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Why is the initiation nick site of an AT-rich rolling circle plasmid at the tip of a GC-rich cruciform?

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pT181 and other closely related rolling circle plasmids have the nicking site for initiation of replication between the arms of a GC-rich inverted repeat sequence adjacent to the binding site for the dimeric initiator protein. Replication is initiated by the initiator-induced extrusion of this sequence as a cruciform, creating a single-stranded region for nicking by the protein. Nicking is followed by assembly of the replisome without relaxation of the secondary structure. Following termination, the initiator protein is released with a short oligonucleotide attached to one subunit, which prevents it from being recycled, a necessary feature of the plasmid's replication control system. The modified initiator can cleave single-stranded substrates and can nick and relax supercoiled plasmid DNA weakly. Although it can bind to its recognition sequence in the leading strand origin, the modified protein cannot induce cruciform extrusion, and it is proposed that this inability is the key to understanding the biological rationale for having the nicking site at the tip of a cruciform: the need to provide the functional initiator with a catalytic advantage over the modified one sufficient to offset the numerical advantage and metabolic stability of the latter.

Keywords: cruciform/initiation nicking site/recycling/RepC/rolling circle

Introduction

Rolling circle (RC) initiators have a strong catalytic preference for a single-stranded substrate, which may be satisfied by local superhelix-driven melting of an AT-rich region containing the nick site. A well-studied example of this is coliphage ϕX174 (Baas, 1987; Hanai and Wang, 1994). In striking contrast to this rule, the initiation nick site for pT181 is at the tip of an inverted repeat element (IR II) that has a much higher G+C content than the rest of the plasmid genome (Gruss and Ehrlich, 1989; Novick, 1989) (Figure 1) and is therefore disfavored for spontaneous superhelix-driven melting. However, binding of the homodimeric pT181 initiator protein, RepC/C, melts the IR II loop region, resulting in extrusion of the IR II element as a cruciform (Noirot *et al*., 1990; Jin *et al*., 1996), thereby generating the necessary single-stranded substrate. On the basis of results presented here, we suggest that, concomitantly with nicking, the entire cruciform is converted to a melted region for assembly of the replisome (see Figure 2A and B). We propose that this remarkable cruciform-based initiation mechanism is not merely a curious accident of sequence organization but has a substantive biological rationale that is derived from the plasmid's need to prevent recycling of the initiator because recycling would obviate control of plasmid copy number.

Recycling is prevented by a modification of the initiator, consisting of the attachment to one subunit of a short oligonucleotide representing sequences 3′ to the nick site in the double strand origin (DSO) (Rasooly and Novick, 1993). The resulting heterodimer, RepC/C*, is present in great excess, is metabolically stable, can bind to the pT181 DSO and can inhibit but not initiate replication (Jin *et al*., 1997). We have proposed that this oligonucleotide is generated by a short 3′ extension of the leading strand at the end of the replication cycle and becomes attached to the protein via the normal termination mechanism (Rasooly and Novick, 1993).

Since RepC/C* is isolated from pT181-containing cells, there must always be at least a small fraction of RepC/C, corresponding to material that has not yet been used for replication, and accounting for the *in vitro* replication activity originally observed in extracts of pT181-containing staphylococci by Khan *et al.* (1981). In our initial studies of RepC/C* (Rasooly and Novick, 1993; Rasooly *et al*., 1994a,b), we attributed the weak nicking, relaxing and replication activity seen in these preparations to this small fraction of RepC/C, which was estimated on theoretical grounds to be 5–10%. The more recent results reported here and elsewhere (Jin *et al*., 1997) suggest that the level of RepC/C present in these preparations is considerably lower than 5% and although it can account for the observed *in vitro* replication activity, it cannot be responsible for any of the other activities observed.

Thus, in this study, we show that the modified initiator is fully active for the cleavage of single-stranded oligonucleotides and for the re-ligation of free and bound oligonucleotides—activities that would be expected for the bound initiator during the termination of replication. However, it cannot induce cruciform extrusion. Consequently, it has only very weak nicking and relaxing activity on supercoiled DNA, and cannot generate the melted region that we believe to be required for assembly of the replisome. Additionally, it can transfer its attached oligonucleotide to the plasmid DNA, generating a nicked form with a 3′ extension of the plus strand. Because of the high concentration of the modified initiator, even weak nicking/relaxing activity could seriously interfere with normal replication. Thus the cost of this very convenient means of preventing recycling of the initiator is the creation of a Frankenstein—a monster that, if not carefully controlled, can wreak havoc on the system. We suggest that this is the biological rationale for placing the initiation

