oriD structure controls RepD initiation during rolling-circle replication

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Bacterial antibiotic resistance is often carried by circular DNA plasmids that are copied separately from the genomic DNA and can be passed to other bacteria, spreading the resistance. The chloramphenicol-resistance plasmid pC221 from *Staphylococcus aureus* is duplicated by a process called asymmetric rolling circle replication. It is not fully understood how the replication process is regulated but its initiation requires a plasmid-encoded protein called RepD that nicks one strand of the parent plasmid at the double-stranded origin of replication (oriD). Using magnetic tweezers to control the DNA linking number we found RepD nicking occurred only when DNA was negatively supercoiled and that binding of a non-nicking mutant (RepDY188F) stabilized secondary structure formation at oriD. Quenched-flow experiments showed the inverted complementary repeat sequence, ICRII, within oriD was most important for rapid nicking of intact plasmids. Our results show that cruciform formation at oriD is an important control for initiation of plasmid replication.

Mechanisms that control initiation of DNA replication are essential to ensure DNA synthesis occurs at the correct time and at the correct level in order to maintain the genetic integrity of the organism. The pT181 family of plasmids contain antibiotic resistance genes and replicate by a mechanism known as rolling circle replication. One family member is pC221 and the initiation of its replication, occurs when RepD, a homodimeric, topoisomerase I-like enzyme, binds at the double-stranded origin of replication, oriD, which contains three inverted complementary repeat sequences (ICRs), termed ICRI, ICRII and ICRIII. It binds tightly and specifically to ICRIII ($K_d = 10^{-8}$ M) and rapidly nicks one DNA strand within ICRII and, as a result, DNA supercoiling is relaxed due to free rotation around single bonds in the backbone structure of the single-stranded DNA. The exposed single-stranded region allows a helicase and DNA polymerase to bind and together unwind and copy the leading strand (Fig. 1). During nicking, the active site Tyr188 of RepD forms a covalent bond with the 5′-end of the DNA and it remains bound until the termination of replication. In the final stage, DNA is supercoiled again by gyrase activity.

In vitro experiments have shown that the RepD nicking reaction is very slowly reversible (rate constant $0.004$ s$^{-1}$) and religation gives rise to a closed, relaxed, plasmid. At equilibrium the nicked and religated plasmid products are present in roughly equal amounts and the equilibrium constant is close to unity. This means the rate at which RepD nicks a relaxed plasmid is similar to the rate of religation and much slower than the nicking rate measured on supercoiled plasmid. Therefore DNA topology, in some way, has a profound effect on RepD nicking activity.

More generally, we know that supercoiling-induced changes of DNA topology have significant and diverse effects on protein-DNA interactions. Build-up of positive supercoiling ahead of DNA replication or transcription machinery must be relieved in order to prevent such activities from stalling and negative twist tends to open the DNA duplex, which accelerates processes requiring access to the DNA bases. Another consequence of negative twist is to stabilize alternative DNA structures, such as cruciforms and hairpins, which can form at regions where there are inverted complementary repeats, or ICRs, in the DNA.

It has long been suspected that secondary structure formation at the ICRs of oriD might be important for site recognition and nicking by RepD, in particular, hairpin formation at ICRII would expose the nick site on a single-strand loop. A recent structural study provides strong support for this idea as the reported 3-dimensional structure of RepD indicates oriD would need to be bent in order to accommodate simultaneous binding at ICRII.
and positioning of ICRII close to the active site Tyr188. Recently, the activity of an homologous protein, RepC, was shown to be supercoiling-sensitive. However, direct evidence explaining the supercoiling-sensitivity is still missing.

In the current work, we have investigated the effect of supercoiling and associated structural changes on the activity of RepD by applying known amounts of DNA supercoiling and monitoring RepD nicking at the single molecule level using magnetic tweezers. We found that RepD nicking activity is controlled by DNA supercoiling and is blocked by positive supercoiling and activated by small amounts of negative supercoiling. Our results also show that regions of DNA within oriD form secondary structures when negative supercoiling is applied and the structures are stabilized by RepD binding. We used bulk rapid-reaction methods (quenched-flow) to investigate the effect of oriD mutations on RepD nicking. We conclude that the ICRII within oriD serves both as a unique start site for replication and because of its propensity to form secondary structures, as a mechanical checkpoint that ensures replication can only commence when the plasmid DNA is supercoiled, an indication that DNA is not damaged or a previous cycle of replication has fully finished.

