

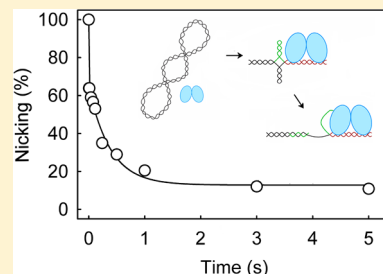
Kinetic Mechanism of Initiation by RepD as a Part of Asymmetric, Rolling Circle Plasmid Unwinding

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S Supporting Information

ABSTRACT: Some bacterial plasmids carry antibiotic resistance genes and replicate by an asymmetric, rolling circle mechanism, in which replication of the two strands is not concurrent. Initiation of this replication occurs via an initiator protein that nicks one DNA strand at the double-stranded origin of replication. In this work, RepD protein from the staphylococcal plasmid pC221 carries this function and allows PcrA helicase to bind and begin unwinding the plasmid DNA. This work uses whole plasmid constructs as well as oligonucleotide-based mimics of parts of the origin to examine the initiation reaction. It investigates the phenomenon that nicking, although required to open a single-stranded region at the origin and so allow PcrA to bind, is not required for another function of RepD, namely to increase the processivity of PcrA, allowing it to unwind plasmid lengths of DNA. A kinetic mechanism of RepD initiation is presented, showing rapid binding of the origin DNA. The rate of nicking varies with the structure of the DNA but can occur with a rate constant of $>25 \text{ s}^{-1}$ at $30 \text{ }^\circ\text{C}$. The equilibrium constant of the nicking reaction, which involves a transesterification to form a phosphotyrosine bond within the RepD active site, is close to unity.



Bacterial plasmids are extrachromosomal genetic elements, characterized by an autonomous replication mechanism when transferred into a suitable host. Understanding how such plasmids transfer and replicate provides important information about how such genetic material might be controlled. In this context, it is important to understand control elements that differentiate plasmid replication from that of chromosomal DNA, for example, to provide potential targets for drugs to impede the spread of these plasmids. Some bacterial plasmids use a mechanism of replication different from the bidirectional mechanism that is normal for the chromosomal DNA. In the latter, the double-stranded DNA (dsDNA), separated at the origin, is simultaneously unwound in each direction by helicases, allowing DNA replication on both strands moving away from the origin. An alternative mechanism is termed asymmetric, rolling circle replication and is used by plasmids, including those belonging to the pT181 family, for example, those from *Staphylococcus aureus* that contain antibiotic resistance genes.¹ These plasmids are transferred readily between Gram-positive organisms, thereby transmitting selective advantage.

Asymmetric, rolling circle replication starts with the interaction between a replication initiator protein (Rep), which is encoded on the plasmid, and the double-stranded origin of replication (DSO) sequence on the plasmid. In this work, the particular protein RepD from staphylococcal plasmid pC221 interacts with the DSO sequence, *oriD*.² RepD binds as a dimer with high affinity to an inverted complementary repeat, ICRIII, that is contained within the DSO (Figure 1A), so RepD is brought into the proximity of the neighboring ICRII sequence.³ The latter is often assumed to have a cruciform structure, as shown in the cartoon in Figure 1A, although its actual structure may depend on the degree of DNA supercoiling and the

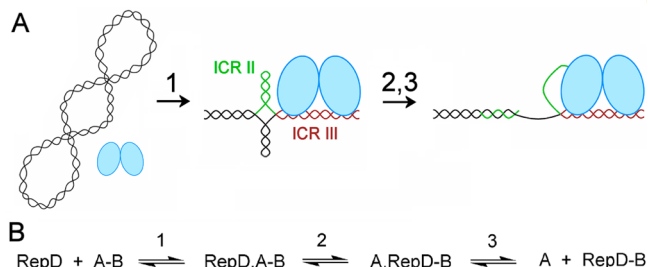


Figure 1. Schematic representation of the first steps of asymmetric replication of the T181 family plasmid. (A) Interaction of RepD with *oriD* (step 1, with numbering equivalent to the scheme in panel B) followed (steps 2 and 3) by nicking to produce a single-stranded region to allow PcrA to bind and to release the free 3'-end for the polymerase to begin replication. The strand of ICRII that is nicked is colored green; ICRIII is colored brown. (B) Simplified reaction scheme for RepD binding an appropriate DNA structure (A-B with the nicking site between sequences A and B), nicking, and then dissociation of the 3'-end of nicked DNA product (A) from RepD. Steps are numbered so that for step i , the rate constants for the forward and reverse reactions and the equilibrium constant are k_{+i} , k_{-i} , and K_i , respectively.

binding of initiator proteins.³ RepD nicks one strand [the (+)-strand] of the DNA within the ICRII sequence, resulting in a covalent phosphodiester between the tyrosine within the active site of RepD and the 5'-end of the nicked, (+)-strand DNA.^{4,5} (+)-Strand elongation is initiated by PcrA helicase and

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DNA polymerase III from the host. PcrA binds to the exposed single-stranded DNA (ssDNA) of the (–)-strand and unwinds in the 3′- to 5′-direction, relative to this strand. RepD then remains part of the replication complex during the complete synthesis of a new (+)-strand of the plasmid DNA, using the parental (–)-strand as the template for the polymerase.⁴ RepD remaining associated with PcrA during this process results in displacement of a ssDNA loop. The (+)-strand extension continues around the plasmid until it reaches the DSO again. At this point, RepD performs a series of transesterifications to produce strand exchange, to resolve the complex,⁵ probably using nicking sites on each monomer of the dimer. The complete, double-stranded plasmid is released, along with a circular single-stranded plasmid, created from the (+)-strand and RepD as an inactive form, covalently modified with a short length of DNA.⁶ The displaced single-stranded plasmid is able to initiate replication from a region of secondary structure, the single-stranded origin (SSO).¹ Finally, the new plasmid, currently in a relaxed, closed form, is supercoiled by a DNA gyrase.

RepD causes a very large improvement in the ability of PcrA to unwind double-stranded DNA (dsDNA). PcrA is unable to unwind DNA substrates as short as 30 bp on its own, yet in the presence of RepD, PcrA becomes highly processive: a single PcrA molecule can unwind plasmid lengths of DNA without significant, premature termination.⁷ Thus, RepD has significant roles in three main stages of replication. As well as being responsible for nicking at initiation and the strand exchange at termination, RepD seems to be essential for the high processivity of PcrA during unwinding. This study describes the kinetic mechanism of the formation of the initiation complex among *oriD*, RepD, and PcrA and characterizes these separate, albeit linked, functions of RepD.