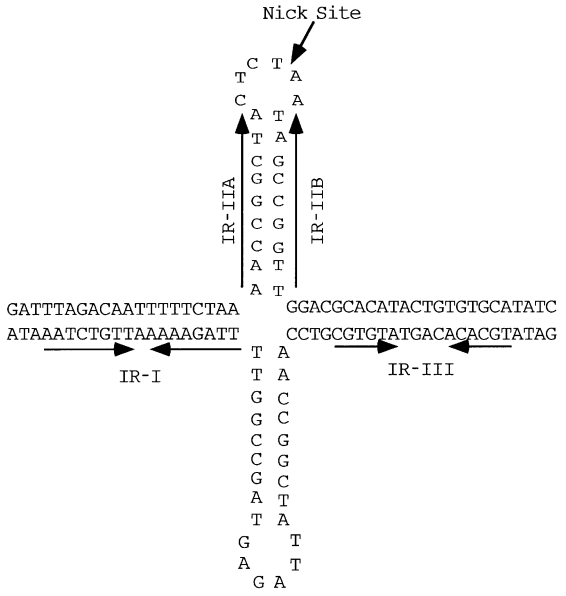

Fig. 1. Structure of the pT181 double-strand origin. The origin region contains three sets of inverted repeats (IR) which form a cruciform structure as shown in the figure. IR II is conserved among the pT181 family; it contains the initial nick site and the tip of its stem–loop structure is thought to serve as the actual substrate for RepC nicking. IR III contains the specificity determinant that varies among the family members.

nick site at the tip of a GC-rich cruciform. By this means, the native protein, which is synthesized at the rate of only one dimer per plasmid replication event (Bargonetti *et al*., 1993), is provided with a sufficient catalytic advantage to offset the high concentration and metabolic stability of the modified one.

Results

To understand the biological strategy underlying the cruciform-based initiation mechanism, we have compared the activities of the two forms of the initiator *in vitro*. Using oligonucleotide substrates, we show that RepC/C* is fully active in reactions that are predicted to occur during termination; using supercoiled plasmid DNA, we demonstrate that it has only weak activity in reactions that could interfere with initiation.

Cleavage of single-stranded oligonucleotides containing the pT181 DSO nick site

As shown in Figure 2, termination is assumed to involve cleavage of the displaced new–old leading strand junction by the free subunit of the attached initiator in steps C to D, followed by cleavage of the extended nascent leading strand as in F to G. If RepC/C* is biochemically analogous to the bound initiator, then it should cleave a single-stranded oligonucleotide containing the DSO nick site. That this is the case is shown in Figure 3. In Figure 3A is shown the time course for the formation of RepC/C**(22) by cleavage of an oligonucleotide with 22 nucleotides 3′ to the nick site [RepC/C**(*N*) is the heterodimer generated *in vitro* by cleavage of an oligonucleotide with *N* residues 3′ to the cleavage site or *N*′, residues 5′ to the cleavage site]. The quantitative results (Figure 3B) indicate that there was an initial rapid cleavage phase followed by

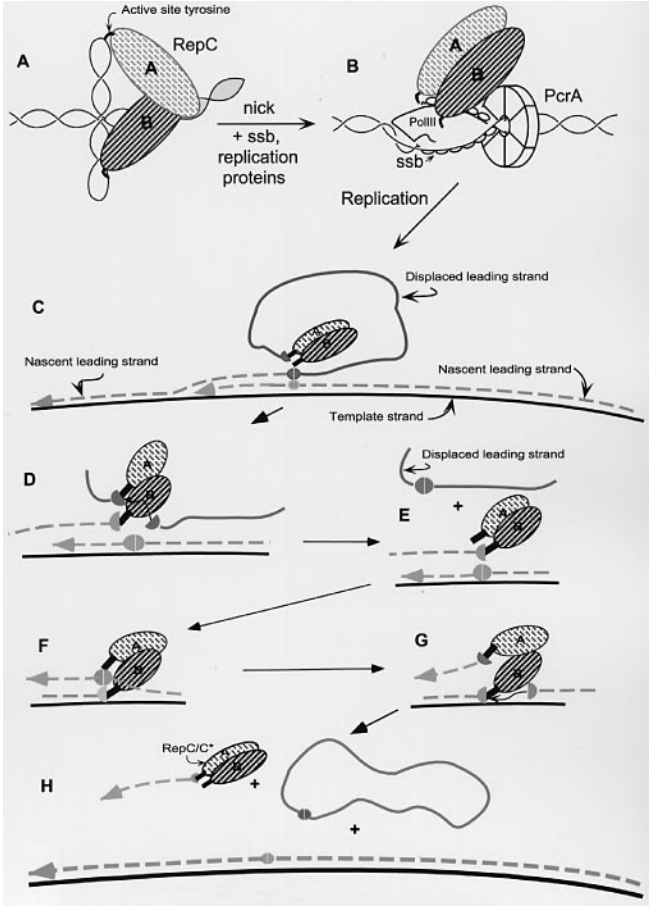

Fig. 2. Model for pT181 replication. (**A** and **B**) Initiation. RepC/C homodimer induces extrusion of IR II cruciform and one subunit binds to the L arm on the lagging strand while the other nicks the leading strand and becomes attached to the 5′ nick terminus. The replisome is assembled concomitantly and is assumed to include polymerase III holoenzyme, single-strand binding protein and PcrA helicase, as well as RepC. (C–H) Termination. Only the region surrounding the DSO is shown. At the end of the replication cycle, the leading strand is extended for a short distance past the nick site, displacing the junction between the old and nascent leading strands (**C**), which is then cleaved by subunit B of the bound initiator. This is followed by a transesterification (**D**) in which the free 3′ OH of the displaced leading strand attacks the protein–DNA bond between the 5′ end of the same strand and subunit A of the bound initiator, releasing the old leading strand as a single-stranded circle and leaving subunit B attached to the 5′ end of the nascent leading strand (**E**). The 3′ end of the leading strand is then displaced by the homologous 5′ end with the attached initiator (**F**), and the displaced leading strand is then cleaved by subunit A of the initiator, followed by a second transesterification (**G**) in which the nascent leading strand is circularized and the initiator released with the short oligonucleotide representing the 3′ extension of the leading strand attached to subunit A (**H**).

