The Inactive pT181 Initiator Heterodimer, RepC/C*, Binds but Fails to Induce Melting of the Plasmid Replication Origin*

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Staphylococcus aureus plasmid pT181 replicates via a rolling circle mechanism. The synthesis of the pT181 initiator protein (RepC) is regulated by antisense RNAs, and RepC is inactivated after usage by the attachment of an oligonucleotide to one of its subunits. The inactivated heterodimeric RepC/C* has been shown to be unable to initiate replication in vitro (Rasooly, A., and Novick, R. P. (1993) Science 262, 1048–1050). The inactive RepC/C* has been found to be very stable and constitute about 90–95% of the total RepC antigen inside the cell. We studied the specific interaction of the RepC/C and RepC/C* complex with the pT181 double strand origin. The results indicated that RepC/C and RepC/C* footprint supercoiled DNA differently although their footprints on linear DNA are similar; we also find that RepC/C is able to enhance cruciform extrusion while RepC/C* cannot. RepC/C* binds and bends the double strand origin much more weakly than does RepC/C. These results suggest that the attached oligonucleotide induces a conformational change in the RepC/C* molecule that is responsible for its lack of activity.

All known bacterial plasmids encode a specific initiator and control their replication primarily by negatively regulating its synthesis. Often, the linkage between replication and the control of initiator synthesis is indirect in that the regulatory factors act in trans and respond to the copy number of the plasmid. This type of replication control requires that the initiator be utilized stoichiometrically rather than catalytically since the latter would obviate such control. Plasmids such as pT181 and its relatives, which replicate by the rolling circle mechanism, inactivate the initiator protein automatically as a part of the termination process (1). ColEI and its relatives, which replicate by the θ mechanism, utilize a long RNA primer as initiator and automatically degrade this primer as replication proceeds. Plasmids such as R1, which also replicate by the θ mechanism, require new protein synthesis for each round of replication (2), suggesting that the initiator is inactivated after use.

The inactivation mechanism for pT181 is unique; the initiator protein, RepC, is a dimer, referred to as RepC/C, and following the completion of a round of replication, an oligonucleotide, representing sequences immediately 3′ to the initiation

nicking site is attached to the active site tyrosine of one of its subunits (1). Our initial experiments suggested that the heterodimeric derivative, RepC/C*, lacked the nicking, relaxation, and replication activities of the native protein. This was puzzling. Since only one of the two subunits was modified, the unmodified subunit would be expected, a priori, to have nicking activity, the reversal of which would result in relaxation, and to be able to initiate but not terminate replication. Testing of these possibilities was complicated by the fact that preparations of total RepC antigen from pT181-containing cells generally consist of 90–95% RepC/C* and 5–10% RepC/C, which represents active protein that has not been used for replication and which we have not succeeded in cleanly removing. Consequently, these preparations generally have low but definite RepC activity, making it difficult to determine with confidence whether RepC/C* itself has any activity. Although any possible activity of RepC/C* would have to be so weak that it could have little or no impact on the primary regulation of replication initiation, even weak activity could have important implications for other aspects of the plasmid life cycle, particularly since RepC/C* is metabolically stable and is present at such a high concentration. As it happens, RepC/C* has very weak nicking and relaxation activities but probably lacks replication activity. Furthermore, we have demonstrated that RepC/C* inhibits the replication and relaxation activities of RepC/C in vitro and question whether it has any negative regulatory activity in vivo.

In an attempt to understand the structural basis for the profound difference in activities of the two forms of RepC, we have compared their interactions with the pT181 leading strand origin by DNase I footprinting, KMnO₄ sensitivity, and binding-bending analysis.

The pT181 double strand origin (DSO)³ of replication consists of three sets of inverted repeats, IR-I, IR-II, and IR-III (3). IR-II contains the initiation nicking site, and IR-III contains the specific initiator recognition site. RepC/C has several distinct activities on pT181 DNA, namely bending (4), enhancement of cruciform extrusion (5), and nicking and topoisomerase I-like relaxation (6) in addition to replication initiation.