Rolling circle plasmid replication is a complex process that requires specific interactions between DNA and three different proteins, RepD, PcrA, and DNA polymerase III, each with essential roles in producing a copy of the (–)-strand template. Although there have been several investigations of the role of Rep proteins, there are considerable gaps in our understanding of their detailed mechanism. There is no crystal structure of a replication initiator of this protein family, but it is known that the different members have specificity for the matching ICRIII sequence and that this part of the DSO is mainly responsible for the affinity of RepD for the plasmid.² RepD mutations have been central to delineating its activity, and the positions of these mutations will be discussed in relation with the sequence for the RepD used here.⁸ The position for formation of the DNA–RepD covalent bond is Y188,⁴ although a mutation at this position, Y188F, did not affect its noncovalent binding to ICRIII.² Another RepD variant, R186K (published as R189K using different numbering), has the mutation two residues from the active tyrosine, meaning it is likely to be within the RepD active site. This variant may form a covalent DNA–protein complex but is religation deficient: in other words it does not allow the final processing to re-form circular DNA.⁹ This study describes the activity of (N189K)RepD with the mutation at a position +1 from the active tyrosine. Because this mutant retains the ability to form a covalent attachment to the DNA, albeit impaired, it is useful to study the nicking and binding properties of RepD.

A transesterification involving primary alcohols in the absence of the protein and plasmid framework might be expected to have an equilibrium constant close to unity: one

phosphodiester bond is broken, but another is formed. In practice, the reaction seems driven toward nicking by the supercoiling of the plasmid,⁴ and in addition, Rep proteins seem to induce formation of a cruciform at the nicking site, thereby ensuring that the DNA at this position is not base-paired.^{3,10} Biochemical evidence supports the model in which PcrA interacts directly with RepD: RepD recruits PcrA helicase at *oriD* and increases the affinity of PcrA for this region.⁹ Although RepD increases PcrA processivity,^{7,11,12} it is possible that this is due to the topology of the DNA at the unwinding junction, a result of the interaction of RepD with the DNA and steric blocking of premature PcrA dissociation. Once DNA is unwound past the ICRIII sequence, the proteins must remain bound even though RepD is no longer interacting with its high-affinity site. During unwinding, the (+)-strand forms a single-stranded loop due to the fact that the 5′-end is tethered to RepD through a covalent bond: such loops have been observed by atomic force microscopy.¹²

Factors relating to RepD that are important for successful initiation and strand separation are described here. As part of this, the kinetic mechanism of the initiation reaction is described in terms of RepD and PcrA loading. In particular, there is reversible binding and nicking, as indicated in Figure 1B. Previous work⁷ described the use of Y-shaped oligonucleotide junctions to mimic part of *oriD* that is needed for initiation and strand separation. Fluorescence labeling gave signals that are correlated with protein binding and dissociation of the nicked DNA 3′-end. Here, this is extended by using the techniques to examine different structural mimics of parts of *oriD* and combined with rapid-mix quenched flow to study the nicking reaction itself. Some of the features that lead to successful initiation, in terms of the structure of the DNA prior to RepD nicking, are investigated. Furthermore, it is demonstrated that there is not an absolute requirement for RepD to nick and covalently bind the DNA. Although this function is required for initiation of plasmid unwinding in terms of producing a site for PcrA to bind, it is not necessary for the unwinding itself.

■ EXPERIMENTAL PROCEDURES

PcrA from *Bacillus stearothermophilus* and RepD from *S. aureus* were prepared as previously described.^{2,7,13} The (N189K)RepD gene was generated using the QuikChange II site-directed mutagenesis kit and protocol (Stratagene) using primers containing the single mutation (CGTGACAGTGATAGATTTATTAGAAATTTATAAAAAAAAAACAAGAACG and GGCGTTATCTTTACGTTCTTGTTTTTTTTATAAATTCTAATAATC), and the newly generated gene was confirmed by sequencing. Diethylaminocoumarin-labeled single-stranded DNA-binding protein (DCC-SSB) was prepared and characterized as described previously.^{14,15}

Oligonucleotides, either unlabeled or labeled with fluorescent dyes, were from Eurofins Ltd. DNA junctions were made by mixing two oligonucleotides as described previously.⁷ A construct containing the whole *oriD* was also generated by annealing two synthetic oligonucleotides [OriD1 and OriD2 (Figure 2)]. DNA plasmids of different lengths, based on the pCERoriD plasmid, which contains *oriD*,¹¹ were prepared with 2437, 3094, 3650, 4907, 6086, and 6642 bp, as described previously.⁷ The 4907 bp plasmid was digested using the NdeI restriction enzyme (New England Biolabs) under the appropriate conditions at 37 °C for 2 h to produce linear dsDNA with *oriD* positioned around the middle of the plasmid length. The digest was run on a 1% (w/v) agarose gel. The DNA fragment was visualized,

Y-junction	
DS-junction	
Hinge-junction	
OriD-1	5'-GCTTTAGACAATTTTTCTAAAACCGGCTACTCTAATAGCCGGTTAAGTGGTAATTTTTTACCACCC-3'
OriD-2	5'-GGGTGGTAAAAAATTACCACTTAACCGGCTATTAGAGTAGCCGGTTTTAGAAAAATTGTCTAAAGC-3'
ICRII-part	5'-CTAATAGCCGGTT-3'
30 bp-F (Cy3/Dabcyl Y-junction without ICRIII)	
20 bp-F (Cy3/Dabcyl Y-junction without ICRIII)	

Figure 2. Structure of DNA junctions. The circled F shows the position of the fluorescein label, when present. The arrow shows the nicking site. The ICRII and ICRIII sequences are labeled, but using a dotted line where only part of ICRII is present. Two lengths of the Y junction are shown with Cy3/Dabcyl labeling but with random sequence in the dsDNA rather than ICRIII, used for the unwinding assay; other lengths have been described previously.⁷

removed from the gel, and purified using the QIAquick gel extraction kit (Qiagen).

Nicking Assay on Agarose Gel Electrophoresis. The nicking reaction was performed at 30 °C, via incubation of 15 nM pCERoriD plasmid and 60 nM RepD in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 1 mM EDTA, and 10% ethanediol and quenching with 50 mM EDTA at different times. The DNA topology of products was analyzed on 1% agarose gels in 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA containing 1 μg/mL ethidium bromide. Visualization was achieved by illumination using a trans-illuminator (Uvitec, Cambridge, U.K.), and gel bands were quantified using Uvitec software.

Nicking Assay via Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Nicking was achieved by mixing 1 μM RepD and 10 μM DNA. Reactions were conducted in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% (v/v) ethanediol, 200 mM KCl, and 10 mM MgCl₂, for 10 min at 30 °C. RepD nicking was analyzed using PAGE (Bio-Rad mini gel system) under SDS denaturing conditions. Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad).

