Replication Origin of the Broad Host Range Plasmid RK2

POSITIONING OF VARIOUS MOTIFS IS CRITICAL FOR INITIATION OF REPLICATION*

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

From the ‡Department of Biology, Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634 and the **University of Gdansk, Department of Molecular and Cellular Biology, 24 Kiadki, PL-80822 Gdansk, Poland

The 393-base pair minimal origin, oriV, of plasmid RK2 contains three iterated motifs essential for initiation of replication: consensus sequences for binding the bacterial DnaA protein, DnaA boxes, which have recently been shown to bind the DnaA protein; 17-base pair direct repeats, iterons, which bind the plasmid encoded replication protein, TrfA; and A + T-rich repeated sequences, 13-mers, which serve as the initial site of helix destabilization. To investigate how the organization of the RK2 origin contributes to the mechanism of replication initiation, mutations were introduced into the minimal origin which altered the sequence and/or spacing of each particular region relative to the rest of the origin. These altered origins were analyzed for replication activity in vivo and in vitro, for localized strand opening and for DnaB helicase mediated unwinding. Mutations in the region between the iterons and the 13-mers which altered the helical phase or the intrinsic DNA curvature prevented strand opening of the origin and consequently abolished replication activity. Insertions of more or less than one helical turn between the DnaA boxes and the iterons also inactivated the replication origin. In these mutants, however, strand opening appeared normal but the levels of DnaB helicase activity were substantially reduced. These results demonstrate that correct helical phasing and intrinsic DNA curvature are critical for the formation of an open complex and that the DnaA boxes must be on the correct side of the helix to load DnaB helicase.

Many plasmid replicons isolated from Gram-negative bacteria contain at their origin clusters of iterated repeats as well as consensus binding sites for the DnaA protein (for review, see Ref. 1). Most have evolved various replication initiation strategies utilizing the concerted action of a plasmid-specific protein and the host DnaA protein. Interestingly, the organization of the origin region of most narrow host range plasmids such as P1, F, pSC101, and R6K as well as the Escherichia coli chromosome, oriC, consists of DnaA boxes immediately adjacent to the A + T-rich region with the polarity of the DnaA boxes corresponding to the direction of replication (see Ref. 2). This is not the case with the broad host range plasmid RK2 (3, 4), where the A + T-rich region of the origin (oriV) is separated from the DnaA boxes by a 180-bp1 sequence consisting of five iterons that bind the RK2 replication initiation protein, TrfA (5–8). It is possible that this organization contributes to an initiation mechanism which allows for replication of this plasmid in a wide range of Gram-negative bacteria.

The TrfA protein exists in two forms, 44 and 33 kDa, as a result of an internal translational start in the trfA gene; both are functional in E. coli (9, 10). Purified TrfA protein is largely dimeric but binds as a monomer to the 17-bp iterons at oriV. The sequence requirement for TrfA binding has been well studied (12, 13). The binding of TrfA to the iteron sequences, with the assistance of either HU or DnaA, results in strand opening in the A + T-rich region specifically at the four 13-mers (L, M, 2, and R) present within this region (14). This thermodynamically unstable 13-mer motif is commonly found in plasmid replicons as well as in oriC (15) and serves as the site of strand opening and the subsequent entry for the DnaB-DnaC complex (16–18). In the case of RK2, once DnaB helicase is loaded at the open 13-mer region, replication proceeds unidirectionally toward the G + C-rich region (19).

The E. coli DnaA protein binds specifically to four 9-bp consensus sequences, DnaA boxes, arranged as inverted pairs in the RK2 origin immediately upstream of the iterons (14). The DnaA protein enhances or stabilizes the formation of the TrfA-mediated open complex (14), but the nature of this interaction in not yet understood. Additionally, DnaA is required to recruit DnaB to oriV during RK2 replication initiation (20). DnaB recruitment to oriV is similar to that observed for oriC which is facilitated through a DnaA-DnaB protein interaction (2). It has been proposed (20) that the initial recruitment of DnaB occurs at the four upstream DnaA boxes and that a rearrangement of this prepriming complex is required for the correct positioning of either the DnaB-DnaC complex or DnaB at the open region of oriV.