A previously published DNase I footprint of RepC/C on a linear fragment containing the pT181 DSO showed protection of the specific RepC binding site (IR-III) and the proximal stem of the IR-II cruciform (7). We have re-analyzed the DNase I footprint, comparing linear with supercoiled DNA as well as RepC/C with RepC/C*. Additionally, we used potassium permanganate to compare the ability of RepC/C and RepC/C* to enhance cruciform extrusion and gel mobility measurements to

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This paper is dedicated to the memory of PeiZhi Wang, a wonderful long time colleague who passed away in November, 1995.

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1 R. Jin, and R. P. Novick, in preparation.
2 R. Jin, A. Rasooly, and R. P. Novick (1996) J. Bacteriol., in press.
3 The abbreviations used are: DSO, double strand origin; IR-I, -II, and -III, inverted repeats I, II, and III, respectively; TBE, Tris borate-EDTA.
determine the relative binding and bending activities and the binding constants for the two forms. We find that RepC/C and RepC/C* footprint supercoiled DNA differently although their footprints on linear DNA are similar; we also find that RepC/C* is unable to enhance cruciform extrusion and that it binds and bends the DSO much more weakly than does RepC/C. Our results suggest that the attachment of an oligonucleotide not only blocks the active tyrosine of one subunit but also causes an allosteric modification of the protein that reduces its ability to bind to the DSO and eliminates its ability to enhance cruciform extrusion, resulting in the observed loss of activity.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Staphylococcus aureus strains RN8601 and plasmids pRN6397 and pRN6921 were from this laboratory. Plasmid pSK1284, which contains a tandem duplication of repC, was kindly provided by Dr. Saleem Khan. All strains were grown in CY broth (8) with vigorous aeration at different temperatures. Growth was turbidimetrically monitored using a Klett-Sumner colorimeter with a green (540 nm) filter.

Purification of RepC Protein—N-terminal histidine-tagged RepC/C protein1 was purified from S. aureus strain RN8601 containing pRN68921. pRN68921 was constructed by cloning repC-his6 (a histidine codons fused to the N terminus) to pRN5458 (9) so that its expression is driven by the β-lactamase promoter. In this construct, the pT181 DSO, which is located within repC, was inactivated by a synonymous substitution of 4 nucleotides surrounding the nick site (10). RepC/C* (with a small amount of RepC/C) was purified from a strain containing pRN68921 plus pRN6397, which contains the functional pT181 DSO cloned to pE194. Cells were grown to a density of 100 Klett units in 1 liter of CY medium, 2% carboxyphenyl benzoyl-6-aminopenicillanic acid was added to final concentration of 5 μM/ml, growth was continued for another 2 h, and cells were then harvested by centrifugation. The cell pellet was resuspended in 20 ml of buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM KCl, and 5% ethylene glycol. Lysostaphin was then added to a final concentration of 150 μg/ml, and the suspension was incubated on ice for 1 h. After 2 freeze-thaw cycles, the suspension was centrifuged at 100,000 × g (Beckman SW 40 Ti) for 30 min. Streptomycin sulfate was added to the supernatant to a final concentration of 150 μg/ml, and the mixture was incubated at 4°C for 1 h with gentle shaking. The agarose beads were washed with the above buffer four times, and proteins were then eluted with a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, 5% ethylene glycol, and 30% saturation fraction was pelletized by centrifugation at 25,000 × g for 15 min. The pellet was dissolved in 20 ml of buffer containing 10 mM Tris-HCl (pH 8.0), 1 M KCl, and 5% ethylene glycol. Lysostaphin was then added to a final concentration of 150 μg/ml, and the suspension was incubated on ice for 1 h. After 2 freeze-thaw cycles, the suspension was centrifuged at 100,000 × g (Beckman SW 40 Ti) for 30 min. Streptomycin sulfate was added to the supernatant to a final concentration of 150 μg/ml, and the mixture was incubated at 4°C for 1 h with gentle shaking. The agarose beads were washed with the above buffer four times, and proteins were then eluted with a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, 5% ethylene glycol, 250 mM imidazole, and 0.5 mM diithiothreitol. The eluate was subjected to ammonium sulfate fractionation, and 30–40% saturation fraction was pelletized by centrifugation at 20,000 × g and then dissolved in 10 mM Tris-HCl (pH 8.0), 150 mM KCl, 0.1 mM EDTA, 0.5 mM diithiothreitol, and 5% ethylene glycol. This material was usually >95% pure RepC antigen.