Gel Shift Assay. The extent of RepD binding to Y junction DNA (as in Figure 2) was determined by incubating 1 μM DNA with 0, 1, 3, 5, 10, or 15 μM RepD at 30 °C for 10 min in binding buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM KCl, and 5 mM EDTA. Samples were then applied to a 6% polyacrylamide native gel (Bio-Rad mini gel system). The gel was run in 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA (pH 8.0) at 200 V for 30 min and then stained with SYBR gold (Invitrogen). Visualization was achieved using a UVitec trans-illuminator.

Stopped-Flow Kinetic Measurements. These were performed using a Hi-Tech stopped-flow apparatus with a xenon–mercury lamp (TgK Scientific). Solution conditions and concentrations for measurements are indicated in the figure legends. In all the experiments described, the stated concentrations are those in the mixing chamber and reactions were conducted at 30 °C. For DCC-SSB fluorescence, the excitation wavelength used was 436 nm with a 455 nm cutoff filter (Schott glass). Cy3 was excited at 548 nm, with a 570 nm cutoff filter (Schott glass) on the emission. Anisotropy was measured with the instrument in the T format, allowing simultaneous acquisition of parallel (*I*_{||})

and perpendicular (I_{\perp}) components. This allowed anisotropy, $(I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, and intensity, $I_{\parallel} + 2I_{\perp}$, to be calculated from the same set of data. Because the fluorescence intensity change was very small, anisotropy data were not corrected for different species having different intensities. Fluorescein was excited at 497 nm with a 515 nm cutoff filter. Fluorescence data were analyzed on Kinetic studio (TgK Scientific) or Grafit.¹⁶

Quench-Flow and High-Performance Liquid Chromatography (HPLC) Analysis of Nicking. Quench-flow experiments were performed using the RQF-63 Hi-Tech Rapid Quench Flow system (TgK Scientific) to elucidate the extent and kinetics of nicking of various DNA constructs by RepD. For oligonucleotide-based junctions, labeled with fluorescein, RepD (3 μM) was mixed with DNA (1 μM) (concentrations after mixing) in 50 mM Tris-HCl (pH 7.5), 200 mM KCl, 10 mM MgCl_2 , 1 mM EDTA, and 10% ethanediol. Concentrations are those after mixing. Quench-flow products were analyzed with a gradient HPLC system (Waters), equipped with the Waters 2475 multiwavelength fluorescence detector and the Waters 2487 dual λ absorbance detector. The DNA was analyzed on a 1 mL Resource Q ion-exchange column, run at 1 mL/min with a linear gradient (15 mL) from 250 mM to 1 M NaCl in 10 mM NaOH. Fluorescein was excited at 497 nm with emission at 519 nm. Peaks were integrated using the Waters HPLC software package, Empower 2, including correction for any sloping baseline.

For quench-flow measurements using a DNA plasmid, 3650 bp pCERoriD (30 nM) was mixed with 150 nM RepD (concentrations after mixing) in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl_2 , 1 mM EDTA, and 10% ethanediol, and then the reaction was quenched with 25 mM EDTA (pH 7.5) at 30 °C. Products were separated on an agarose gel, as described above, and bands were quantified using UVIpro (UVItec, Cambridge, U.K.).

RESULTS

Nicking Kinetics. It has been shown previously that Y junctions, made from two partially base-paired oligonucleotides and shown in Figure 2, are substrates for RepD and PcrA with similar unwinding kinetics as a whole plasmid.⁷ These junctions have ICRIII within the duplex region. The single-strand 5'-arm contains part of the ICRII sequence, including the nicking site. The other arm is dT₁₀, providing a site to which PcrA can bind. In particular, junctions with fluorescein attached gave signals that allowed the measurement of RepD binding kinetics.⁷ The rapid binding is accompanied by an increase in fluorescence anisotropy, consistent with formation of a larger complex,

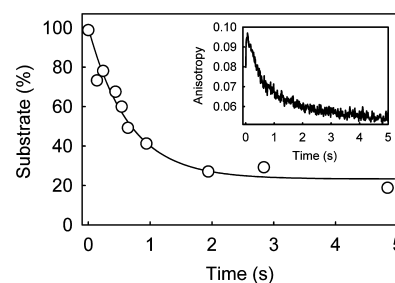


Figure 3. Time course of nicking the fluorescein Y junction with excess RepD. Quench-flow time course of nicking using EDTA as the quench and HPLC to analyze product formation as described in Experimental Procedures. The line is the best fit exponential to the data, giving a rate constant of $1.5 \pm 0.2 \text{ s}^{-1}$ for nicking. Reproducibly $\sim 80\%$ of the DNA is nicked. The inset shows the fluorescence anisotropy at 800 nM RepD and 100 nM DNA for comparison.

including the fluorophore with a concomitant increase in rotational correlation time. There was then a slow decrease in anisotropy, which likely correlates with dissociation of the small nicked DNA, containing the fluorescein.

Such fluorescence measurements did not allow measurement of the nicking reaction kinetics. To obtain those directly, we mixed RepD and the fluorescent Y junction in a quench-flow instrument, to obtain rapid mixing and reaction for a short time. We quenched the reaction by mixing with 50 mM EDTA at specific times, which rapidly reduces the free Mg^{2+} concentration and stops the nicking reaction.⁴ The quenched solution was then analyzed for fluorescent DNA species by HPLC (Figure S1 of the Supporting Information). Quenching at different times gave the time course of the nicking reaction (Figure 3). This measurement does not distinguish between bound and dissociated fluorescein–DNA forms because HPLC is conducted under denaturing conditions. This shows that the nicking reaction at 1.5 s^{-1} is very similar to the slow decrease of the anisotropy, shown in the inset of Figure 3, suggesting that the chemical step, nicking, is rate-limiting and is followed by rapid release of the nicked DNA. The amplitude of the quench-flow transient shows $\sim 80\%$ of the DNA is nicked (Table 1). A gel shift assay showed that for the conditions of this measurement and others described below with DNA junctions, such as quench-flow or other nicking assays ($\leq 3 \mu\text{M}$ RepD), only a single RepD dimer is bound to the DNA (Figure S2 of the Supporting Information).