**Results**

**Positive supercoiling blocks RepD activity.** In order to determine the effect of DNA supercoiling on the kinetics of the RepD nicking reaction we constructed 10-kb and 4-kb, double-stranded DNA (dsDNA) linear templates with centrally located oriD sequence, flanked on either side by lengths of dsDNA "handles" that allowed specific attachment at one end to the surface of a microscope coverslip and at the other to a super paramagnetic bead (~1 μm diameter). Experimental conditions were optimized such that 5–15 beads, each tethered by a single DNA molecule via both DNA strands could be observed and recorded simultaneously by bright-field video microscopy. When a polarized magnetic field was applied, the easy axis of the paramagnetic beads became aligned with the magnetic dipole so that when the field was rotated the beads also turned and the attached DNA became supercoiled. At low stretching force (<0.5 pN), supercoiling was initially taken up by DNA twist but after a few turns the molecule buckled and underwent writhe with consequent plectoneme formation causing shortening of ~70 nm for each additional turn and this behavior was roughly symmetrical for positive and negative supercoiling (Fig. S1). When RepD was added to the experimental chamber and DNA was negatively supercoiled, the nicking reaction caused sudden loss of supercoiling (Fig. 2B). The DNA relaxed back to its rest length and further magnetic field rotation no longer caused it to supercoil. The nicking reaction could therefore be monitored directly from changes in DNA length (i.e. z-displacement of the attached paramagnetic bead) or because the DNA molecule was no longer able to be supercoiled.

The striking initial observation was that when the 10 kb-DNA template was positively supercoiled by +50 turns (superhelical density, σ = +5%), it was completely resistant to RepD nicking even after several hours incubation. Conversely, when the DNA was subjected to physiological levels of negative supercoiling (−50 turns, σ = −5%) it was nicked within a few seconds of RepD addition (Table 1).

We tested for nick site religation by repeatedly rotating the magnetic field through 50 positive turns at five minute intervals over a period of up to 2 hours and scored the number of DNA molecules for which torsional
Figure 2. Magnetic tweezers RepD nicking assay. (A) A 1 μm diameter, paramagnetic bead is attached by multiple biotin-streptavidin linkages to one end of a dsDNA (4 kb or 10 kb) molecule that has the initiation site, oriD, within its central region. Multiple digoxigenin-anti-digoxigenin linkages attach the other end to a glass microscope coverslip. Application of a magnetic field, generated by a pair of permanent magnets, causes the bead to rise from the microscope coverslip surface, extending the DNA molecule. Rotation of the magnetic field causes bead rotation and DNA supercoiling. At low force, the dsDNA first becomes twisted and then undergoes plectoneme formation (DNA writhe) resulting in reduction in DNA end-to-end length and bead motion towards the coverslip surface. When RepD nicks the DNA, supercoiling is relaxed and the bead moves rapidly upwards, towards its extended length. (B) The upper trace shows rotation of the magnetic tweezers (MT) and the lower trace shows bead displacement relative to its rest height plotted as a function of time. At the start of the experiment (t = 50 s), the DNA is supercoiled positively by 50 turns, increasing its super-helical density, $\sigma$, by $(50 \times (10.5/10\text{ kb})) = +5\%$ which causes plectoneme formation and bead height to reduce. RepD (1 nM) is then flowed into the experimental chamber (down-arrow, t = 80 s) and after 250 s the DNA remains intact and supercoiled. The magnetic tweezers are then rotated counter-clockwise by 100 turns (at t = 350 s) so that the DNA is then negatively supercoiled by $-50\%$ ($\sigma = -5\%$). After a stochastic delay (here, ~50 s) the DNA is nicked (diagonal arrow) and the bead moves rapidly back to rest height. After a further 100 s (at t = 525 s), rotation of the magnetic tweezers causes the magnetic bead to rotate but no longer causes the DNA to become supercoiled. The applied force due to the magnetic field was 0.4 pN and temperature was 23°C.

| Supercoiling | Force = 0.4 pN | Force = 0.8 pN |
|--------------|---------------|---------------|
| +50 turns ($\sigma = +5\%$) | 0% (n = 10) | 0% (n = 36) |
| −50 turns ($\sigma = -5\%$) | 100% (n = 10) | 100% (n = 21) |

Table 1. Effect of DNA supercoiling on RepD nicking activity. 10-kb dsDNA templates with oriD were held using the magnetic tweezers at fixed amounts of either positive or negative supercoiling at two different forces (0.4 and 0.8 pN) while incubated with RepD (100 nM). The proportion of nicked DNA molecules was then counted by noting the change in DNA length after 10 minutes’ incubation.

continuity had been restored so that the DNA molecule could again be supercoiled (Fig. 3A). We found the half-time for nick religation was ~24 minutes (Fig. 3B), which is about seven times slower than measured in bulk assays (previously performed at 30°C and the current experiments which were at 23°C). The discrepancy may be explained by the temperature difference and also the fact that we hold the DNA under a small amount of tension (0.5 pN) in the magnetic tweezers experiments, potentially making it less favorable for the DNA 3′ end to
bind RepD and undergo the phophodiester exchange reaction. Saturating concentrations of RepD (100 nM) were used, and several flow-cell volumes were exchanged during addition, so it is unlikely that depletion of RepD by non-specific binding to surfaces would have a significant affect, since its affinity for DNA is ~6 nM.