Potassium Permanganate Probing of RepC/C-DNA and RepC/C* DNA Complexes—2 μg of pT181 plasmid DNA (either supercoiled or linearized by NdeI digestion) was incubated with 10 pmol Rep C/C or Rep C/C* protein in binding buffer (10 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA) without Mg2+ in a total volume of 50 μl at 37°C. After 30 min, 2.5 μl of 80 mM KMnO4 was added to the reaction for 1 min at 37°C. The reaction was stopped by the addition of 2.5 μl of β-mercaptoethanol, and plasmids were recovered by using Qiagrep spin plasmid miniprep columns. NaOH (200 mM) was used to break the backbone of DNA at the sites of KMnO4 attack and to denature the double-stranded plasmid DNA. The reaction was allowed to proceed at room temperature for 5 min and then neutralized by adding 3 M NaOAc (pH 5.5) to a final pH <7.0. DNA was recovered by ethanol precipitation. Primer extension reactions were carried out using 32P 5'-end-labeled primers hybridized to either of the plasmid strands and 3 units of Sequenase for 15 min at 43°C. The reactions were stopped by the addition of stop dye mix and heated at 90°C for 2 min prior to denaturing gel electrophoresis.

DNA Binding Analysis—A set of DNA fragments of the same length, but with the pT181 DSO located at different positions, were isolated from pSK1284 by digestion with different restriction enzymes. Equimolar amounts of protein (RepC/C or RepC/C*) and DNA fragment (final volume of 15 μl) were incubated at room temperature for 10 min in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, and 5% ethylene glycol. The reaction mixtures were loaded onto a 5% polyacrylamide gel (acylamide:biacylamide, 70:1) and separated by electrophoresis for 4 h at 10 V/cm in TBE buffer. Gels were stained in ethidium bromide and photographed.

Kd Determination—Quantitative gel mobility shift assays were used to determine the dissociation constants (Kd) of the Rep-DNA complexes. Fixed amounts of RepC/C or RepC/C* (250 pmol) were titrated with increasing concentrations of radiolabeled pT181 DSO-containing DNA fragment. The fragment used was the HindIII fragment of plasmid pSK1284 (see Fig. 4). After incubation at room temperature for 10 min in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, and 5% ethylene glycol (total volume of 2 μl), the reaction mixtures were loaded onto a 5% polyacrylamide gel (acylamide:biacylamide, 70:1) and separated by electrophoresis for 4 h at 10 V/cm in TBE buffer. Gels were analyzed with a Molecular Dynamics PhosphorImager using Image Quant software.

RESULTS

Potassium Permanganate Probing of RepC/CDNA and RepC/C* DNA Complexes—Previous studies have demonstrated that RepC enhances or stabilizes the IR-II cruciform (5) (Fig. 1). At the tip of the cruciform is a single-stranded region that contains the RepC nicking site and has been proposed to be the