In this study we examine how the positioning of various regions in the RK2 minimal origin effect its replication activity in E. coli, specifically the initiation of replication including the key steps of localized strand opening and DnaB helicase loading. The data indicate that helical phasing and intrinsic DNA curvature are critical factors for the formation of a functional nucleoprotein structure and advance our understanding of the mechanism of RK2 replication initiation.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Proteins, DNA, and Reagents

The E. coli strains used in this study were XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F’ proAB lacZAM15 Tn10)) from Stratagene, Inc., C600 (thr leu thi lacY supE44 tonA), CC118

* This work was supported in part by National Institutes of Health Research Grant AI-07184. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a National Science Foundation Graduate Fellowship.

‡‡ Supported by Polish State Committee for Scientific Research Grant 6P04A01712.

** To whom correspondence should be addressed. Tel.: 619-534-3638; Fax: 619-534-7073.

† The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction.
(araD139 Δara, leu7697 ΔluxX74 phoA230 galE galIK thy rpsB rpoB argF, recA1), and CC118Δpir (CC118 (Δpir)), the latter two strains were kindly provided by Dr. Ken Timmis. Proteins were purified as described: DnaB (21), DnaC (22), and His6-TrfA 254D/267L (23). DnaA protein was provided by Dr. Alessandra Blasina. Commercially available proteins were: DNA gyrase, HU, and SSB from Enzyco, Inc.; bovine serum albumin (fraction V) and creatine kinase, Sigma; restriction endonucleases, T4 DNA ligase, and the Klenow fragment of E. coli DNA polymerase from Promega; Pfu DNA polymerase and DpoI from Stratagene, Inc.; T4 polynucleotide kinase, New England Biolabs; AmpliTaq DNA polymerase from Perkin-Elmer. Plasmid pKD19L1 is a dual RK2-R6K mini-replication derived from pTJS42 (12), pSP6 (12), and pUTKM (25) (provided by Dr. Ken Timmis). The Hpall fragment containing the minimal five iteron RK2 origin (6) along with the multiple cloning sites of pUC19 was subcloned as an EcoRI-HindIII fragment from pSP6 into pTJS42, replacing the existing minimal oriV sequence to yield pTJS42*. The R6K γ origin from pUTKM was cleaved with EcoRI-BamHI, blunted, and inserted into the filled in EcoRI site of pTJS42* to yield pKD19L1. Commercially available reagents were from the following sources: creatine phosphate, radiolabeled ATP, and potassium permanganate (KmO₄) from Sigma; dNTPs from Pharmacia; [methyl-³²P]ATP 6,000 Ci/mmol from NEN Life Science Products; Ultra Clean DNA purification kit, MoBio Laboratories. All oligonucleotide primers used for PCR or primer extension were synthesized by OPERON, Inc.

**Origin Mutant Construction and Transformation Assay**

Mutagenesis was performed following the protocol of either ExSite or QuickChange PCR-based Site-directed Mutagenesis Kit (Stratagene, Inc.) except that Pfu DNA polymerase (26) was used in every case. Primers used for mutagenesis were designed according to manufacturers recommendations and included the recognition sequence for either EcoRI or BamHI. Amplification on template DNA, pSP6, was for 9–18 cycles at 95 °C for 1 min, 56 °C for 1 min, and 68 °C for 8 min. The KpnI-XbaI fragment from pSP6 containing the mutated oriV sequence was subcloned into pKD19L1 replacing the wild-type origin. Unlike the other derivatives, oriV ΔGC was not generated by PCR but by cutting the pKD19L1 oriV 4–6 (which has an additional BamHI site due to the insertion) with BamHI and religating which resulted in a deletion of 65 bp. Plasmids containing a mutated oriV in pKD19L1 were obtained and maintained in E. coli strain CC118 (Δpir) on Luria-Bertani agar plates supplemented with 12.5 µg/ml tetracycline. To test for oriV function in vivo, plasmids were purified through two CaCl₂ buoyant density gradients and between 200 and 500 ng was used for the transformation of the parent E. coli strain, CC118, where replication can be initiated only from the RK2 origin.