Dependence of Nicking on DNA Structure. This Y junction allows successful initiation and unwinding, even though

Table 1. Summary of Rate Constants for Binding and Nicking^a

	binding ($\mu\text{M}^{-1} \text{ s}^{-1}$)	nicking			
		fast phase		slow phase	
		s^{-1}	% of signal	s^{-1}	% of signal
Y junction	109 ± 3	1.5 ± 0.2	77 ± 4	—	—
DS junction	259 ± 38	15 ± 3	41 ± 4	0.56 ± 0.12	31 ± 2
hinge junction	175 ± 4	>0.5	15 ± 5	—	—
plasmid with wild-type RepD	not determined	>25	35 ± 2	2.6 ± 0.4	52 ± 3
plasmid with (N198K)RepD	not determined	0.0031 ± 0.0001	86 ± 1	—	—

^aBinding was measured by anisotropy with fluorescein-labeled DNA; nicking was measured by quenching and gel or HPLC analysis of product DNA. Wild-type RepD was used unless otherwise stated. Only single phases in the nicking reaction were determined with the Y junction and hinge junction and with the plasmid nicked by (N189K)RepD. In all cases, a small fraction of the DNA did not react. See the text for details. The data are for a single set of measurements, but all sets were repeated.

only a partial ICRII sequence is present as a single strand. Several different structures were examined to see how the form of ICRII, presented to RepD, affects the nicking by either altered kinetics or thermodynamics. The reactions with various DNA structures were followed by a combination of fluorescence stopped-flow, quench-flow, and gel assays. As shown previously,^{2,17} only a short ssDNA containing part of ICRII is required for cleavage. Thus, oligonucleotide CTAATAGCCGGTT [ICRII-part (Figure 2)], incubated with RepD, results in quantitative formation of RepD-AATAGCCGGTT, as shown by the gel in Figure 4A and identified by mass spectrometry.

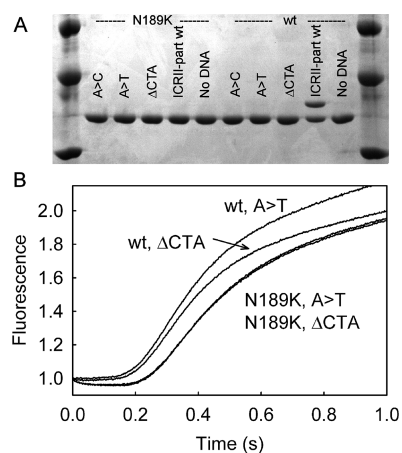


Figure 4. RepD nicking and translocation activities on DNA containing mutations in the nicking site. (A) Nicking activity of the ICRII-part oligonucleotide (Figure 2) analyzed by SDS–PAGE (see Experimental Procedures for details). Variants of ICRII-part were obtained by deleting the first three bases (mutation Δ CTA) or changing the nick site A to T (mutation A > T) or to C (mutation A > C). The molecular mass standard bands in order from top to bottom are 66, 45, and 31 kDa, respectively. Note that only one subunit of the dimer becomes DNA-bound. The control with the wild-type sequence of ICRII-part is also shown. (B) Comparison of unwinding by wild-type RepD and (N189K)RepD using the “20 bp-F” Cy3-Dabcyl-labeled junction (Figure 2). Nick site variants of this junction were Δ CTA and A > T, as described above. See Figure S8 of the Supporting Information for concentrations and conditions. The traces are normalized to an initial value of 1.

The binding of RepD and nicking of three types of oligonucleotide junctions were compared by measuring the kinetics of fluorescence anisotropy changes and the quench-flow reaction, as described above. The results are summarized in Table 1. These junctions, whose structures are in Figure 2, differ only in the form of the ICRII, and all share the same dsDNA sequence containing ICRIII. The Y junction is described above. The hinge junction contains the complete ICRII sequence on one arm and so is likely to have the hairpin structure shown in the representation of *oriD* in Figure 1. The DNA can form a hinge at the connection between ICRII and ICRIII. The DS junction is a completely complementary double strand containing part of the ICRII sequence, so the nicking site is presented as a double strand, not hinged. These therefore represent three classes of possible structures of ICRII that might be present in the plasmid, albeit lacking supercoiling.

With fluorescein labeling, both the hinge junction and DS junction showed a similar rapid increase in fluorescence anisotropy upon being mixed with RepD (Figure 5), as described previously for the Y junction⁷ and shown in the inset of Figure 3.

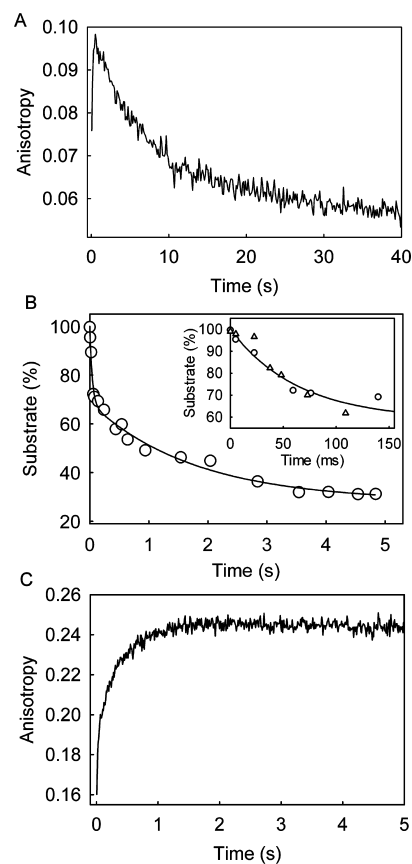


Figure 5. Time course of fluorescence anisotropy and quench-flow analysis on mixing the fluorescein hinge and DS junctions with excess RepD. Solution and experimental conditions are as in the legend of Figure 3. (A) Fluorescence anisotropy at 800 nM RepD with the DS junction (100 nM). (B) Quench-flow time course of DS junction nicking with EDTA quench. The line shows a best fit double exponential. Following a burst, the slow phase has a rate constant of $0.56 \pm 0.12 \text{ s}^{-1}$. The inset shows shorter time points to define the rapid phase. Circles are time points using EDTA as the quench, and triangles are from a quench by 5% perchloric acid. The solution was then neutralized with sodium acetate. The two sets of data are the same within experimental error, and the line is the best fit single exponential with a rate constant of $15 \pm 3 \text{ s}^{-1}$. (C) Fluorescence anisotropy at 800 nM RepD with 100 nM hinge junction.

Analysis of this binding phase involved measuring the observed rate constant as a function of RepD concentration under pseudo-first-order conditions (Figure S3 of the Supporting Information). This gave rate constants of $109 \mu\text{M}^{-1} \text{ s}^{-1}$ for the Y junction, $259 \mu\text{M}^{-1} \text{ s}^{-1}$ for the DS junction, and $175 \mu\text{M}^{-1} \text{ s}^{-1}$ for the hinge junction (Table 1). In addition, a junction totally lacking the ICRII arm or having random sequence in this arm gave a rate constant of 85 or $124 \mu\text{M}^{-1} \text{ s}^{-1}$, respectively (data not shown). This suggests that all these junctions have similar, rapid binding kinetics, presumably dominated by binding of RepD to ICRIII, which is common to all the junctions. In contrast, the second phase of the anisotropy traces showed kinetics that differed greatly among the three junctions (Figures 3 and 5).