RepD is activated by small amounts of negative supercoiling. To determine the threshold of supercoiling required for RepD nicking to occur, we developed an experimental protocol that allowed the nicking reaction to be monitored at very low levels of DNA supercoiling, where bead z-displacement is minimal: First, all of the DNA molecules within the field of view were positively and then negatively supercoiled by rotating the magnetic field by +50 through to −50 turns. This allowed the thermally-driven starting variation in linking number for each DNA molecule to be accurately determined (Fig. S1). The DNA molecules were then positively supercoiled by applying +20 turns of the magnetic field and RepD (100 nM) was added to the experimental chamber. The supercoiled DNA molecules were then successively unwound by rotating the magnetic field to test points of +6, +4, +2, 0, −2, −4, etc. turns, relative to the nominal zero starting value where, on average, the DNA molecules were in their relaxed B-state. This therefore gave a spread of DNA supercoiling values at each test point due to the dispersion in starting offset values for each molecule across the field of view. The DNA molecules were held at each test point for 2 minutes and then rewound by +20 turns so that the number of intact (un-nicked) molecules could be counted by checking which of the DNA molecules underwent plectoneme formation and characteristic reduction in bead-height (z-displacement). When wild-type, oriD-containing, DNA molecules were held either in a positively supercoiled or relaxed state they were completely resistant to nicking by RepD (as found above). However, when small amounts of negative-supercoiling were applied, the RepD nicking reaction was activated and 50% of the DNA molecules were nicked with 4.5 turns of negative supercoiling (σ ~ −1.18%) (Fig. 4).

The shape and position of the curve, relating RepD nicking to DNA supercoiling, gives information about the energetics of the system. If we assume that negative supercoiling, which tends to unwind the dsDNA helix, causes local melting[10] then regions of dsDNA duplex containing ICRs may separate, allowing complementary

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**Figure 3.** DNA nick-religation activity by RepD. (A) Upper trace (solid line) shows rotational position of the magnetic tweezers and the lower trace (filled circles) shows the bead height as a function of time. The 10-kb dsDNA was first positively supercoiled (by +50 turns) and then RepD (100 nM) was added (down-arrow). The magnetic field was then rotated (by −100 turns) driving the bead and associated DNA molecule toward 50 turns of negative supercoiling. In this example, the DNA molecule was nicked (diagonal arrow) almost as soon as it started to enter the regime of negative supercoiling. Because the DNA had been nicked it could no longer undergo the characteristic length changes associated with supercoiling. Repeated cycles of 50 positive turns of field rotation were then applied to test if the DNA molecule was “supercoilable”. Here, after 54 minutes, the DNA spontaneously religated, and could again be supercoiled. (B) Cumulative frequency plot showing the number of nicked DNA molecules remaining as a function of time (n = 8). The mean time for religation to occur was 1,420 seconds (~24 minutes), giving a rate constant (dotted line) of 5 × 10^−4 s^−1. The horizontal lines indicate timing uncertainty due to gaps between applications of the +50 turn test protocol (see (A) above). Experiments were at F = 0.4 pN and 23 °C.
DNA was held at the same degree of positive supercoiling (\(\sigma \sim -0.2\))

Depending on the supercoiled state of the DNA this will either reduce negative supercoiling (from \(-2.5\%\) to \(-4.2\%\)) in the absence of RepD, they exhibited large amplitude length fluctuations that occurred on a timescale of a few seconds (Fig. 5). With increased force and more negative supercoiling, DNA spent more time in these extended-length alternative states. When the experiment was conducted at higher temperature (26 °C) and using a low-salt buffer (containing no added KCl or MgCl₂), fluctuations did not occur when the DNA was held at the same degree of positive supercoiling (\(\sigma \sim -2.5\%\)).

This phenomenon has been observed before and can be explained by formation and collapse of secondary structure at the ICRs with concomitant loss and gain of DNA writhe. Each turn of the B-form DNA backbone helix (10.5 bases) converted into the hairpin structure relieves one turn of negative supercoiling (or generates an additional turn of positive supercoiling). When the DNA is negatively supercoiled and in writhe, conversion of B-form DNA to a hairpin structure results in a large change in end-to-end length, because each turn of writhe that is lost causes a ~70 nm vertical bead movement at 0.4 pN force. 