**In Vitro Replication Assay**

Preparation of E. coli C600 crude extracts (FII) competent for RK2 replication and reaction conditions were essentially as described (27). Reactions contained 0.3 µg of DNA template and the indicated amounts of His6-TrfA 254D/267L protein. Incubation was at 32 °C for 60 min.

**Strand Opening Using Permanganate Footprinting**

Permanganate (KmO₄) footprinting and primer extension using PCR were carried out as described previously (14) with some modifications. A 24-mer primer (5’-GGGATCCGATGCTGAGTGCACGCTC-3’) was used for the top strand analysis and a 25-mer primer (5’-GAATTCTAATTCACGGACTTGGGTT-3’) for bottom strand analysis. Where indicated, reactions contained 420 ng of TrfA (His6-TrfA 254D/267L) protein and 625 ng of DnaA protein. 8 ng of HU protein was present in all reactions.

**Assay of DnaB Helicase Activity**

**F1⁺ Formation**—Assays were performed as essentially as described previously (20). Reactions (25 µl) contained 40 mM HEPES-KOH, pH 8.0, 25 mM Tris-HCl, pH 7.4, 4% (w/v) sucrose, 4 mM dithiothreitol, 80 µM bovine serum albumin, 11 mM magnesium acetate, 2 mM ATP, 500 mM (each) CTP, GTP, UTP, 20 mM creatine phosphate, 20 µM dNTPs, and 160 ng of BSF C. DNA gyrase, 125 ng of DnaB, 625 ng of DnaA, 24 ng of DnaC, 300 ng of DNA template (containing wild-type or mutated oriV), and 560 ng of His6-TrfA 254D/267L. Incubation was at 32 °C for 30 min and reactions were stopped by the addition of EDTA to 10 mM and SDS to 2.5% followed by incubation at 65 °C for 2 min. The samples were photographed at 25 V for 20–22 h in a 1% agarose gel in 0.5x Tris borate, pH 8.3, and 2 mM EDTA, then stained with ethidium bromide. The percent of F1⁺ conversion was measured using densitometry scans.

**Bending Assay—Static bending of oriV containing fragments was determined directly by mobility in 5% polyacrylamide gels run at 100 V for 4 h at 4 °C.** 1 µg of plasmid template (pKD19L1) was cleaved with KpnI-XbaI to generate fragments containing wild-type or mutated origins. The apparent molecular weight of each fragment was determined using the Alpha Innotech Imaging System.

**RESULTS**

**Origin Function Is Effected by Altered Spacing**—The DNA sequence required for the binding of the TrfA protein to the iteron region has been well characterized (12, 13). It has also been shown that the DnaA protein binds to four DnaA boxes just upstream of the iterons (14). However, little is known about the importance of the spatial arrangement of the DnaA boxes, iterons, and the A + T-rich (including 13-mers) and G + C-rich regions within the RK2 minimal origin. In this work mutants with altered spacing were constructed in four different areas of oriV: between the iterons and the A + T-rich region, oriV 1; within the A + T-rich region itself, oriV 2; between the DnaA boxes and the iterons, oriV 3; and between the A + T- and G + C-rich regions, oriV 4 (Fig. 1). Mutants were generated by PCR and subcloned into a dual origin (RK2-R6K) plasmid, pKD19L1. All mutations were confirmed by sequence analysis (data not shown). pKD19L1 derivatives can replicate by the R6Kγ origin in E. coli strain CC118 (Δpir) which supplies the replication protein required for R6K replication (25); however, in the parent strain, CC118, replication can only be initiated by the RK2 origin. Thus, the ratio of transformation frequencies obtained in CC118 versus CC118 (Δpir) was used to quantitate oriV function in vitro. Ratios obtained for oriV mutants were normalized against the ratio obtained for pKD19L1 (wild-type oriV) (Table II). The insertions of 6 bp, approximately half of one helical turn, in the first three regions (oriV 1, oriV 2, and oriV 3) inactivated or greatly reduced replication activity. Only the insertion at the start of the G + C-rich region (oriV 4–6) had little to no effect on plasmid replication.