The DS junction had a much slower decrease in anisotropy (0.13 s^{-1}) (Figure 5A), but like that of the Y junction, the anisotropy returned to approximately its starting value. Note that the long time-base traces shown in Figures 3 and 5 do not show this starting value clearly because of the rapid initial increase. The slow decrease in anisotropy presumably

represents slow release of the four-nucleotide product containing the fluorescein from the protein complex. Quench-flow–HPLC analysis (Figure 5B and Table 1) showed a rapid phase of nicking (15 s^{-1}) with an $\sim 40\%$ amplitude. There was then a slow phase at 0.56 s^{-1} , resulting in a further 30% reaction. A potential explanation is that for this junction, there is rapid nicking to form the equilibrium amount ($\sim 50\%$ for these conditions) of the RepD–DNA–CTCT–fluorescein complex. There is then slow release of product, resulting in further nicking. The difference between the kinetics of the anisotropy and quench-flow time courses will be reviewed in Discussion.

Note that the quench-flow data of the DS junction at short times (Figure 5B, inset) showed that there is little or no difference between using EDTA or acid as a quencher. This supports the use of EDTA as an effective, rapid-quench reagent for this reaction and the fact that release of Mg^{2+} from the reactive site of RepD is rapid.

The hinge junction shows a similar rapid, initial increase in anisotropy, but this is followed by a further, large increase at 2.4 s^{-1} (Figure 5C). Finally, there is a very slow, small decrease at 0.07 s^{-1} (data not shown). For this junction, presumably not much product dissociates, although the physicochemical basis of the second phase increase is not clear. Quenching the reaction at times between 5 and 120 s showed a constant $\sim 15\%$ nicking by HPLC (data not shown). These data suggest that nicking ($>0.5\text{ s}^{-1}$) occurs but only to a small extent (Table 1), so a time course was not attempted by the quench-flow method. This is consistent, at least qualitatively, with the anisotropy time course of this type of junction, described above.

For all three junctions, the kinetics of PcrA-driven unwinding were measured, as previously described,⁷ using the signal provided by Cy3 and Dabcyl labels at the distal end of the duplex, which now has random sequence rather than ICRIII to mimic unwinding of the plasmid away from *oriD* (data not shown). There were relatively small differences in translocation rates: 62 bp/s for the Y junction, 34 bp/s for the DS junction, and 86 bp/s for the hinge junction. There were also differences in the amplitudes, but it is difficult to correlate this between different preparations of DNA, which, apart from structure, may have different percent labeling.⁷

Plasmid Nicking. Nicking was also measured for a supercoiled plasmid (3650 bp) containing the whole *oriD* sequence.⁷ Manual mixing shows rapid and essentially complete nicking to form the open, relaxed structure as visualized on an agarose gel (Figure 6A).⁶ This simple assay gives a topological analysis of the plasmid: the supercoiled plasmid, substrate for RepD, runs differently from the relaxed form produced by RepD nicking. Quench-flow mixing was then used to obtain greater time resolution. RepD and the supercoiled plasmid were mixed, and the reaction was then quenched with 50 mM EDTA at different times. The reaction products were separated on an agarose gel and the bands quantified to obtain a time course of the nicking reaction (Figure 6C). This shows a rapid burst at $>25\text{ s}^{-1}$ (too fast to obtain a rate constant) with an $\sim 35\%$ amplitude followed by a slow phase at $\sim 2.5\text{ s}^{-1}$ with a further $\sim 50\%$ reaction (Table 1).

On a longer time scale (Figure 6A), a new gel band appears, corresponding to a closed, circular plasmid form,⁶ formed by religation of the DNA ends. This slow reaction results in 23% closed, relaxed plasmid at apparent equilibrium with nicked, open DNA. This is likely to represent the equilibrium constant for the nicking reaction of the relaxed plasmid–RepD complex in the absence of supercoiling. Quantitation of the gel bands as

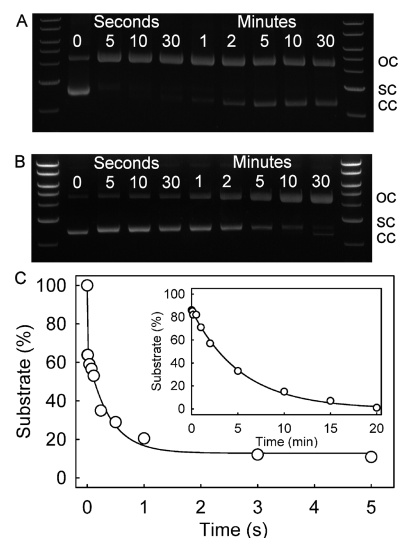


Figure 6. RepD nicking–closing activity on the 3650 bp pCERoriD plasmid for (A) wild-type RepD and (B) (N189K)RepD. Reactions were initiated by addition of 60 nM RepD to 15 nM pCERoriD in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl_2 , 1 mM EDTA, and 10% ethanediol at 30°C . At the time points indicated (in seconds and minutes), samples were removed and quenched by addition of 50 mM EDTA. Samples were analyzed on a 1% agarose gel. The plasmid topology is indicated by the bands (SC, supercoiled; OC, open, circular, nicked RepD complex; CC, closed, circular). Molecular mass markers are in order from top to bottom 10, 8, 6, 5, 4, 3, and 2 kb, respectively. (C) Quench-flow time course, analyzed on a 1% agarose gel of wild-type RepD nicking on the 3650 bp supercoiled plasmid. See Experimental Procedures for concentrations and conditions. The line is the best fit exponential for the slow phase that follows a burst. The burst at $>25\text{ s}^{-1}$ has an $\sim 35\%$ amplitude followed by a slow phase at $\sim 2.6 \pm 0.4\text{ s}^{-1}$ with a further 50% reaction. The inset shows the equivalent time course with (N189K)RepD, but using manual mixing. The line is the best fit exponential giving a rate constant of $0.0031 \pm 0.0001\text{ s}^{-1}$.

a function of time gives a rate constant of 0.0037 s^{-1} for this religation (Figure S4 of the Supporting Information).

Requirement for Nicking. RepD binding and nicking on the ICRII sequence represent the first events in the plasmid replication. The following step is the recruitment of PcrA helicase onto the (–)-strand of the *oriD* sequence. Nicking is presumably essential for loading of PcrA on the plasmid, as this is the way in which ssDNA is created in the correct position for this helicase to bind.