a somewhat slower phase, which reached a plateau at 60% RepC**(22) and 40% RepC. This suggests that the modified heterodimer RepC/C**(22) can cleave the oligonucleotide further to produce RepC**(22)/C**(22) homodimers. At least 20% of the total RepC molecules must therefore be in the form of RepC**(22)/C**(22) homodimers. The observed cleavage of single-stranded oligonucleotides is typical of an RC initiator (Pansegrau *et al*., 1993; Moscoso *et al*., 1995), except that our results imply the formation of doubly derivatized dimers, which have not been reported previously. In Figure 3C and D is shown a parallel reaction in which RepC/C* has been

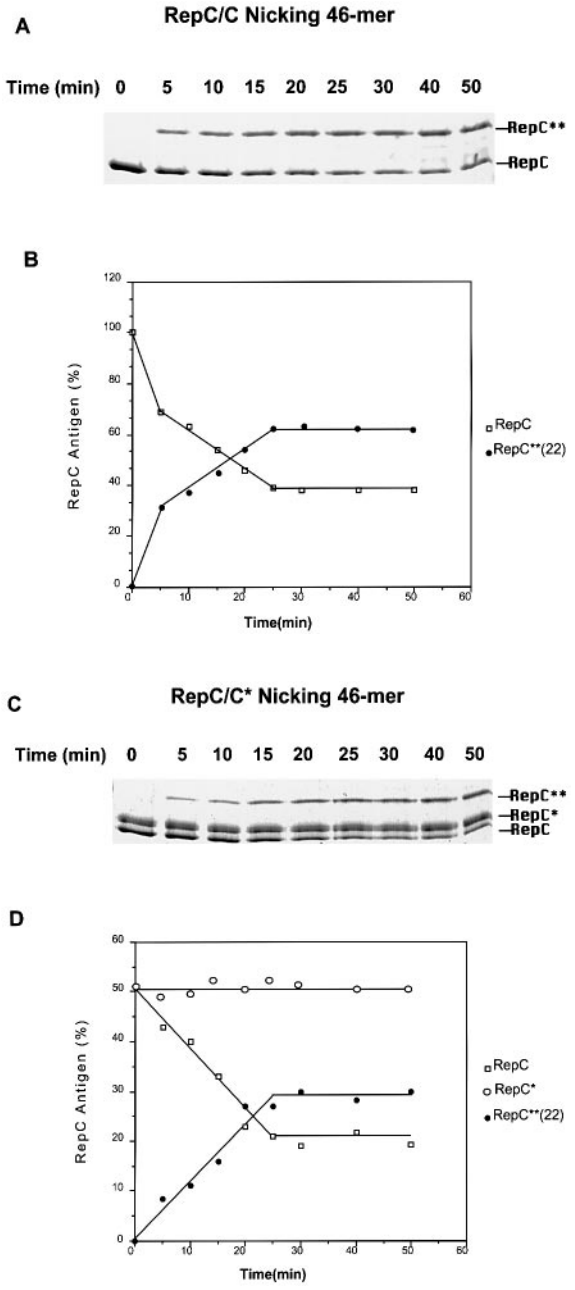

Fig. 3. Formation of RepC** by RepC/C- or RepC/C*-induced cleavage of single-stranded oligonucleotide. Purified RepC/C (**A** and **B**) and RepC/C* (**C** and **D**) were tested for cleavage of a single-stranded 46mer containing the RepC nick site as indicated by the formation of RepC/C** or RepC*/C**. Samples taken at different time points were resolved on SDS–PAGE and gels were stained with Coomassie brilliant blue, (A) for RepC/C and (C) for RepC/C*. Bands were quantitated using the IS-1000 Digital Imaging System (Alpha-Innotech) and plotted aganist time, (B) for RepC/C and (D) for RepC/C*.