**Figure 4.** dsDNA is nicked by RepD only when it is subjected to negative supercoiling. Beads that were tethered to the surface by a single, 4-kb, dsDNA molecule were identified by the characteristic change in DNA length upon supercoiling (\(n_{obs} \sim -70\)). The DNA molecules were then positively supercoiled by +20 turns before 100 nM RepD was added to the experimental flow-cell. All DNA molecules remained intact until they were subjected to small levels of negative supercoiling. After \(-4.5 \pm 0.1\) turns (±SEM) of negative supercoiling (\(\sigma \sim -1.2\%\)), 50% of the DNA molecules with the wild type oriD sequence (circles) were nicked by RepD. The oriD mutant mut2/3 (squares), had the 50% nicking threshold at \(-5.6 \pm 0.2\) turns. Experiments were at F = 0.4 pN and 23 °C. The exact level of supercoiling for each DNA molecule was corrected for its initial starting offset due to thermal motion (see main text for details). At low levels of supercoiling, the elastic energy (\(\Delta E_{\text{loop}}\)) work) due to thermal motion (see main text for details). At low levels of supercoiling, the elastic energy (\(\Delta E_{\text{loop}}\)) due to thermal motion (see main text for details). At low levels of supercoiling, the elastic energy (\(\Delta E_{\text{loop}}\)) due to thermal motion (see main text for details). At low levels of supercoiling, the elastic energy (\(\Delta E_{\text{loop}}\)) due to thermal motion (see main text for details). At low levels of supercoiling, the elastic energy (\(\Delta E_{\text{loop}}\)) due to thermal motion (see main text for details).  

**Negative supercoiling causes DNA length fluctuations.** To explore the structural stability of the ICRs at oriD a 4-kb DNA template was used in order to increase the signal-to-noise ratio of our measurements and also to eliminate other, non-oriD, ICRs. When the DNA molecules were held at 0.67 to 0.91 pN load and subjected to negative supercoiling (from \(-8\) to \(-16\) turns, i.e. \(\sigma \sim -2.5\%\)) in the absence of RepD, they exhibited large amplitude length fluctuations that occurred on a timescale of a few seconds (Fig. 5). With increased force and more negative supercoiling, DNA spent more time in these extended-length alternative states. When the experiment was conducted at higher temperature (26 °C) and using a low-salt buffer (containing no added KCl or MgCl₂), fluctuations occurred at lower force 0.4 pN (Fig. S2). Notably, the fluctuations did not occur when the DNA was held at the same degree of positive supercoiling (\(\sigma \sim +2.5\%\)).

This phenomenon has been observed before and can be explained by formation and collapse of secondary structure at the ICRs with concomitant loss and gain of DNA writhe. Each turn of the B-form DNA backbone helix (10.5 bases) converted into the hairpin structure relieves one turn of negative supercoiling (or generates an additional turn of positive supercoiling). When the DNA is negatively supercoiled and in writhe, conversion of B-form DNA to a hairpin structure results in a large change in end-to-end length, because each turn of writhe that is lost causes a ~70 nm vertical bead movement at 0.4 pN force.
oriD-dependent length fluctuations are stabilized by RepD binding. We next used a non-nicking variant, RepDY188F, which retains sequence-specific binding to oriD, to explore whether RepD binding at oriD might affect the stability of alternative DNA structures. The supercoiling and force conditions were chosen so alternative structure formation just starts appearing and non-specific (less-stable) structures are not yet stabilized. When the 4-kb dsDNA template was held at 0.67 pN force and negatively supercoiled by −10 turns (σ ~ −2.6%) it exhibited length fluctuations, spending ~14% of its time in the longer state (Fig. 6). After addition of RepDY188F the proportion of time spent in the extended state increased to 68%. Under these conditions dwell times were sufficiently long to allow the fluctuation amplitudes to be measured directly from the raw data and histogrammed (Fig. 7). The mean amplitude, 71.5 nm, is equivalent to 1.2 turns of writhe consistent with ~12 base pairs interconverting between conventional backbone dsDNA helix and an alternative hairpin structure which implies that at least one of the ICRs at oriD forms a hairpin structure as RepD binds.

To verify that DNA length fluctuations were due to oriD-specific binding of RepDY188F, a DNA template with no oriD sequence was tested and no length fluctuations were observed either before or after addition of RepDY188F, supporting the idea that RepD binding stabilizes DNA structural rearrangements that occur specifically at oriD. The ~10-fold change in equilibrium constant, between B-state and alternative structure in oriD,
brought about by RepDY188F addition implies the binding energy is around $-6.3 \text{ kJ.mol}^{-1}$ (10.7 pN.nm per molecule).

When the experiment was repeated at lower force (0.4 pN) the noise level was higher, therefore accurately measuring the time spent in both states was challenging (Fig. S3). However, a similar trend was observed and after addition of RepDY188F the DNA-length histograms were skewed towards the extended lengths.