The N189K mutant of RepD has been used to investigate further the interaction of RepD with DNA. (N189K)RepD bound to the Y junction with kinetics similar to those of wild-type RepD (wtRepD), as shown using a fluorescent junction⁷ (Figure S3 of the Supporting Information). The activities of wild-type and mutant RepD on the supercoiled pCERoriD plasmid were initially compared using agarose gel electrophoresis (Figure 6). wtRepD has high nicking activity with supercoiled plasmid, as described above: almost all the DNA was converted to the open circular form within 5 s of mixing (Figure 6A). Then some closed, relaxed, circular plasmid formed slowly by religation. In contrast, the nicking of supercoiled plasmid by (N189K)RepD was much slower (Figure 6B): integration of the bands gives a rate constant of 0.003 s^{-1} (Figure 6C, inset), ~ 1000 -fold slower than that of wtRepD (Table 1). A faint band was detected for the closed circular form after 30 min, and after further incubation, the amount of this band (24%) remained

constant (data not shown). An equilibrium was reached between closed relaxed and open nicked DNA, similar to that of wtRepD.

After incubation with either wild-type RepD or (N189K)-RepD, PcrA-driven plasmid unwinding was followed in real time using the previously described assay.⁷ Different lengths of supercoiled pCERoriD plasmids were unwound with detection of the ssDNA product by fluorescently labeled single-stranded DNA binding protein, DCC-SSB¹⁴ (Figure 7A). Qualitatively,

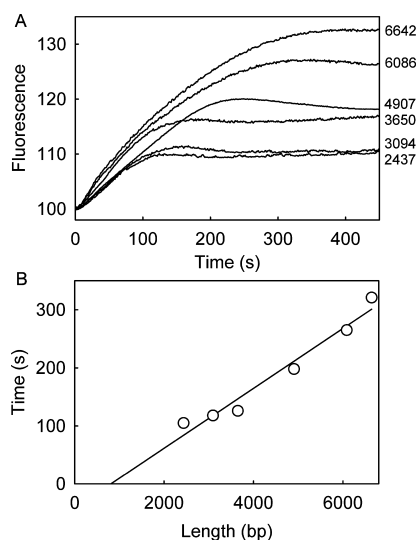


Figure 7. Unwinding of supercoiled plasmids monitored in real time using the SSB fluorescence assay. (A) Fluorescence time course for unwinding of plasmids of different lengths (shown in base pairs) with PcrA and (N189K)RepD. RepD was added to the solution containing the plasmid and left for 5 min before the addition of PcrA; the solution was then mixed with ATP in a stopped-flow apparatus. The solution contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 1 mM EDTA, and 10% ethanediol at 30 °C. Final concentrations after mixing were 0.5 nM plasmid, 95 nM PcrA, 2 nM RepD, 1 mM ATP, and 200 nM DCC-SSB tetramers. (B) Dependence of unwinding time on plasmid length, as described previously.⁷ The linear fit gives a rate constant of 19 ± 2 bp/s for unwinding.

the traces are similar to those obtained with wtRepD,⁷ a lag followed by a fairly constant rate of increase in fluorescence as the plasmid is unwound and then the fluorescence is constant. The translocation rate could be obtained by plotting the unwinding time as a function of length (Figure 7B): the mutant gives a slightly slower rate (19 bp/s) than the wild type (30 bp/s⁷).

A feature of wtRepD unwinding is that intensities seen in these assays decrease if the RepD is preincubated for long times with the plasmid and PcrA before initiation of unwinding by mixing with ATP (Figure S5A of the Supporting Information). This is consistent with the plasmid partly religating over the time frame of a few minutes as shown in the gel assay described above, albeit that in this translocation assay PcrA is also present. This religation then reduces the concentration of active, nicked DNA. Unlike that of wild-type RepD, the fluorescence change obtained for (N189K)RepD is fairly constant for the different aged complexes (Figure S5A of the Supporting Information), suggesting that the mutant performs nicking on *oriD* to give a complex more stable than the wild type over the time range of the aging. This is consistent with the gel assay of plasmid nicking and religating in Figure 6.

Linearized plasmids were not unwound by PcrA when (N189K)RepD was used, although there was some unwinding

with wtRepD (Figure S5B of the Supporting Information). Linearized plasmids are less favored than the supercoiled plasmids as a nicking substrate,¹² so with wtRepD, less DNA was available for unwinding and a smaller amplitude was observed relative to that with circular plasmids (Figure S5 of the Supporting Information). Nicking with nonsupercoiled substrates was tested by SDS-PAGE, in which RepD monomers bound to DNA migrate behind the unbound monomers. When wtRepD was incubated with a DNA construct, containing the complete *oriD* sequence (Figure S6 of the Supporting Information), an adduct formed with the DNA, albeit substoichiometrically. The mutant RepD showed little or no adduct, consistent with the lack of unwinding with the linear DNA. It seems that nicking is required before dsDNA can be fully unwound. This is likely to be due to the need to open a single-strand region at the correct location for PcrA to bind.

(N189K)RepD was also analyzed for its nicking activity on ssDNA oligos and Y junctions, containing a single-stranded ICRII (Figure S6 of the Supporting Information). SDS-PAGE assays as described above showed that these Y junctions were nicked by wtRepD but not the (N189K)RepD mutant, even at very long incubation times [>20 min (data not shown)]. The anisotropy measurement with the fluorescein-labeled Y junction, as shown in Figure 3 with wtRepD, was also conducted with (N189K)RepD (Figure S7 of the Supporting Information). This showed a rapid rise in anisotropy consistent with binding of the fluorescent Y junction, but no subsequent decrease, supporting the idea that it was not being nicked.

Despite not being nicked, (N189K)RepD allowed PcrA-driven junction unwinding, presumably because the ssDNA region is always present for PcrA binding. This was demonstrated using the Cy3-Dabcyl method using the Y junctions (Figure 2),⁷ outlined above (Figure S8 of the Supporting Information). Note that these junctions do not contain the ICRIII sequence, rather random dsDNA sequence to mimic translocation during pseudo-steady-state plasmid unwinding. The rate of unwinding is 47 bp/s (Figure S8 of the Supporting Information), identical to that of the wild type for identical junctions.⁷

As an alternative approach to limit nicking, mutations were introduced into the ICRII region. Nicking was tested by SDS-PAGE, using the single oligonucleotide, ICRII-part (Figure 2), and unwinding the 20 bp-F junction by the Cy3-Dabcyl assay (Figure 4). One mutation deleted the 5'-side of the nicking site [Δ CTA, removing positions 1–3 of 20 bp-F and ICRII-part (Figure 2)], so that there is no phosphodiester in the correct position for transesterification by RepD. The others replaced the A on the 3'-side of the nicking site (A changed to T, A > T, or to cytosine, A > C), which is the base that should form the phosphodiester with the RepD tyrosine. Both types of mutation prevented nicking by RepD (Figure 4A). However, both mutant DNAs supported unwinding with similar activity as shown by the similar fluorescence traces (Figure 4B). This supports the idea that the nicking activity and PcrA translocation activity are separate, but nicking is essential in natural plasmid substrates to provide the helicase with a binding site.