The mutants that were effected by the altered spacing (oriV 1–6, oriV 2–6, and oriV 3–6) were further analyzed for repli-
culation activity in vitro using an in vitro replication assay that has been described previously (27). 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 initiator protein, TrfA. Fig. 2 shows DNA synthesis for wild-type and mutant oriV templates in response to increasing amounts of TrfA protein. In this assay as well as all other in vitro experiments performed here and described previously (11, 14), the histidine-tagged version of the largely monomeric 33-kDa TrfA mutant protein (His₆-TrfA 254D/267L) which exhibits elevated iteron binding (23) was used. Wild-type oriV template reached maximal DNA replication levels with 0.56 μg of His₆-TrfA 254D/267L protein (Fig. 2) and was inhibited by greater amounts of protein (data not shown). Replication reactions with oriV 1–6 and oriV 2–6 remained at background levels, while oriV 3–6 responded somewhat to higher levels of His₆-TrfA 254D/267L. This is consistent with the in vivo transformation results where oriV 3–6 did allow the establishment of a low level of transformants which exhibited a slower growth rate than wild-type transformants.

To rule out the possibility that the lack of function of mutated origins observed in vivo and in vitro was due to the inability of the TrfA initiator protein to bind to the iterons in the mutant constructs, we utilized a gel mobility shift assay with fragments containing wild-type and mutant origins and the His₆-TrfA 254D/267L protein. Each of the mutant oriV derivatives displayed binding of all five iterons at comparable protein levels as observed for wild-type oriV (data not shown).

Effect of Insertion Mutations in oriV on DnaB-mediated Template Unwinding—Following strand opening at the A + T-rich region, DnaB helicase is recruited through a protein-protein interaction with DnaA (20) and then must be loaded at the open region of oriV to facilitate further template unwinding. In the presence of DNA gyrase this unwound form is converted to a highly negatively supercoiled DNA, FI*, which migrates more rapidly on an agarose gel than normally supercoiled DNA, FI (17). Previously it has been demonstrated that unwinding of wild-type oriV supercoiled templates requires TrfA, DnaA, DnaB, DnaC, DNA gyrase, HU, SSB, and ATP (20). The same assay was used to compare wild-type and mutant oriV templates for their ability to serve as substrates for DnaB helicase unwinding. Upon the addition of His₆-TrfA 254D/267L protein to the reaction, 50% (using densitometry scans) of the wild-type oriV template was converted to the faster migrating FI* form. This mutant reproducibly showed significantly less FI* formation than the wild-type template. This mutant reproducibly showed significantly less FI* formation than the wild-type template. Additionally, when the assay was performed in the presence of varying amounts of the DnaB and DnaC proteins, FI* formation occurred at a lower level of the two proteins for the wild-type oriV template than for the oriV 3–6 template (data not shown). These observations indicate that the 6-bp insertion in the oriV 3 region, between the DnaA boxes and the iterons, affects the efficiency of DnaB loading at the open complex.

Correct Helical Phasing Restores Origin Function—To deter-

<table>
<thead>
<tr>
<th>Origin template</th>
<th>CC118</th>
<th>CC118 pir</th>
<th>CC118/CC118 pir</th>
</tr>
</thead>
<tbody>
<tr>
<td>oriV wt</td>
<td>1.1 × 10⁶</td>
<td>2.6 × 10⁴</td>
<td>100</td>
</tr>
<tr>
<td>oriV 1–6</td>
<td>0</td>
<td>1 × 10⁵</td>
<td>0</td>
</tr>
<tr>
<td>oriV 2–6</td>
<td>5 × 10⁵</td>
<td>5.8 × 10⁴</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>oriV 3–6</td>
<td>3 × 10⁵</td>
<td>3.4 × 10⁴</td>
<td>0.2</td>
</tr>
<tr>
<td>oriV 4–6</td>
<td>8.2 × 10⁴</td>
<td>2 × 10⁴</td>
<td>97</td>
</tr>
</tbody>
</table>

* Both mutant and wild-type origins are contained on plasmid pKD19L1.

† Ratio of transformants of CC118 (pir−) and CC118 pir (pir+) normalized to wild-type.

† Transformants exhibited a slower growth rate.