RepD nicking is ~10-fold slower when ICRII sequence is scrambled. In order to understand how the structure of oriD affects RepD activity we performed a series of rapid-mixing quenched-flow experiments to measure the RepD nicking rate using intact, natively-supercoiled, plasmids. The plasmids contained either wild-type oriD or variants in which ICRII (mut2) or ICRIII (mut3) were disrupted (see Table 2). The nicking reaction was initiated by rapidly mixing intact, supercoiled, plasmid with RepD using the same buffer (K100) used for our magnetic tweezers experiments. The reaction was then stopped (i.e. quenched) at various time points by addition of excess EDTA. It took $\sim 150 \text{ ms}$ for RepD to nick 50% of the wild-type oriD plasmid and the mut3 mutant (Fig. 8). However, nicking of the ICRII disrupted mutant (mut2) was ~10-fold slower, taking $>1.5 \text{ s}$ for 50% nicking, and after 5 seconds only 60% of the plasmids had been nicked. Thus the main effect of the mutant oriD (mut2/3) used in the magnetic tweezers measurements is likely to be due to disruption of ICRII; the ICRIII mutation may affect tightness of binding, but at the RepD concentrations used in these experiments, binding is likely to be quantitative.

**Discussion**

The work presented here defines the relationship between supercoiling and effectiveness of RepD nicking at oriD. Because DNA nicking by RepD is an absolute requirement for initiation of rolling-circle plasmid replication, this relationship provides important information on a critical control point. We conclude that negative supercoiling...
Figure 7. Fluctuations in DNA length with central oriD sequence after addition of RepDY188F. The amplitude of DNA length fluctuations due to alternation between B-state DNA and hairpin secondary structure formation was determined from the distance between the means used to fit the twin Gaussian distributions to the histogrammed bead height data. The average amplitude of the length fluctuations is 71.5 nm and standard deviation is 22.4 nm (least-squares fit to the data, dashed line). Given that 1 turn of writhe produces a 60 nm length change at 0.67 pN; the number of base pairs involved in the structural rearrangement = (71.5/60) * 10.5 = 12.5 ± 3.4 bp. Experiments were conducted at 0.67 pN, K100+ buffer and 23°C.

Figure 8. Kinetics of supercoiled plasmid nicking. (A) Intact natively-supercoiled (10 kbp) plasmid was rapidly mixed with RepD in K100 buffer at 23°C. The reaction was quenched by addition of excess EDTA at the time points noted above the gel lanes. The reaction products were electrophoresed on 1% agarose gels in the presence of ethidium bromide. Lower band was the fast-migrating intact, supercoiled plasmid (sc), the upper band, is the slow-migrating nicked, open circular form (oc). (B) Quantitation of RepD nicking by gel densitometry gave the proportion nicked (normalized as oc/(oc+sc)) as a function of time. The least-squares fitted lines are to a single exponential function, giving rate constants, 6 s⁻¹, 7 s⁻¹ and 1.2 s⁻¹ (wt oriD, filled-circles solid-line; mut2 open-triangles, dotted-line, and mut3, open-squares, dotted-line). The inset plot shows data on a faster timescale. RepD nicking of wt oriD and mut3 was complete whereas mut2 had an amplitude of 60%. Experiments were conducted at 23°C.
causes ICRs at oriD to form secondary structure and that RepD binding shifts the equilibrium in favor of such structures. Although our data suggest that only one ICR is stabilized upon RepD binding, more ICRs can fold upon higher level of negative supercoiling. Because the ICRs are mechanically linked in series with one another, when one ICR motif converts into a stem-loop structure it relieves supercoiling, stabilizing the turn and the ICRs. Although activation of RepD nicking has a relatively low requirement for supercoiling (just 4 turns applied to a 4kbp DNA molecule, $\sigma = 1.2\%$) in fact the amount of energy needed to reach that degree of supercoiling is still 10-fold greater than thermal energy. Thermal fluctuations would rarely reach such an extreme value and the energy barrier would give $>1000$-fold slower reaction rate. Although the equilibrium constant for nicking and religation of relaxed plasmid is close to unity, nicking appears inefficient because of the slow forward rate ($\sim 0.004 \text{ s}^{-1}$). Negative supercoiling acts as a strong control/regulatory factor because it stimulates the rate of RepD nicking by $>1000$-fold. We show this both in our quenched-flow (using natively supercoiled plasmid) and magnetic tweezers experiments (where we precisely control supercoiling).

The dependence of the extent of nicking on the amount of supercoiling is well-fitted by a simple (non-cooperative) model of hairpin formation (Fig. 4) that is thermodynamically favored when DNA is negatively-supercoiled. Our finding strongly suggests that hairpin structure formation is a prerequisite for nicking. The idea that RepD can only nick effectively when these secondary structural motifs have formed is supported by recent structural studies of RepD (pdb:4CWE15). In fully double-stranded DNA templates caused the DNA to bend about $\sim 70$ nm per turn of writhe (z-displacement of $\sim 70$ nm per turn of writhe).