DISCUSSION

RepD is essential for the initiation of asymmetric, rolling circle plasmid replication of plasmids containing the DSO, *oriD*, prior to PcrA helicase binding. In this work, most of the data relate to the interaction of RepD with DNA, although there is also information about how such interaction affects the PcrA-driven unwinding. The data do not address directly the interactions of

PcrA with RepD, although this remains an unclear aspect of RepD. Because of this, it remains valid to use *B. stearothermophilus* PcrA, although the RepD is from *S. aureus*. This PcrA allows comparison of unwinding with different RepD variants and different DNA constructs, even though it is possible that precise protein–protein interactions are not completely optimal. The role of RepD (and similar proteins) can be considered in several distinct but related parts.

First, it ensures that copying the (–)-strand starts and ends at the DSO.^{2,4,18} This is achieved by specific interaction with the inverted complementary repeat sequence, ICRIII, carried by the plasmid that encodes RepD. Other pairs of Rep initiators and ICRIII provide their own specificity for this family of plasmids.^{2,19,20}

The second part of the function of RepD is to nick one strand within ICRII with three particular consequences. First, nicking removes the negative supercoiling so the plasmid is accessible to the replicative enzymes. Second, the 5'-end of the nicked strand, covalently bound to RepD, is carried around the plasmid with the protein and so is already in position to take part in the strand exchanges to form the closed, circular parental (+)-strand. Lastly, this nicking also opens up a short stretch of ssDNA on the (–)-strand, allowing PcrA to bind. Another function of RepD is to enhance the processivity of PcrA, allowing the complete plasmid to be unwound. In the absence of a protein such as RepD, PcrA is a poor helicase.^{7,11,12} Finally, RepD modulates more chemistry to terminate replication by processes including strand exchange.¹⁸ The work, described above, addresses the factors that are involved in ensuring that RepD successfully initiates the replication, in particular by examining the kinetics of the nicking reaction and how this is related to subsequent unwinding.

Inverted complementary repeats are essential both for RepD recognition and for formation of the initiation complex. These elements were introduced into DNA junctions that were prepared from oligonucleotides and used in several biochemical assays. In particular, real-time measurements of nicking kinetics show how features of the DNA are important for different steps of formation of the initiation complex. In addition, some measurements were taken on the whole plasmid using discontinuous real-time assays, for example, using the quench-flow technique. Real-time translocation assays were also performed on each type of initiation complex to confirm that the complexes were fully competent in this regard.

The scheme in Figure 1B shows elementary steps of the nicking process. RepD binding (step 1) is followed by the chemical step 2. The resulting intermediate is shown as a noncovalent complex between the RepD–DNA complex and the nicked DNA. There may be interactions of RepD with the nicked DNA as well as potential remnant base pairing of the nicked DNA with the covalently bound part of ICRII. Finally, in step 3, the 3'-end of the nicked DNA dissociates to provide the structure with a stretch of ssDNA to which PcrA binds. This dissociation could be complete separation in the case of some oligonucleotide junctions, or just removal from the RepD binding site in the case with the complete *oriD*. Apart from inhomogeneities that may occur in any biochemical reactions, such as partially damaged proteins, further inhomogeneity could arise in this type of reaction because DNA with inverted complementary repeats might take up various structures, particularly hairpins. Such inhomogeneities could add to the complexity of the measured kinetic transients.

The binding kinetics of RepD that were measured by fluorescence anisotropy (Figures 3 and 5 and Figure S3 of the Supporting Information) depend largely on the presence of ICRIII and vary little with the structure of the neighboring ICRII sequence. This binding [k_{+1} (Figure 1B)] is fairly rapid [$>10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1)]. This binding step is also independent of whether subsequent nicking occurs, as shown using (N189K)RepD (Figure S7 of the Supporting Information).

The direct measurement of nicking kinetics, using quench flow, suggests that both thermodynamic equilibria and kinetics play a part in the observed time courses (Table 1). The extent of reaction was almost quantitative when a single-stranded ICRII is used as substrate, either as a ssDNA oligonucleotide or as part of a Y junction (Figure 4A). Note that only one of the RepD monomers becomes covalently linked via its active site tyrosine to the 5'-end of the DNA. The kinetics with this DNA were not measured, but the reaction of the Y junction (Figure 3) indicates that single-stranded ICRII is nicked slowly (1.5 s^{-1}). In these cases, it is likely that nicking is followed by rapid dissociation of the small DNA product (step 3, Figure 1B). This is advantageous for using this type of Y junction in translocation assays: the loading yield is high, and it seems fully functional in such assays.⁷

The nicking time courses of other junctions and whole plasmids were more complex because of the slow release of the nicked DNA end from RepD and reversibility of the nicking step. The hinge and DS junctions mimic to some degree the extremes of whether the plasmid substrate contains ICRII as a hairpin or whether it is a continuous double strand. The nicking reaction of the DS junction shows burst kinetics (Figure 5B and Table 1). There was an initial rapid phase (15 s^{-1}), likely to represent k_{+2} (Figure 1B). There was then a slow phase (0.56 s^{-1}) of similar size, which might represent the dissociation rate constant, k_{+3} , with K_2 close to unity: only upon dissociation of the product is the nicking reaction pulled to completion. The linear structure of the DS junction may require a bending or contouring of the ICRII region to allow the nick site to be located at the RepD active site.

In contrast, the hinge junction has only a small nicking burst (~15%), based on HPLC analysis (data not shown). This is consistent with the anisotropy signal (Figure 5C), which shows an increase following binding of RepD, suggesting little release of the small DNA product. It seems likely that the substrate structure causes K_2 to be small, favoring substrate. Release of the nicked product is very slow, with the anisotropy only decreasing a small amount at 0.07 s^{-1} . This is possibly due to the remnant base pairing, although for this junction the fluorophore may also interact with bases in the rest of the DNA.

The plasmid nicking kinetics are also multiphasic (Figure 6), a rapid phase ($>25 \text{ s}^{-1}$) with a 35% amplitude and then a slow phase ($\sim 2.5 \text{ s}^{-1}$) (Table 1). The plasmid preparations exhibit some heterogeneity as there is a variable amount of relaxed, open plasmid at time zero. The nicking kinetics are qualitatively similar to those of the DS junction, described above, with K_2 close to unity and k_{+2} fast, followed by slow product release.