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* A. Rasouly, and R. P. Novick, unpublished data.
actual substrate for RepC nicking and initiation (5). To determine whether there is any difference between RepC/C and RepC/C* with respect to cruciform formation, we used potassium permanganate to probe the melting of DNA in Rep-DNA complexes. Potassium permanganate is a strong oxidant that reacts preferentially with unpaired thymidine residues. The DNA lesions caused by permanganate attack can be detected with alkaline treatment followed by primer extension (11). Negatively supercoiled or linear pT181 cop623 plasmid DNA was incubated with purified RepC/C or RepC/C* in the absence of magnesium. Mg^{2+} is required for nicking but not for DNA binding by RepC. Any secondary structure promoted by RepC binding and interaction can, therefore, be seen only in the absence of Mg^{2+}. The reaction mixtures were treated with KMnO_{4} to attack unpaired nucleotides, and the DNA lesions were detected by primer extension from both directions. The results, shown in Fig. 2, indicate that on supercoiled DNA, RepC/C dramatically promotes DNA melting at the region corresponding to the tip of the IR-II stem-loop structure while RepC/C* showed much weaker activity in this respect. Primer extension from both directions revealed the same region of KMnO_{4} hypersensitivity. Primer extension on the bottom strand (nicking strand) showed enhanced bands at nucleotide positions 67, 68, 69, and 70, which corresponds to hyper-sensitivity of nucleotides 67 (T), 68 (A), 69 (T), and 70 (T) of the top strand to KMnO_{4} treatment; whereas, primer extension on the top strand showed enhanced bands at positions 68, 71, 73, which also correspond to the three thymidine residues on the bottom strand in the tip of the IR-II stem-loop. Since these experiments were done with a protein:DNA ratio of 15:1, it is possible that the weak melting activity seen with RepC/C* was due to the small amount of native RepC/C that is ordinarily present in these preparations. When the experiment was repeated with a protein:DNA ratio of about 1:1, essentially the same activity was seen with RepC/C, but there was no detectable activity with RepC/C* (not shown), supporting the above interpretation. On linear plasmid DNA, neither RepC/C nor RepC/C* induced any detectable KMnO_{4} sensitivity. This result is consistent with a requirement for plasmid superhelicity for the initiation of replication, given that the nicking site must be single-stranded and assuming that cruciform extrusion is responsible. These results, however, do not rigorously rule out simple melting of the nucleotides comprising the tip of the IR-II hairpin. Note the region of KMnO_{4} hypersensitivity around positions 90 and 130 on the top (leading) strand, corresponding to T-runs in an AT-rich region of the plasmid. This AT-rich region may enhance replication by facilitating melting in the IR-II region.5

**DNAase I Footprinting Analysis**—It has been shown that RepC interacts with pT181 DSO in a sequence-specific manner using DNase I and neocarzinostatin footprinting on linear double-stranded pT181 DSO DNA (7). Since RepC/C binding on supercoiled DNA affects the secondary structure of the DSO, we considered it important to compare the binding of RepC/C and RepC/C* on supercoiled with that on linear DNA. Accordingly, DNase I footprints were determined for both proteins and for linear versus supercoiled DNA. RepC/C or RepC/C* was incubated with supercoiled plasmid DNA, leaving out Mg^{2+} to prevent RepC/C-induced nicking. After 30 min at 37 °C, DNase I premixed with Mg^{2+} was added to the incubation mixture for 20 s, and primer extension was used to detect the areas protected by protein binding. The results shown in Fig. 3 indicate that both RepC/C and RepC/C* complexes were able to protect the inverted repeat sequence III (IR-III) of the double strand origin covering nucleotides 41–55. However, RepC/C* showed weaker protection of this region than did RepC/C, which suggested weaker binding. This is consistent with the dissociation constant data shown in Fig. 5. In these experiments, the RepC/C* concentration appears to be close to the determined K_{d}, which indicates incomplete DNA-protein complex formation. Furthermore, primer extension on the bottom strand, which reflects the protection pattern of the non-nicking strand,

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5 P. Z. Wang and R. P. Novick, unpublished data.
indicated that RepC/C bound more tightly and footprinted a more extensive region on supercoiled than on linear DNA, particularly in the IR-II region (60–79), consistent with its ability to enhance cruciform extrusion on supercoiled DNA. On the other hand, RepC/C* showed a very similar footprint on linear versus supercoiled DNA, including relatively weak protection of the distal arm of IR-III (41–45) and also of positions 52 and 53 in the proximal arm (plus a strong enhancement at nucleotide 60), none of which are seen with RepC. Surprisingly, primer extension on the top strand, which reflects the protection pattern of the nicking strand, showed very similar footprints by both RepC/C and RepC/C*. Only the IR-III region, but not the IR-II region, was protected by either protein. RepC/C showed stronger protection on supercoiled relative to linear DNA, and both RepC/C and RepC/C* showed enhanced bands at positions 35 and 36. No enhanced band at position 70 (nick site) was observed, indicating that there was no significant RepC/C-induced nicking following the addition of Mg$^{2+}$ along with the DNase I. The above results suggest that on supercoiled DNA, RepC/C binds to IR-III and promotes or enhances DNA melting, that the resulting stem-loop structure in turn interacts with RepC/C, and that this interaction stabilizes the cruciform and reciprocally enhances the binding of RepC to IR-III.