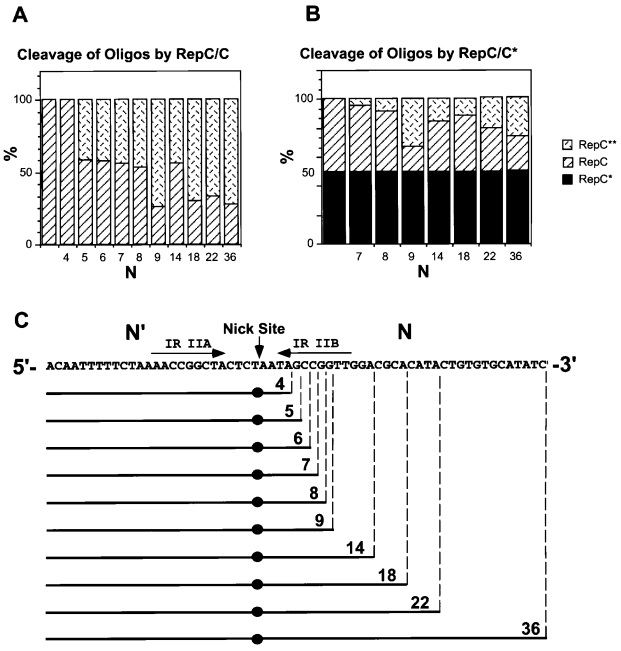

Fig. 4. Effect of oligonucleotide length on cleavage by RepC/C and RepC/C*. Purified RepC/C (**A**) and RepC/C* (**B**) were used to cleave single-stranded oligonucleotides with different numbers of nucleotides 3′ to the nick site. Rep protein (40 pmol) was incubated with 200 pmol of each oligonucleotide at 30°C for 1 h, samples were resolved on 12% SDS–PAGE. Gels were analyzed as in Figure 3. (**C**) Diagram showing the single-stranded oligonucleotides used in the experiments.

used to cleave the same oligonucleotide. As above, there was an initial rapid cleavage, reaching a plateau at 30% of the total RepC antigen, accompanied by a corresponding decrease in the level of the unmodified subunit. The level of RepC*, however, was unchanged. This experiment shows that the unmodified subunit of RepC/C* can cleave an oligonucleotide efficiently and that the product of this reaction is assumed to be a heterodimer consisting of RepC* and RepC**(22) subunits—which would correspond to the product of the first step of termination. Since measurable amounts of unmodified RepC are present and since there is excess oligonucleotide substrate, the observed plateaus in the reaction must represent equilibria between the various reactants and products. This would be consistent with participation of the doubly derivatized dimer in the transesterification reactions that are required during the two stages of termination, as proposed in Figure 2. *In vivo*, we generally see 50% RepC*, never more (Rasooly *et al*., 1994b), precisely what would be expected if the RepC/C* heterodimer is generated and released during the last stage of termination and never re-enters the replication cycle.

A subtle difference between RepC/C and RepC/C* was detected when activities with oligonucleotides with different numbers of units 3′ to the nick site were compared, as shown in Figure 4. A diagrammatic summary of the results for RepC/C is shown in Figure 4A, and for RepC/C* in Figure 4B. As can be seen, RepC/C was able to cleave an oligonucleotide with $N = 5$ with high efficiency, but failed to cleave the substrate with $N = 4$. Thus, the minimum sequence requirement 3′ to the nick site for RepC is five nucleotides, which is much shorter than the oligonucleotide attached to RepC/C* (see below). In Figure 4B, RepC/C* was used to nick substrates from $N = 7$ to $N = 36$ (there was no detectable activity for $N < 7$; not shown). With RepC/C* as well as with RepC/C, there seemed to be a bimodal activity response, reaching a sharp peak at $N = 9$, diminishing for $N = 14$ and 18, then increasing again for $N = 22$ and 36. In all cases, the content of RepC* monomer remained at 50%, corresponding to the proportion present in the starting material

(shown at left), and the level of RepC monomer decreased in proportion to the amount of RepC** formed. The ability of RepC/C but not RepC/C* to cleave the $N = 5$ and $N = 6$ substrates is thought to be the result of an allosteric modification of the heterodimer owing to the presence of the attached oligonucleotide. The peak of activity at $N = 9$, which represents the most frequent oligonucleotide attached *in vivo* (see below), could be a reflection of the high efficiency of cleavage *in vivo* needed for instantaneous stopping of leading strand extension during termination. Note that the value for RepC**(9) in Figure 4B was obtained by subtraction on the assumption that RepC* is a constant 50% of the total RepC antigen.

Re-ligation of oligonucleotides catalyzed by RepC/C and RepC/C***

Completion of the first stage of termination would entail a transesterification reaction in which the newly released 3′ OH of the displaced leading strand would attack the bond between the 5′ end of the same strand and the initially attached subunit of the initiator (Figure 2). This prediction was found to be correct for RepB (Moscoso *et al*., 1995) and for both RepC/C* and RepC/C**(18), as shown in Figure 5A. Here, the free 45mer (N') was 5′-end-labeled prior to incubation with RepC/C* (lane 1) or RepC/C**(18) (lane 4). In both cases, a labeled species was detected that corresponds to the sum of the attached and added oligonucleotides. The results are consistent with the ability of a RepC dimer with both subunits derivatized to catalyze the re-ligation presumably required for termination as shown in Figure 2. Although we have not tested this directly, the equilibrium shown in Figure 3D implies that it must occur. Note that the re-ligation product obtained with RepC/C**(18) was a single band at 63 nucleotides, whereas that obtained with RepC/C* consisted of a family of bands, the strongest with sizes of 54 and 55 plus weaker ones with sizes of 56–58 nucleotides. This means that RepC/C* is not a single species; assuming that the oligonucleotide attached *in vivo* corresponds to the leading strand extension that occurs at the time of termination, these results indicate that the termination point is staggered over a distance of 4–5 nucleotides. The signal that determines stopping and thus sets the length of the leading strand extension is presently unknown. Note that there was no reaction with RepC/C (Figure 5A, lane 2) or with a non-specific oligonucleotide (data not shown), i.e. RepC/C cannot attach an N' oligonucleotide to one of its active tyrosines unless the starting substrate contains the nick site in a cleavable form. As shown above, this means that N must be $\geqslant 5$. The minimum length of the attached oligonucleotide for re-ligation, however, is greater than that for cleavage. Oligonucleotides with $N \leqslant 7$ do not show detectable re-ligation with the above-mentioned 45mer, whereas an attached oligonucleotide with $N = 8$ had nearly the same re-ligation activity with the 45mer as does RepC/C* (not shown). These results probably indicate that the shortest attached oligonucleotide in RepC/C* is nine nucleotides; if there were a species with $N = 8$, a corresponding band would have been expected in Figure 5A, lane 1. We additionally have observed that Mg^{2+} is not required for this re-ligation reaction (not shown), although it is required for all reactions involving cleavage of the intact recognition sequence.