**A. Toukarian and D. R. Helinski, manuscript in preparation.
mine whether the loss of function observed by altering spacing within mutants oriV 1–6 and oriV 3–6 was due to the disruption of a defined helical positioning or a specific sequence requirement, we constructed additional mutants in the regions of oriV 1 and oriV 3 (see Fig. 1). Mutations were made using PCR as described previously in the exact position, unless noted, as the original 6-bp insertion incorporating the same restriction enzyme recognition sequence (see “Experimental Procedures”). New mutations in oriV are shown in Table II and include; oriV 1–6P, a 6-bp insertion just 7 bp upstream of oriV 1–6; oriV 1s, a 6-bp substitution; oriV 1–11, an 11-bp insertion; oriV 3–11, an 11-bp insertion; and oriV 3–17, a 17-bp insertion. Also a mutant was constructed which contained a deletion of almost the entire G + C-rich region, oriV Δ GC. Since the original 6-bp insertion in this region had no effect (Table I), it was of interest to assess the importance of this region. All mutant derivatives were analyzed for origin function in vivo by the transformation assay and in vitro using the in vitro replication assay (Table II).

In the region between the DnaA boxes and the iterons the insertion of half of a helical turn, mutant oriV 3–6, reduced origin activity, but the insertion of a full helical turn, mutant oriV 3–11, restored origin activity close to that of wild-type. Also, the insertion of one and a half helical turns, mutant oriV 3–17, inactivated oriV function (Table II). Additionally, we observed no DnaB activity on the oriV 3–17 template as evidenced by the absence of FI* formation (data not shown). This suggests that the helical phasing in this region is critical for the loading of DnaB helicase at the open region. It should be noted that the DnaA protein was still capable of binding to oriV mutant derivatives using a gel retardation assay (data not presented).

Mutations in the region between the iterons and the 13-mers, oriV 1, showed that specific helical positioning as well as a specific sequence contribute to origin function. A 6-bp insertion at a different position, mutant oriV 1–6P, again resulted in a complete inactivation of oriV. The insertion of a full helical turn in the oriV 1 region, oriV 1–11, partially restored activity, although only 55–78% that of wild-type (Table II). Mutant oriV 1s, which has a substitute sequence with wild-type spacing, surprisingly was only somewhat active compared with wild-type oriV, suggesting that in addition to helical phasing there is an actual sequence requirement in this region.

The deletion of the G + C-rich region had little to no affect on origin function as results showed that the activity of oriV Δ GC was close to that of oriV wild-type. It is important to note that this region is 80% G + C-rich in wild-type oriV, while the
Insertion, sequence substitution or deletion mutations were constructed in the same regions (oriV 1-oriV 4, see Fig. 1) as described under “Experimental Procedures.” Plasmid templates were analyzed as described previously for origin function by transformation and in vitro replication assays.

<table>
<thead>
<tr>
<th>Origin template&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence of mutants 5’ to 3’&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Spacing&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Relative transformation frequency&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Relative activity&lt;sup&gt;e&lt;/sup&gt; in vitro&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>oriV wt</td>
<td></td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>oriV 1–6</td>
<td>AAAGAATTCCATCCAG</td>
<td>Plus 0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>oriV 1–6&lt;sup&gt;p&lt;/sup&gt;</td>
<td>AGGTTTCGCAAATCCAG</td>
<td>Plus 0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>oriV 1s</td>
<td>AAAGAATTTC</td>
<td>0</td>
<td>29</td>
<td>40</td>
</tr>
<tr>
<td>oriV 1–11</td>
<td>AAAGAATTCCATCCAG</td>
<td>Plus 11</td>
<td>55</td>
<td>78</td>
</tr>
<tr>
<td>oriV 2–6</td>
<td>AGGTTATCCAA ATAT</td>
<td>Plus 6</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>oriV 3–6</td>
<td>GCGAATTGCTACG</td>
<td>Plus 6</td>
<td>0.2</td>
<td>19</td>
</tr>
<tr>
<td>oriV 3–11</td>
<td>GCGAATTGGCTAGC</td>
<td>Plus 11</td>
<td>87</td>
<td>88</td>
</tr>
<tr>
<td>oriV 3–17</td>
<td>GCGAATTGCAGTGATGCAGC</td>
<td>Plus 17</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>oriV 4–6</td>
<td>GCGGATTCCTGCG</td>
<td>Plus 6</td>
<td>97</td>
<td>87</td>
</tr>
<tr>
<td>oriV ΔGC</td>
<td>GGGCAGGCAGCC</td>
<td>Minus 62</td>
<td>112</td>
<td>92</td>
</tr>
</tbody>
</table>