**DNA Bending Analysis of RepC/C and RepC/C* on the pT181 DSO**—The pT181 DSO has been shown to contain a static bend that is enhanced by RepC/C binding (4). Static bends in DNA may facilitate recognition of the DNA by specific binding proteins; protein-induced or enhanced bending of DNA has been shown to play an important role in facilitating gene expression, DNA replication, and other biological processes. Using a set of circularly permuted fragments containing the pT181 DSO, we have qualitatively and quantitatively measured the relative mobility of the RepC/C-DNA and RepC/C*-DNA complexes on a polyacrylamide gel. The results, shown in Fig. 4B, demonstrate clearly that RepC and RepC/C* have dramatically different DNA bending abilities. In our experiments, we found that RepC/C purified from *S. aureus* produced the same mobility shift patterns as that from *E. coli* as described by Koepsel *et al.* (7). RepC/C*-DNA complexes showed a significantly smaller mobility shift than the RepC/C-DNA complexes. As have been shown in the permanganate probing as-

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**FIG. 4.** Gel mobility shift assay showing the DNA binding and bending ability of RepC/C and RepC/C* complexes. A, schematic diagram showing the DNA fragments used in the gel mobility shift assays. B, gel shift analysis of permuted pT181 DSO containing DNA fragments in the presence of RepC or RepC/C* complexes. C, the above results are plotted against the position of the restriction cleavage sites (measured from the right end of the fragment). [ ], RepC; [ ], RepC**.
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saying, no cruciform formation was detected on linear DNA in the presence of either RepC/C or RepC/C*. We conclude that the differential mobility shifts are merely due to their differential bending ability although cruciform formation has been known to change the mobility of DNA fragments. In Fig. 4C are shown the results from Fig. 4B plotted according to the positions of the restriction sites used to generate the fragments (Fig. 4A). This plot shows that the bending site for RepC/C* is the same as that for RepC/C, consistent with the observation that RepC/C footprinted the same region (IR-III) of the DSO. In contrast, the RepC/C* heterodimer was shown to bend DNA to a much lesser extent than RepC, which accounts for the smaller mobility shifts observed with the former. In this gel mobility shift experiment, the intensity of the retarded RepC/C* DNA bands were much weaker than the RepC/C-DNA bands. This is consistent with the footprinting experiments in which RepC/C* showed weaker affinity for the DSO than RepC.

**Dissociation Constants (Kd) of Rep-DNA Complexes**

As shown above, RepC/C* can bind and bend the pT181 DSO similarly to RepC/C although less strongly. Additionally, it can inhibit RepC/C activities in vitro and is ordinarily present at 10× the concentration of RepC/C (1).²

These results have raised the question of whether RepC/C* has any role in pT181 replication in vivo, for example, as a negative regulator. As the beginning of an investigation of this possibility, we have determined the relative binding affinities of RepC/C and RepC/C* for the pT181 DSO. This was done by calculating the respective dissociation constants on the basis of gel mobility analysis. Fixed amounts of the two Rep proteins were titrated with increasing amounts of a DSO-containing fragment, and polyacrylamide gels were used to separate the Rep-DNA complex from free DNA. Measurement of the radioactivity of the free and retarded DNA bands gave the results shown in Fig. 5. Kₘ for RepC/C-DNA and RepC/C*-DNA complexes were estimated to be 40.5 and 158.2 pm, respectively, by Scatchard plot (Fig. 5).

**DISCUSSION**

The objective of this study was to determine whether there are particular differences in specific properties of RepC/C versus RepC/C* that might help to define the in vivo role of RepC/C* and pinpoint the basis for the ability of RepC/C but not RepC/C* to initiate replication. Three different methodologies were used, namely, in vitro footprinting, KMnO₄ sensitivity, and gel mobility analysis. Each of the three methods revealed one or more critical features that together have enabled us to formulate a model for the initiation of replication and an appreciation of the key defect in RepC/C*.