The re-ligation reaction of an exogenous single-stranded oligonucleotide with RepC/C* or RepC/C** is predicted to regenerate active RepC/C. To test this prediction, we incubated a preparation of RepC/C* with the same 45mer as used in the above re-ligation mixture, then tested the product for its ability to nick and relax supercoiled pT181 DNA. The results of this experiment (Figure 5B) were that RepC/C*, after incubation with the 45mer, showed much greater nicking and relaxing activity on supercoiled pT181 DNA than did the same RepC/C* preparation that had not been pre-incubated with the 45mer. These results confirm the prediction that active RepC/C is recovered following the re-ligation reaction, although this reaction is not expected to happen *in vivo*, since there would be no substrate corresponding to the above 45mer.

Given that re-ligation is a necessary stage of RC termination, we have considered the possibility that this reaction involves the IR II stem–loop structure of the leading strand DSO. To begin a test of this possibility, we determined the effect of blocking formation of the IR II hairpin on the re-ligation reaction. As shown in Figure 5C, oligonucleotides complementary to either arm of the IR II stem, excluding loop nucleotides, strongly inhibited the re-ligation reaction between RepC/C* and the same 5′-end-labeled 45mer as that used in Figure 5A. Complete inhibition was observed only when the oligonucleotides were complementary to the four nucleotides of the loop 5′ to the nick site as well as to the arms of stem (oligos 4 and 5). As a further test of this possibility, we used an oligonucleotide containing only the four loop nucleotides 5′ to the nick site, plus a random 13 nucleotide sequence. This oligonucleotide, which cannot form any secondary structure with the attached oligonucleotide, showed barely any re-ligation activity with RepC/C* (not shown). Therefore, the single-strandedness of the nucleotides of the loop 5′ to the nick site is required for re-ligation, and pairing between the two arms of IR II facilitates the reaction. It has been shown previously by Khan and co-workers (Koepsel and Khan, 1987) that secondary structure is not required for the cleavage reaction, and it is confirmed here in an experiment in which RepC/C was used to nick a series of partially or completely double-stranded DNA substrates. RepC was able to cleave efficiently partially double-stranded substrates with the nicking site exposed but unable to form any secondary structure (Figure 5D, lanes 3 and 4). This experiment also shows that the requirement for single-strandedness can be satisfied by a double-stranded linear substrate with a nick in the complementary strand opposite the nicking site (lane 5). The corresponding substrate without the nick was not cleaved detectably (lane 6), confirming the strong preference of RepC for a single-stranded substrate. It is predicted that RepC/C* will show similar activities with these partially double-stranded substrates; tests are in progress.

Activity of RepC/C on supercoiled pT181 DNA*

Since both RepC/C* and RepC/C** cleave single-stranded oligonucleotides containing the RepC nick site as efficiently as does RepC/C, using the unmodified subunit, it seemed likely that both would show activity on supercoiled DNA. In Figure 6 are shown the results with RepC/C*.