<sup>a</sup> Both mutated and wild-type origins are contained on plasmid pKD19L1.
<sup>b</sup> Sequence that was inserted or substituted is shown in bold underline.
<sup>c</sup> The spacing change is expressed relative to the wild-type origin.
<sup>d</sup> Transformation frequency and in vitro replication activity are normalized to that of the wild-type origin.
<sup>e</sup> Mutation is an insertion 7 bp upstream of the oriV 1–6 insertion site.

To investigate the effect of the various insertion mutations on origin DNA structure, fragments containing wild-type and mutant oriV were analyzed using polyacrylamide gel electrophoresis. The fragment containing wild-type oriV migrated at an apparent size that was greater than its actual size due to the positioning of an adjacent high G + C-rich sequence which might have a compensatory effect.

**Intrinsic DNA Curvature Contributes to the Structural Requirement for Initiation of oriV—**In the wild-type origin sequence (oriV 1 region) there are 56 bp separating the last iteron sequence and the first 13-mer of the open region. Analysis of this sequence revealed the presence of dA-dT tracts which have been implicated in static DNA bending in several systems (28–30). The insertion in mutant oriV 1–6 is located in the first A-tract. It was noted during gel retardation experiments (data not presented) that a fragment containing oriV 1–6 migrated with a faster mobility in a 5% polyacrylamide gel than a fragment containing wild-type oriV, suggesting that it may have an altered DNA confirmation. It has been well documented that bent DNA fragments exhibit anomalously slow electrophoretic mobility (31).

To investigate the effect of the various insertion mutations on origin DNA structure, fragments containing wild-type and mutant oriV were analyzed using polyacrylamide gel electrophoresis. The fragment containing wild-type oriV migrated at an apparent size that was greater than its actual size due to the positioning of an adjacent high G + C-rich sequence which might have a compensatory effect.

**Intrinsic DNA Curvature Contributes to the Structural Requirement for Initiation of oriV—**In the wild-type origin sequence (oriV 1 region) there are 56 bp separating the last iteron sequence and the first 13-mer of the open region. Analysis of this sequence revealed the presence of dA-dT tracts which have been implicated in static DNA bending in several systems (28–30). The insertion in mutant oriV 1–6 is located in the first A-tract. It was noted during gel retardation experiments (data not presented) that a fragment containing oriV 1–6 migrated with a faster mobility in a 5% polyacrylamide gel than a fragment containing wild-type oriV, suggesting that it may have an altered DNA confirmation. It has been well documented that bent DNA fragments exhibit anomalously slow electrophoretic mobility (31).

To investigate the effect of the various insertion mutations on origin DNA structure, fragments containing wild-type and mutant oriV were analyzed using polyacrylamide gel electrophoresis. The fragment containing wild-type oriV migrated at an apparent size that was greater than its actual size due to the positioning of an adjacent high G + C-rich sequence which might have a compensatory effect.

**Intrinsic DNA Curvature Contributes to the Structural Requirement for Initiation of oriV—**In the wild-type origin sequence (oriV 1 region) there are 56 bp separating the last iteron sequence and the first 13-mer of the open region. Analysis of this sequence revealed the presence of dA-dT tracts which have been implicated in static DNA bending in several systems (28–30). The insertion in mutant oriV 1–6 is located in the first A-tract. It was noted during gel retardation experiments (data not presented) that a fragment containing oriV 1–6 migrated with a faster mobility in a 5% polyacrylamide gel than a fragment containing wild-type oriV, suggesting that it may have an altered DNA confirmation. It has been well documented that bent DNA fragments exhibit anomalously slow electrophoretic mobility (31).

