements for helicase delivery, loading, and activity at the RK2 oriV.

**RK2 Open Complex Formation**—The results of the KMnO₄ modification footprinting experiments clearly show that the TrfA protein opens the RK2 origin. KMnO₄ sensitivity was observed along a 46-bp sequence in the top strand and a 13-bp sequence in the bottom strand. Differences in susceptibility to single strand-specific reagents between the top and bottom strands have also been observed in the case of opening in the oriC and P1 origins (15, 48). The top strand of RK2 with respect to the direction of replication likely serves as the template for lagging strand polymerization. In the case of plasmid P1, the major KMnO₄ reactivity also occurs within the lagging strand (16). Open complex formation at the RK2 oriV is localized within the A+T-rich region and corresponds to the region of lowest internal stability within the minimal origin, which includes four 13-mer repeat sequences (Fig. 6). These four 13-mers (L, M₁, M₂, R) exhibit a DNA consensus sequence (TAA-AACnTnTTTT) that is similar to the 13-mer consensus sequence determined for oriC (GATCTnTnTTTT) (10). The location of the four 13-mers coincides precisely with the site for oriV duplex opening (Fig. 6). Since a specific sequence and spacing within the 13-mer region of oriC are required for replication activity (10, 12–14), it is of interest to determine if all four oriV 13-mers are required for RK2 replication. The TrfA-induced opening does not absolutely require the presence of nucleotides; however, the formation of the open complex was somewhat enhanced by ATP or ATP·S. Similar observations have been reported for strand destabilization at the origin of bacteriophage λ (17). In contrast, ATP is required for *E. coli* oriC open complex formation mediated by the DnaA protein (10, 12).

DnaA or HU protein is required for full opening of oriV by TrfA (Figs. 3 and 4). Similarly, the formation of an open complex at the replication origin of the F plasmid by the RepE protein requires the HU protein and is enhanced by DnaA (18). HU protein also has been shown to enhance DnaA-mediated opening of the origin of *E. coli* (12). The mechanism by which the HU or the DnaA protein enhances open complex formation is not known. Recently it has been shown that binding of DnaA protein induces a bend in oriC DNA (8). Similarly, HU protein binds nonspecifically to double- or single-stranded DNA, and this binding either induces bending and folding or alters the superhelicity of supercoiled DNA (42). Thus, it is possible that DnaA or HU-dependent DNA bending is responsible for the observed increase and/or stabilization of TrfA-mediated oriV opening. Interestingly, although DnaA can perform a role similar to that of HU in the formation of the open complex at the RK2 origin, it is unable to open oriV by itself or in the presence of HU. This is in contrast to the origin regions of oriC and plasmid P1. The addition of the RepA protein increases the DnaA-induced strand opening at the P1 origin region, but RepA alone does not form an open complex (16). The ability of the plasmid-specifid initiation protein, in the case of RK2, to bring about the formation of an open complex in the absence of a specific host protein may be a contributing factor accounting for the different replication activities observed for plasmids RK2 and P1. RK2 is a broad host range replicon, whereas the P1 replicon appears to have a more limited host range.
Although the DnaA protein by itself does not induce open complex formation, it is required for RK2 replication in vivo and in vitro (1), and it does enhance or stabilize the TrfA-induced open complex formation. Recently, it has been shown that the DnaA protein plays a critical role in the delivery of the E. coli DnaB (helicase) to the RK2 origin. In the absence of TrfA protein, an oriV-DnaA-DnaB-DnaC complex can be isolated using a gel filtration method. These observations, along with results presented in this paper, indicate at least two roles for the DnaA protein during RK2 replication initiation: (i) enhancement of the formation of an open complex; and (ii) helicase delivery to the origin region. We propose that the TrfA protein in turn forms an open complex at the 13-mer region and that the DnaA protein induces specific nucleoprotein structural changes at the origin, which allows proper positioning of the DnaB helicase within the open complex. The helicase is activated by these events, and replication is allowed to proceed. Finally, the formation of a specific nucleoprotein structure containing four DnaA boxes (which potentially can form a cruciform structure) that bind DnaA protein and 17-bp iterons that bind the TrfA protein appears to be a critical factor in the initiation of RK2 replication and may also contribute to the unique broad host replication properties of this plasmid.

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