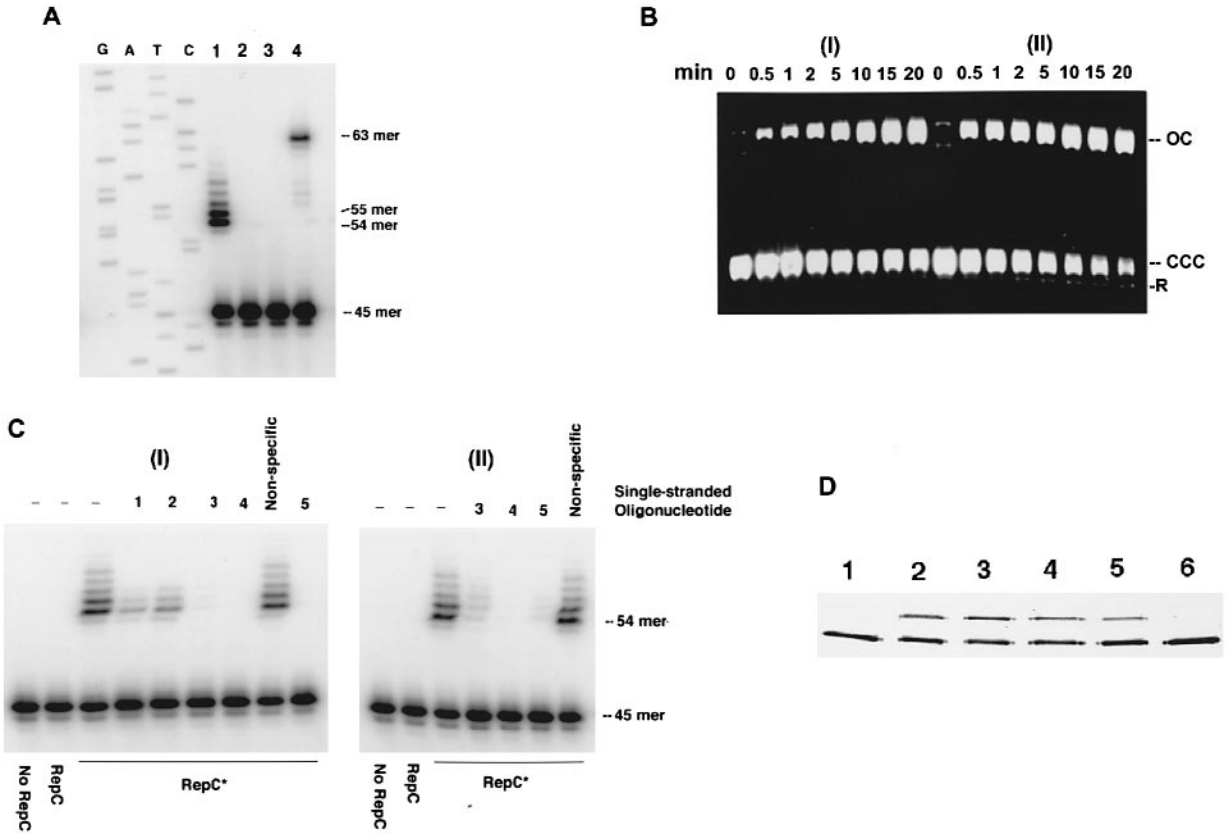

Fig. 5. Activities of RepC/C and RepC/C* with various substrates. (**A**) Re-ligation (transesterification) catalyzed by RepC/C*. Purified protein was incubated with a 5′-end-labeled 45mer representing sequences 5′ to the nick site on the pT181 leading strand. After a 30 min incubation, the reactions were analyzed on a sequencing gel along with a set of Sanger sequencing reactions covering the same region, then analyzed by a phosphoimager. Lane 1, RepC/C*; 2, RepC/C; 3, no protein; 4, RepC/C**(18). (**B**) Recovery of functional RepC/C from RepC/C* by re-ligation. One hundred pmol of purified RepC/C* were incubated with 400 pmol of a non-specific oligonucleotide (I) or with the same 45mer as in (A) in a buffer containing 10 mM Tris–HCl (pH 8.0), 100 mM KCl and 5% ethylene glycol at 30°C for 30 min. Then 100 pmol of supercoiled pT181 DNA and sufficient $Mg(OAc_2)$ to give a final concentration of 5 mM were added and incubation at 30°C continued. Samples removed at the indicated time points were resolved on 1% agarose containing 1 μg/ml ethidium bromide. (**C**) Inhibition of RepC/C*-catalyzed re-ligation by DSO-specific oligonucleotides. RepC/C* was incubated with the 5′-end-labeled 45mer as above. Oligonucleotides complementary to all or part of IR II (sequences listed below left) were added to the reaction in amounts corresponding to the RepC/C* concentration. Oligonucleotide numbers correspond to lane numbers; lanes with a (–) sign represent reactions in which no complementary oligonucleotide was added. Panel (I), 10 pmol of the indicated oligonucleotide were added simultaneously with RepC/C*; (II) 10 pmol of the oligonucleotide were pre-hybridized with the the 45mer before the reaction with RepC/C*. (**D**) Cleavage by RepC/C of partially double-stranded oligonucleotide substrates. Purified RepC/C (50 ng) was incubated with 10 pmol of each of the substrates whose sequences are shown below right. Partially double-stranded substrates were prepared by annealing the separate oligonucleotides. Note that #(5) was prepared by annealing the two shorter oligonucleotides to the one longer and therefore has a nick in the minus strand corresponding to the RepC recognition site. After 30 min at 30°C, samples were separated by SDS–PAGE and analyzed by Western immunoblotting with rabbit polyclonal anti-RepC antiserum. Lane numbers correspond to the numbers of the substrates, except that lane 1 had no added oligonucleotide.