When DNA is negatively supercoiled, elastic energy is stored in both twist and writhe and this stored energy is dissipated by hairpin structure formation. Conversely energy stored when DNA is positively supercoiled acts against hairpin formation. At low levels of supercoiling the DNA becomes twisted and energy associated with its torsional distortion has a quadratic dependence on the number of supercoiling turns, $n$; given by: $\frac{1}{2} \kappa (2 \pi n)^2$, where $\kappa$ is the torsional stiffness (see Fig. 3 legend). At a critical torque force, DNA buckles (called the buckling transition) and then undergoes writhe whereby the DNA backbone wraps around itself to produce plectonemes. In our magnetic tweezers experiments, DNA tension is held constant and energy stored as writhe increases linearly with the number of additional turns. At low force, each additional turn of writhe results in a change in DNA end-to-end length of $\sim 70$ nm (Fig. S1C) which at 0.4 pN load gives an energy change of $\sim 28$ pN nm (16.5 kJ. mol$^{-1}$). When a hairpin structure forms, entropic energy stored as both twist and writhe is lost from the DNA backbone and this counteracts (or pays) the enthalpic cost of forming unstacked and unpaired bases in the hairpin loop regions. We found RepD nicking activity hairpin formation was activated by 4.5 turns of negative supercoiling applied to a 4-kb length of DNA ($\sim 8$ nm). This is much greater than the separation of the corresponding amino acid residues on the proposed RepD structure ($\sim 4.5$ nm). Furthermore, the near orthogonal geometry of the corresponding regions in the RepD structure also argues that there should be a matching $90^\circ$ bend between ICRII and ICRIII (as proposed in reference20). Together this suggests there is a structural rearrangement at oriD allowing RepD to bind at ICRIII while contacting the nick site within ICRII.

We found that at critical levels of tension and supercoiling the DNA molecules showed large amplitude length fluctuations (measured by changes in bead displacement). Fluctuations occurred when DNA was negatively but not positively supercoiled and only when the ICRs at oriD were present. The finding is most easily explained by transient secondary (hairpin) structure formation19 at the inverted complementary repeat regions of oriD that gives rise to stochastic loss and gain of DNA writhe. The increased dwell-time of the extended state in the presence of the non-nicking RepD mutant protein (RepDY188F) can be explained by hairpin structure stabilization as the protein binds (Figs 5, 6 & S3). The change in equilibrium constant gives an estimate of the binding energy of RepD, $-6 \text{ kJ/mol}$, which would likely be insufficient to drive hairpin formation on relaxed DNA.

Although activation of RepD nicking has a relatively low requirement for supercoiling (just 4 turns applied to a 4kbp DNA molecule, $\sigma = 1.2\%$) in fact the amount of energy needed to reach that degree of supercoiling is still 10-fold greater than thermal energy. Thermal fluctuations would rarely reach such an extreme value and the energy barrier would give $>1000$-fold slower reaction rate. Although the equilibrium constant for nicking and religation of relaxed plasmid is close to unity, nicking appears inefficient because of the slow forward rate ($\sim 0.004 \text{ s}^{-1}$). Negative supercoiling acts as a strong control/regulatory factor because it stimulates the rate of RepD nicking by $>1000$-fold. We show this both in our quenched-flow (using natively supercoiled plasmid) and magnetic tweezers experiments (where we precisely control supercoiling).

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When DNA is negatively supercoiled, elastic energy is stored in both twist and writhe and this stored energy is dissipated by hairpin structure formation. Conversely energy stored when DNA is positively supercoiled acts against hairpin formation. At low levels of supercoiling the DNA becomes twisted and energy associated with its torsional distortion has a quadratic dependence on the number of supercoiling turns, $n$; given by: $\frac{1}{2} \kappa (2 \pi n)^2$, where $\kappa$ is the torsional stiffness (see Fig. 3 legend). At a critical torque force, DNA buckles (called the buckling transition) and then undergoes writhe whereby the DNA backbone wraps around itself to produce plectonemes. In our magnetic tweezers experiments, DNA tension is held constant and energy stored as writhe increases linearly with the number of additional turns. At low force, each additional turn of writhe results in a change in DNA end-to-end length of $\sim 70$ nm (Fig. S1C) which at 0.4 pN load gives an energy change of $\sim 28$ pN nm (16.5 kJ. mol$^{-1}$). When a hairpin structure forms, entropic energy stored as both twist and writhe is lost from the DNA backbone and this counteracts (or pays) the enthalpic cost of forming unstacked and unpaired bases in the hairpin loop regions. We found RepD nicking activity hairpin formation was activated by 4.5 turns of negative supercoiling applied to a 4-kb length of DNA ($\sigma = 1.3\%$), which, coincidentally, is close to the buckling transition. Using hairpin loop energy cost values from Table 4 in ref.21 the free energy change associated with hairpin loop formation at ICRII, II and III (with loop sizes 7, 6, 5 bases resp.), is: $+17.6$, $+16.8$, $+13.9 \text{ kJ/mol}$, which in units of mechanical work per dsDNA molecule gives $+59$, $+56$, $+46 \text{ pN nm}$ for each loop pair (resp.). If we consider, for example, the enthalpic cost of forming the two loop regions at the ICRII cruciform, $E_{loop} = +56 \text{ pN nm}$, compared to the entropic energy available from loss of negative supercoiling due to the change in linking number when the 24bp making up ICRII are transferred from the linear DNA backbone to the hairpin stem structure (24/10.5 = 2.29 turns); we obtain $\Delta \text{ work} \sim 60 \text{ pN nm}$ (at 0.4 pN load, and z-displacement of $\sim 70$ nm per turn of writhe).