As noted above, the leading strand replication origin of pT181 plasmids consists of two adjacent inverted repeats (IR-II and IR-III), which are involved in RepC recognition (3). The IR-III region is divergent in sequence among the members of the pT181 family and distinguishes the origin region of one member from that of the others. Only the IR-III proximal arm is required for sequence specific recognition (12). Thus, unlike many other dimeric DNA-binding proteins, RepC/C binds asymmetrically, and its sequence-specific binding to IR-III is stabilized by its interaction with the IR-II region. The IR-II region is conserved among the six members of the pT181 family; it contains the nick site for replication initiation and forms a cruciform structure in vivo as well as in vitro (5). Like other RC initiators, RepC/C has much higher nicking activity on single-stranded than on double-stranded DNA. Extrusion of the IR-II cruciform is thought to provide the necessary single-stranded region in the GC-rich pT181 DSO (5).

Our in vitro permanganate probing assays have shown that RepC/C is able to melt the DSO DNA on a supercoiled but not on a linear substrate. Sequence considerations make it likely that the observed melting pattern represents a cruciform; however, proof of this structure requires definitive experiments with cruciform-specific enzymes that are planned. The importance of plasmid superhelix for cruciform extrusion is correlated with the importance of superhelix for the initiation of replication on all circular prokaryotic replicons, including pT181. It is suggested that the inability of RepC/C* to induce melting/cruciform extrusion is the basis for its inability to initiate replication.

Bending of DNA in response to regulatory protein binding is a widely observed phenomenon. Bending may facilitate the interaction of the regulatory protein with upstream or downstream DNA sequences, it may facilitate protein-protein interactions, it may introduce favorable conformational changes in
DNA structure, and it may play an energetic role for biological function. RepC has been shown to enhance the bending of pT181 DSO, and the center of the induced bend corresponds to the RepC binding site (4, 7) although the exact biological significance of the RepC enhanced bending is still not clear. Gel mobility shift analysis with the two dimeric forms of RepC showed clearly that both proteins bind in a similar manner to the DSO and both enhance the previously observed bend in the same way. The bending center for both RepC/C and RepC/C* is the same. RepC/C*, however, produces a considerably smaller gel shift than RepC/C because it enhances the natural bend to a considerably smaller extent. It is notable that no binding pattern was observed that might indicate dissociation of either of the two dimeric forms. The weaker activity of RepC/C* on DNA bending might contribute to its inability to enhance melting and to initiate replication.

DNase I footprinting analysis also revealed a critical difference between RepC/C and RepC/C*. Both forms of the protein showed similar protection patterns on linear DNA (confined to the IR-III region), though weaker for RepC/C*. Very weak protection in the IR-II region by RepC/C on the lagging strand was sometimes detected. This result is different from that reported previously for RepC/C by Koepsel, et al. (7), who observed protection of IR-II on linear DNA but with a much higher protein:DNA ratio (300:1) than that used here. This additional protection presumably represented much weaker or even nonspecific binding, owing to the very high protein concentration used. On supercoiled DNA, RepC/C* had the same protection pattern as on linear DNA; whereas, RepC/C additionally and strongly protected the proximal arm of IR-II. Interestingly, this protection involved the lagging strand but not the leading strand, which contains the nick site. The results confirm the asymmetric binding of RepC to the DSO as previously proposed on the basis of mutational analysis (12). The structure of the RepC/C-DNA complex is predicted to show one subunit binding to IR-III plus the proximal arm of IR-II on the lagging strand and the second subunit positioned to nick the leading strand. Recent observations, suggesting that a RepC heterodimer with one subunit defective in nicking the other defective in IR-II binding has weak replication activity, suggest that one subunit is responsible for binding and the other for nicking.6

The critical differences between RepC/C and RepC/C* are thus the greater affinity of RepC for the DSO, differential binding to supercoiled versus linear DNA, and differential ability to enhance cruciform extrusion. We suggest that the strong binding of RepC/C to IR-III (plus bending) coupled with the free energy of superhelix formation initiates melting (and, presumably, cruciform extrusion) in the IR-II region. RepC/C then binds to the proximal arm of the IR-II cruciform, which stabilizes the secondary structure and facilitates nicking.

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