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                    nick site
                  ⟶  ⇓  ⟵
   5′-TTTCTAAAACCGGCTACTCTAATAGCCGGTTGGACGC-3′  (+) DSO
   3′-AAAGATTTTGGCCGATGAGATTATCGGCCAACCTGCG-5′  (−)
1  3′----------------------ATCGGCCAACC-----5′  (−)
2  3′--------------------TTATCGGCCAACC-----5′  (−)
3  3′----GATTTTGGCCGAT----------------------5′  (−)
4  3′----GATTTTGGCCGATGAGA------------------5′  (−)
5  3′----GATTTTGGCCGATGAGATTATCGGCCAACC-----5′  (−)
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                    nick site
                  ⟶  ⇓  ⟵
(2) 5′-CTAAAACCGGCTACTCTAATAGCCGGTTGGAC-3′  (+)
(3) 5′-CTAAAACCGGCTACTCTAATAGCCGGTTGGAC-3′  (+)
          3′-TTATCGGCCAACCTG-5′              (−)
(4) 5′-CTAAAACCGGCTACTCTAATAGCCGGTTGGAC-3′  (+)
    3′-GATTTTGGCCGATGAGA-5′                 (−)
(5) 5′-CTAAAACCGGCTACTCTAATAGCCGGTTGGAC-3′  (+)
    3′-GATTTTGGCCGATGAGATTATCGGCCAACCTG-5′  (−)
(6) 5′-CTAAAACCGGCTACTCTAATAGCCGGTTGGAC-3′  (+)
    3′-GATTTTGGCCGATGAGATTATCGGCCAACCTG-5′  (−)
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Similar results were obtained with RepC/C**(18) (not shown) and have been reported for the closely related RepD protein (Thomas and Jennings, 1995). As can be seen, RepC/C* nicks supercoiled pT181 DNA, but with much lower efficiency than RepC/C. A plot of the time course of both reactions showed that the initial nicking rate of RepC/C* was ~1/12 that of RepC/C. Similarly, RepC/C* can catalyze the formation of relaxed covalent closed circular (CCC) DNA, but again, at a much slower rate than RepC/C. The re-ligation step with RepC/C* may be somewhat different from that with RepC/C, as illustrated in Figure 7A. Here, we predict the formation of an intermediate in which both subunits are attached to the DNA (IV). This reaction would be reversible, except

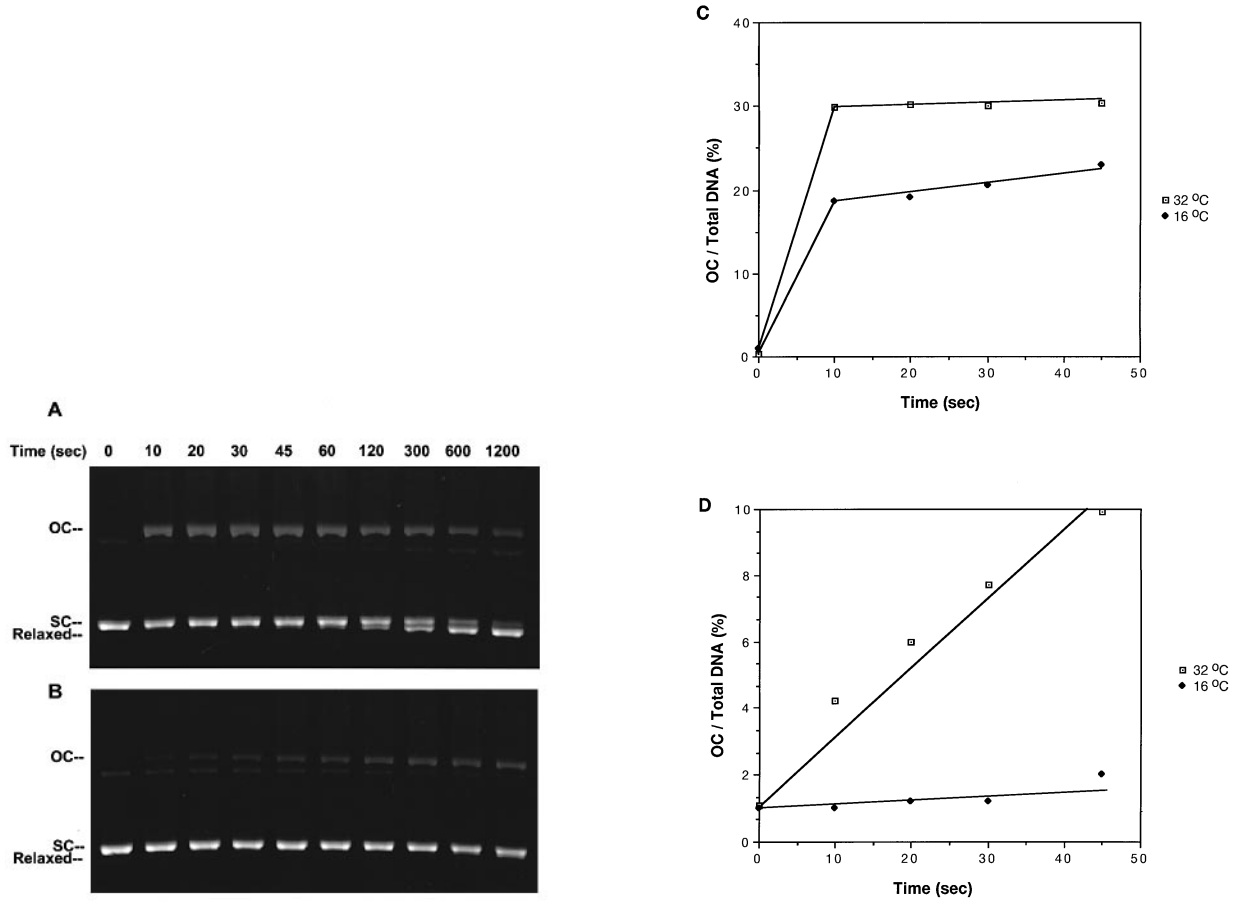

Fig. 6. Nicking and relaxing activity of RepC/C and RepC/C*. (**A** and **B**) Time course of the reaction at 32°C analyzed by agarose gel electrophoresis. Reactions were performed with equimolar amounts of protein (20 ng) and supercoiled pT181 DNA (0.8 μg) and stopped at different time points with EDTA. Samples were resolved on 1% agarose gels containing 1 μg/ml ethidium bromide. (**C** and **D**) Graphic representation of the initial rates of nicking by RepC/C (C) and RepC/C* (D) at 32 and 16°C. Agarose gels such as those shown in (A) and (B) were analyzed by scanning densitometry and the fraction of nicked circular DNA plotted as a function of incubation time. Note the difference in scales.