Binding of a homologous initiator, RepC, to oriC was found to protrude single-stranded DNA regions consistent with hairpin formation22. Also, RepC binding to short oriC DNA templates caused the DNA to bend about oriC23. Recent work using magnetic tweezers showed RepC supercoiling-sensitivity24. Our results are consistent with these studies and extend their findings by directly showing the extrusion of cruciform structures in oriD.

Our data support the formation of ICRII upon RepD binding. These results strongly suggest the important role of cruciform extrusion in controlling the rolling circle replication. We note that other regulation points in initiation of replication may include control of RepD synthesis24 and that RepD is inactivated after the replication cycle is complete25. The energy of supercoiling can drive the initiation process to completion prior to the NTP-driven processes of DNA unwinding and DNA synthesis. RepD can only nick when the DNA is negatively supercoiled, hence replication can commence only when the plasmid is topologically intact. Furthermore, if the plasmid retains rotational freedom behind the replication fork it would not be negatively supercoiled until the leading strand had been fully replicated and single strand nick resealed by the termination reaction. This means replication cannot be erroneously initiated at the newly synthesized oriD site until the previous replication cycle is complete and the intact daughter plasmid has become negatively supercoiled. This is important to ensure a faithful copy of the original plasmid is created.
Materials and Methods

All reagents were sourced from Sigma-Aldrich Co. (Gillingham, Dorset, UK) unless stated otherwise; TFS (ThermoFisher Scientific, Hemel Hempstead, Herts, UK), NEB (New England Biolabs, Hitchin, Herts, UK), Agilent (Agilent Technologies, Stockport, Cheshire, UK).

DNA constructs. The 10-kb and 4-kb dsDNA templates were each constructed from three separate pieces of dsDNA that were made by PCR. A 500-bp digoxigenin-labelled “handle”; a 500-bp biotin-labelled “handle” and a central fragment of either 9.5-kb or 3.6-kb which had oriD located approximately in the middle. All three pieces of DNA were created from a synthetic, 10-kb plasmid based on pCerOriD\(^\text{+}\). The central fragments of DNA, used for the magnetic tweezers experiments, were generated from pCerOriD using suitable primers and PfuUltra II Fusion HS DNA polymerase (Agilent) according to the manufacturer’s instructions. The products were purified and digested with FastDigest AciI and FastDigest Apal simultaneously. The 4-kb DNA fragment, without oriD, was prepared from a pCerOriD 10-kb plasmid which previously had oriD deleted using the QuikChange technique (Agilent) and suitable primers. In order to create the differentially end-labelled dsDNA templates the two differentially-labelled DNA handles (below) were mixed with the chosen central fragment (10-kb or 4-kb) at a molar ratio of 20:20:1 and ligated using T4 DNA ligase (NEB), for 2.5 h at room temperature in T4 ligase buffer. The final product was gel-purified and stored in small aliquots at ~80 °C in 1 mM EDTA, 10 mM Tris.HCl pH 8.0.

Differentially-labelled DNA handles. In order to create the digoxigenin- and biotin-labelled dsDNA handles a ~1000-bp piece of DNA was generated by PCR, using 0.5 ng/µl pCerOriD DNA, 1.25 u/µl recombinant Taq Polymerase (TFS), Taq polymerase buffer with 2 mM MgCl\(_2\), 0.2 mM dNTP (each) and 1 µM of suitable primers and in the presence of either 0.02 mM digoxigenin-11-dUTP or biotin-16-dUTP. The PCR product was purified with a PCR purification kit (QIAGEN, Manchester, UK), and treated with a FastDigest (TFS) restriction enzyme; using Apal for the digoxigenin-labeled handle and AciI for the biotin-labeled handle, according to the manufacturer’s instructions. After digestion the enzyme was inactivated at 60 °C for 20 min and DNA was purified again. By using this protocol 1000-bp and 500-bp handles were produced for 4-kb and 10-kb DNA constructs respectively.

Mutant oriD plasmids. 10-kb pCerOriD with wild type (wt) oriD was mutated 10-kb pCerOriD with wt oriD was mutated by excising the oriD sequence (AvaI and HindIII (NEB)) and ligating the required, 5’ phosphorothiolated, mutant oriD oligoduplexes (Integrated DNA Technologies, Leuven, Belgium), with T4 ligase (NEB). This gave three variants; mut2 with disrupted ICRII, mut3 with disrupted ICRIII, and mut2/3 with both ICRII and ICRIII regions disrupted. The inverted complementary repeat sections that form stem structures were mutated but the central loop regions were left unchanged (see Table 2).