**DISCUSSION**

RK2 as a broad host range plasmid has evolved the ability to adapt to the replication machinery of many different bacterial hosts. This ability could be mediated by the plasmid-encoded replication protein, TrfA, as some hosts vary in their requirement for the two alternate forms (33 or 44 kDa) of TrfA protein (9). Also, the fact that TrfA is absolutely required for opening the Δ + T-rich region is consistent with a broad host range replication strategy in that it does not rely solely on a bacterial host protein(s) to perform this key function, as is the case with the narrow host range plasmid, P1 (33). However, other narrow host range plasmids such as R6K and F also utilize the plasmid-encoded Rep protein to initiate strand opening (34, 35). In

**FIG. 5.** Analysis of bending of origin fragments. Polyacrylamide gel electrophoresis was performed on oriV templates (see “Experimental Procedures”). The apparent size based on mobility is compared with the actual size of the origin containing fragment. The closer the mobility ratio is to 1, the less bent the DNA fragment, while a mobility ratio farther away from 1 indicates that the DNA is of a more bent conformation. The sequence of each mutation is described in Table II.
fact, the opening strategy observed with the F plasmid (35) is very similar to that of RK2 (14). This suggests that Rep protein-mediated strand opening is not a mechanism specific to broad host range plasmids. Alternatively, the architecture of the RK2 plasmid itself may be a germaine factor in ensuring proper initiation in different bacterial hosts. The data presented here support the idea that a specific DNA structure facilitates the nucleoprotein structure required for initiation of RK2 replication.

The Importance of Helical Phasing for the Formation of a Functional Open Complex—In the RK2 origin there are 56 bp, approximately five helical turns, separating the site of TrfA protein binding (iterons) and the site of the open region (13-mers). An insertion of 6 bp at the beginning of this region, mutant oriV 1–6, completely abolished replication activity by disrupting open complex formation. Restoring the proper helical phase by inserting 11 bp in the same region, mutant oriV 1–11, did restore replication activity 55–78% to that obtained for wild-type (see Table II). This is in contrast with what has been observed for replication initiation at the E. coli chromosome, oriC, where there are only 13 bp separating the 13-mers and the closest DnaA box and the loss or addition of even 1 bp as well as the addition of 11 bp greatly reduced replication activity (36). The melting of the three 13-mers repeated in the A + T-rich region of oriC is initiated by the binding of DnaA protein to the DnaA boxes (37–39) and requires precise spacing of the DnaA boxes relative to the 13-mers (36). The combined evidence that DnaA binds 13-mer sequences (40), and the differential requirement of the 13-mers observed by mutational studies, suggests that DnaA may interact with the middle and rightmost 13-mer sequence (39). For RK2 replication it is unclear if a similar type of interaction between TrfA and one or more of the 13-mers helps to initiate the formation of the open complex. We show that helical phasing is important in this area which may suggest that this type of interaction exists. In addition, because the insertion of a complete helical turn between the iterons and 13-mers did not completely restore replication activity (mutant oriV 1–11), there may also be an optimal spacing requirement. We investigated this possibility by making a 6-bp substitution in the same area which therefore maintains wild-type spacing. This mutant, oriV 1s, exhibited reduced replication activity. This result indicates that we had disrupted an actual sequence required for maximum initiation.

Intrinsic DNA Curvature Is Required for Optimal Replication Activity—Further analysis of the sequence between the iterons and the 13-mers revealed the presence of adenine tracts which have been shown to direct DNA bending in other systems (28, 30). Static or intrinsic bends as well as protein-induced bends have been shown to contribute to replicative structures in oriC (41), λ (30, 42), R6K (34), P1 (43), pT181 (44), SV40 (29), pSC101 (45), and pLS1 (46). In most of these systems, the binding of the Rep protein induces a bend. For λ and SV40 replication origins it has been shown that the presence of an intrinsic bend is required for origin function (29, 30, 42). For RK2 it was observed previously that while the binding of the TrfA protein did not induce a bend in the origin, there was a slight intrinsic bend in the absence of protein (32). Our analysis of origin containing fragments using polyacrylamide gel electrophoresis (Fig. 5) is consistent with this observation. The wild-type origin fragment exhibited a slightly slower electrophoretic mobility than expected for its size indicating the presence of a bent DNA confirmation. Interestingly, only fragments with mutations in the oriV 1 region displayed different mobility ratios than the fragment containing wild-type oriV correlating a change in DNA curvature to a loss of origin function. Using a program written to calculate bending and curvature in B-DNA (47), we analyzed the entire sequence of oriV. Consistent with our gel mobility results, the bending model predicted an intrinsic DNA curvature in the region between the iterons and 13-mers which was disrupted by the insertion (oriV 1–6) or substitution (oriV 1s) of 6 bp. Thus, the intrinsic DNA curvature in this region appears to also be a contributing factor for proper replication initiation, possibly a critical element in the formation of a specific nucleoprotein structure for open complex formation.