Gel analysis of plasmid nicking on a slower time scale shows the slow religation of the DNA to give a relaxed, closed plasmid at an observed rate constant of 0.0037 s^{-1} with an equilibrium constant of 0.3 (Figure 6A and Figure S4 of the Supporting Information). It seems likely that RepD remains bound to the relaxed, closed plasmid, based on the dissociation constant for dissociation of RepD from the Y junction,⁷ so this is a direct measurement of the equilibrium constant for the nicking

reaction. The reaction is formation and breakdown of a phosphodiester bond, and as described in the introductory section, this equilibrium might be expected in the absence of other factors to be close to unity.

Depending on the structure of the DNA substrate, either release of DNA as a separate molecule or relief of supercoiling results in the high extent of product formation. Both the free energy of supercoiling in the intact plasmid and RepD binding could drive the formation of the cruciform extrusion of palindromic sequences contained within *oriD*. The formation of the cruciform has been shown to be dependent on superhelicity: the torsional stress of supercoiling leads to a decrease in the activation energy of the reaction followed by conformational changes in DNA structure that favor the formation of cruciform structures.²¹ In the absence of enzymes, this occurs only very slowly, even when thermodynamically favored.²¹ However, RepD might enhance and stabilize the extrusion on the supercoiled plasmid through binding, as seen previously with RepC on supercoiled but not linear DNA.^{3,10} This then exposes the nick site as a single-stranded loop as the target site for RepD nicking activity.²² Supercoiling could also be the cause of a larger k_{+3} , thereby shifting the equilibrium toward “product release” by physically separating the 3'-end of the nicked DNA from the RepD active site. In this model, the supercoiling has little effect on step 2 (Figure 1B).

The site-specific nick results in a phosphodiester bond at Tyr188.⁴ (N189K)RepD was examined in detail for nicking activity and its ability to support the helicase activity of PcrA. A range of linear DNA constructs containing ICRIII and the nicking site were not substrates for this mutant (Figure 4A and Figure S6 of the Supporting Information). Only the supercoiled plasmid showed significant nicking, and this was at a rate 3 orders of magnitude slower than that of wtRepD (Table 1). However, when PcrA/RepD-mediated unwinding was tested using this mutant, DNA structures that had a ssDNA region in the appropriate place for PcrA binding were unwound with little or no modification of kinetics. The DNA also required a site for this RepD to bind, containing most of the ICRII sequence on the 3'-side of the nick site, but not necessarily the ICRIII sequence. As with wtRepD, a random sequence can be used here, albeit with a lower affinity for RepD (Figure S8 of the Supporting Information). Complementary data were obtained by introducing mutations into ICRII to prevent nicking. Again, when there is a site for PcrA and RepD (wt or N189K) to bind, then the dsDNA is unwound approximately as with unimpaired ICRII (Figure 4B).

However, DNA structures that did not provide a site for PcrA to bind, such as a linearized plasmid that was not nicked by (N189K)RepD, were also not unwound (Figure S5B of the Supporting Information). If the supercoiled plasmid was allowed sufficient preincubation time with (N189K)RepD to complete the slow nicking, then this was also unwound with kinetics very similar to those of wtRepD (Figure 7 and Figure S5A of the Supporting Information). This again illustrates the importance of supercoiling in driving the nicking to completion. The combined data with this mutant RepD suggest that K_2 and k_{+2} (in the scheme of Figure 1B) are greatly reduced by the mutation. Only the effect of supercoiling is driving the reaction to completion.

The nicking activity per se and the presence of the covalent DNA bonding with RepD are not essential for the role of RepD in enhancing PcrA processivity in DNA unwinding (Figure 7). The speed of unwinding is reduced with (N189K)RepD: PcrA

unwinds junction DNA at a rate of 47 bp/s and plasmid at a rate of 19 bp/s. The DSO is also used as a termination signal, leading to formation of the closed plasmid following replication.¹⁸ RepD nicking and closing activities are part of initiation and termination, respectively. It might be expected that the presence of the 5'-end on RepD is important for the religation, as it brings this DNA end in the correct position to undergo strand exchange. Following termination, RepD is released with a short oligonucleotide attached to one subunit and then cannot initiate replication in vitro.¹⁷ The attached oligonucleotide induces protein conformational changes that weaken its ability to form the cruciform structure,¹⁰ preventing the recycling of the initiator.¹⁷

As conformational changes in the DNA–RepD complex are essential for nicking, it is possible that mutation of N189 might reduce bending activity. RepC is a homologue of RepD but with specificity for another ICRIII sequence, part of *oriC*. The heterodimer, RepC–RepC–oligonucleotide, in which one subunit has a short oligonucleotide bound to its tyrosine, is able to bind but not to induce melting of *oriC*.¹⁰ Thus, a similar situation could occur for (N189K)RepD. This mutant could not efficiently enhance the secondary structure causing a very slow nicking reaction for supercoiled plasmid and an absent nicking for linear DNA. However, this cannot be the complete effect, as the mutation reduces the rate of nicking of ssDNA substrates, suggesting that either the DNA at the nick site cannot assume the correct conformation or the nicking itself is impaired.

In summary, detailed examination of the kinetics of RepD binding and nicking for a variety of DNA structures has shown that, while binding is usually rapid, the nicking rate constant is very dependent on the way in which the nicking site is presented. Although the equilibrium constant for the nicking is close to unity, the extent of overall reaction is considerably affected by the DNA structure, particularly by supercoiling, which favors product formation and increases the overall rate of nicking. The separation of nicking function of RepD from its role in assisting strand separation is clearly demonstrated by the measurement of translocation rates in situations where nicking does not occur. However, it is intriguing that RepD can increase the processivity of PcrA during unwinding, so it presumably must maintain interaction with the PcrA–DNA junction throughout. The fact that covalent attachment of DNA to RepD is not apparently essential means that this factor is not crucial in maintaining RepD at the junction. Furthermore, once unwinding is underway, RepD cannot be in contact with its tight-binding ICRIII site. The interactions of RepD with DNA and PcrA during unwinding remain to be elucidated. What factors maintain tight interaction of RepD with DNA once unwinding has progressed beyond ICRIII? What are the interactions between RepD and PcrA? Do the physical arrangements of the DNA and proteins impose constraints on dissociation of each, for example, by there being a hole in the proteins through which DNA threads?

■ ASSOCIATED CONTENT

📄 Supporting Information

Figures of HPLC analysis of fluorescein-labeled oligonucleotides, gel shift assay of RepD binding, kinetics of binding of RepD to different junctions, and kinetics of formation of the relaxed, closed plasmid from the relaxed, open plasmid. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; DCC-SSB, single-stranded DNA-binding protein from *Escherichia coli*, labeled at G26C with IDCC {N-[2-(iodoacetamido)ethyl]-7-diethylaminocoumarin-3-carboxamide}; wtRepD, wild-type RepD from *S. aureus* plasmid pC221; ICR, inverted complementary repeat; DSO, double-stranded origin of replication.

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