Protein production. We used RepD cloned into pET11a following the method described by Thomas et al.\(^5\). Briefly, the pET11a plasmid with cloned RepD gene was expressed in B834 (DE3) pLysS cells at 30 °C. Cell pellets were sonicated and protein was salted-out using saturated (NH\(_4\))\(_2\)SO\(_4\). The product was cleaned with HiTrap Q FF column and bound to on HiPrep heparin column. Protein was eluted with a KCl gradient (50 mM Tris.HCl pH 8.0, 10 mM KCl, 10 mM MgCl\(_2\), 1 mM EDTA), filtered through a 0.45 µm filter and concentrated using 10,000 MWCO Vivaspin concentrator Millipore (Millipore, Watford, Herts, UK). The final RepD product was quantified using an extinction coefficient of 119,514 M\(^{-1}\).cm\(^{-1}\) at 280 nm for the dimer. The protein solution was stored frozen at ~80 °C in 1 mM EDTA, 10 mM Tris.HCl pH 8.0.

Quenched-flow. RepD (15 µl of 70 nMolar = 10 pmoles) was mixed with an equal volume of 10-kb pCerOriD plasmid (20 ng/µl = 0.045 pmol of oriD sites) in a rapid-mix quenched-flow apparatus (QFM-4000, Bio-logic Science Instruments, Seyssinet-Pariset, France) using a 3.5 µl delay-line. Both reactants were in K100 buffer (100 mM Tris.HCl, pH 7.5, 100 mM KCl, 10 mM MgCl\(_2\), 1 mM EDTA). The reaction was stopped at various times by controlled addition of EDTA (total volume, 15 µl of 150 mM EDTA, 10% (v/v) ethanediol, and 200-500 mM KCl), filtered through a 0.45 µm filter and concentrated using 10,000 MWCO Vivaspin concentrator Millipore (Millipore, Watford, Herts, UK). The final RepD product was quantified using an extinction coefficient of 119,514 M\(^{-1}\).cm\(^{-1}\) at 280 nm for the dimer. The protein solution was stored frozen at ~20 °C. To produce the non-nicking, RepDY188F mutant, we used the QuikChange technique (Agilent) using 58-base primers having the desired, centrally located tyrosine to phenylalanine point mutation (i.e. attCTa.. to attCTta..) on forward and reverse primers.

Magnetic tweezers device. A custom-built magnetic tweezers apparatus was constructed around an Eclipse TE 2000-U inverted microscope (Nikon UK, Kingston-upon-Thames, Surrey, UK) with 100 × 1.4 NA oil immersion two objective lenses and 100 W halogen lamp illumination, fitted with a custom-built two-axis piezo stage (SmearAct, GmbH, Oldenburg, Germany) and piezo objective focusing device (P721, PILOC, Physik Instrumente, Cranfield, Beds UK). To increase the field of view, the camera was mounted on an additional 0.45 C-mount TV lens adaptor (Nikon). Two longitudinally magnetized, 5 mm diameter × 5 mm long cylindrical neodymium magnets (F641, MagnetExpert Ltd, Tuxford, Notts, UK) were mounted on a three axis manipulator with a custom-built stepper-motor driven, rotation mount. The motor was coupled by a drive-belt system to avoid magnetic interference. Video images were usually recorded at 20 Hz with a gated, 10 ms exposure using a 1024 × 1280 CMOS sensor, Prosilica GC1280 GigE camera (Allied Vision,stadtroda, Germany) and custom image capture software.

Magnetic tweezers experiments. The microscope flow-meter was made by sandwiching two coverslips on top of one another held with double-sided sticky tape to give ~10 µl volume channels. The lower coverslip
Data analysis.  

Bead positions were tracked in three-dimensions using custom-written ImageJ macro. The x- and y-pixel dimensions were calibrated using a microscope reticle slide and the computed z-displacement was cross-calibrated against control images obtained by moving the microscope objective in a triangular waveform through a known distance (usually +/−2.5 μm) using the Pifloc focus device. The z-calibration was linear over a range of −5 μm. z-displacement traces were filtered using a 25-point moving average. The vertical force, which extends the DNA molecule, was generated by the magnetic tweezers and was determined from the variance in x and y bead position. Single DNA-bead tethers show a characteristic asymmetric z-dependence (see Data analysis, below) as negative supercoiling melts DNA. This does not happen when the bead is tethered by more than one DNA molecule because multiple DNA molecules become twisted around each other (braided) giving a very different z-dependence on supercoiling.

Availability of materials and data.  

The materials and datasets generated during the current study are available from the corresponding author on reasonable request.

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Author Contributions
A.T., M.R.W. and J.E.M. designed research; A.T. performed research; M.R.W. and J.E.M. contributed reagents/analytical tools; A.T. and J.E.M. analyzed data; A.T., M.R.W. and J.E.M. wrote the article.

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