Requirement of 13-mers for Open Complex Formation—Tandemly repeated motifs (usually 13-mers) are present in the A + T-rich regions of many prokaryotic origins and replicons (15). The DNA helix at these repeats is typically thermodynamically unstable helping to facilitate strand separation (48, 49). Mutational analysis of the 13-mers in E. coli oriC demonstrated that the helical instability of the L 13-mer and the specific sequence of the M and R 13-mers were required for efficient opening of the origin (39, 49). For RK2 the 13-mers present in oriV (L, M1, M2, and R) are very similar to the oriC consensus sequence and have recently been demonstrated to be the exact sites of strand separation (14). The insertion of 6 bp within the M2 13-mer, mutant oriV 2–6, disrupted strand opening specifically at the M1 and M2 13-mers, even though the sequence of the insertion (GAATTC) preserved the A + T richness of the sequence. It is therefore possible that this insertion has disrupted a specific sequence or spatial arrangement of the 13-mers required for strand opening. Interestingly, mutant oriV 2–6 was able to replicate in a topA E. coli strain (data not shown) which has been shown to increase DNA negative supercoiling (50). This is similar to that observed for oriC where DNA supercoiling induced opening complex formation even in the presence of a mutated 13-mer region (49). Further investigation is required to determine the exact contribution of each 13-mer to strand separation at the RK2 origin. It is possible that as proposed for oriC initiation (15), DnaA interacts with a specific 13-mer(s) to stabilize the opening by TrfA which also might be mediated by a TrfA-13-mer interaction.

It should also be noted that in a previous study of the RK2 origin (51), the deletion of 8 bp within the A + T-rich region (the same site of the oriV 2 insertion in our study) resulted in an inactive origin in vivo, while the replacement of the nucleotides (maintaining wild-type spacing) with a sequence containing the same EcoRI site (GAATTC) as used in our study, restored replication activity in vivo. The authors suggested that either correct spacing is required for origin function or that this region normally contains a putative DnaA-binding site and the substituted sequence containing an EcoRI site created a weaker but still functional DnaA box. However, the insertion in mutant oriV 2–6 in our study actually created a stronger DnaA box with respect to the consensus sequence. This argues more for the importance of the correct spacing and sequence of the M2 13-mer.

Position of the DnaA Boxes Is Critical for Origin Function—During RK2 replication initiation, the DnaA protein is responsible for DnaB helicase recruitment to oriV through a physical protein-protein interaction (20). The authors proposed that the initial recruitment site for DnaB is at the DnaA boxes located upstream of the TrfA bound iterons (20). Once DnaB is recruited to the origin it likely must be repositioned to be loaded at the open complex. Possibly the TrfA protein directs this repositioning; however, there is no direct evidence for a physical interaction between TrfA and DnaB proteins. In this study we provide evidence that the four upstream DnaA boxes must be on the same helical face with respect to the rest of the

---

3 K. S. Doran and D. R. Helinski, unpublished results.
Origin Structure Requirements for Initiation of RK2 Replication

This work provides additional support for a working model of RK2 replication initiation which involves separation between two key steps in initiation: strand opening and DnaB helicase loading. The unique organization of the RK2 origin may contribute to its ability to replicate in most Gram-negative bacteria. Insertion mutants oriV 1–6 and oriV 2–6 were also unable to replicate in Pseudomonas putida 3–6, which substantially effected replication in P. aeruginosa 1–6 and

4 K. S. Doran, D. R. Helinski, and I. Konieczny, manuscript in preparation.

Acknowledgments—We thank Dr. A. Blasina and Dr. K. Timmis for generous gifts of purified protein and bacterial strains/plasmids, respectively. We thank R. Neves for help in the preparation of the manuscript.

REFERENCES