that its reversal would generate relaxed plasmid DNA (VI) rather than supercoiled CCC DNA. Alternatively, the intermediate could resolve by re-ligation, generating species (V) in which the plus strand contains a 5′ extension corresponding to the oligonucleotide adduct in RepC/C*. This reaction would also be reversible, leading to form (IV) and (VI). We have succeeded in demonstrating species (V) by restriction analysis (Figure 7B), confirming the proposed mechanism of interaction of RepC/C* with supercoiled DNA. Given that relaxed pT181 DNA with RepC attached is not a substrate for replication *in vitro*, and the reaction from species (IV) to (V) is also reversible, it is considered very unlikely that species (V) could be used as a substrate for replication. Therefore, the formation of this species in any significant quantity is likely to interfere seriously with replication of the plasmid.

RepC/C cannot induce cruciform extrusion*

The striking difference in the reactivity of RepC/C and RepC/C* on a supercoiled substrate suggested that there might be a difference in the relative ability of the two forms to induce extrusion of the IR II cruciform. In fact, preliminary results have suggested that RepC/C* may be deficient in cruciform extrusion activity (Jin *et al.*, 1996). Figure 8A shows the results of a $KMnO_4$ analysis of cruciform extrusion by RepC/C in comparison with RepC/C* on supercoiled pT181 DNA. As can be seen, RepC/C causes strong $KMnO_4$ sensitivity of nucleotides in and around the IR II loop, whereas these nucleotides are not $KMnO_4$ sensitive in the absence of the protein or in the presence of an equimolar quantity of RepC/C*. In this figure, only the results for the bottom (template) strand are shown. Identical results were obtained for the leading strand (not shown). We have shown elsewhere that RepC/C* has no detectable nicking activity on double-stranded linear DNA and so the kinetics of the reactions shown in Figure 6 suggest that RepC/C* may depend for its nicking activity on infrequent spontaneous extrusion events. Since RepC/C* can bind to the adjacent pT181 DSO recognition site (IR III), it may be that the bound molecule immediately relaxes any cruciforms that spontaneously extrude, so that the relaxation rate may provide a measure of the frequency of spontaneous cruciform extrusion over time. If this were the case, one would predict a much stronger effect of temperature on the nicking/relaxing activities of RepC/C* than of RepC/C. An experiment similar to that shown in Figure 6A and B was performed at 16°C and the results are shown in Figure 6C and D. Again, there was extremely rapid nicking by RepC/C, with the reaction essentially complete by the time of the first sample (10 s) whereas the rate of nicking by RepC/C* was reduced to a barely detectable level. Densitometric analysis showed that the

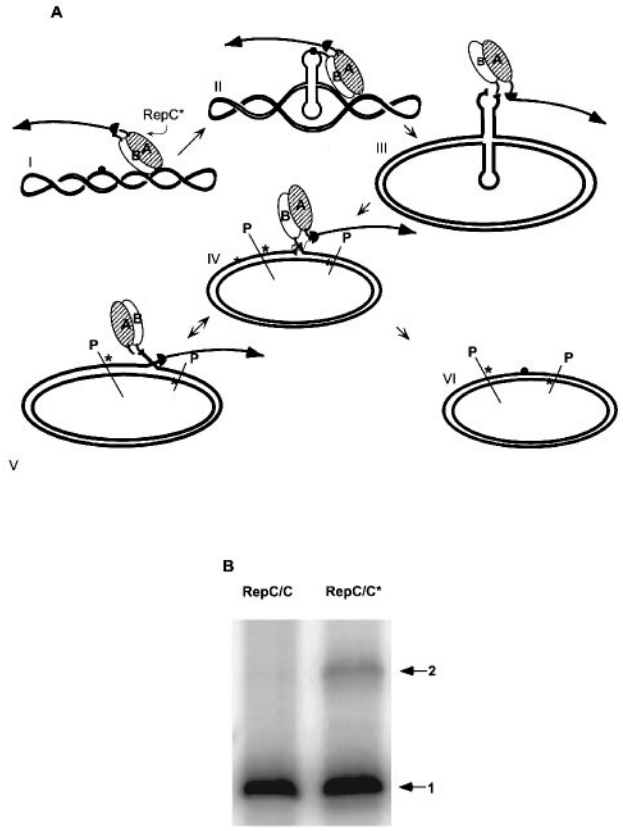

Fig. 7. Nicking of supercoiled DNA by RepC/C*. (**A**) Proposed mechanism. RepC/C* is shown bound to its recognition site on supercoiled pT181 DNA (I). Following spontaneous cruciform extrusion, the molecule is nicked by subunit B which becomes attached (II and III). Transesterification by the free 3′ end to subunit B reverses the nicking reaction, giving rise to a relaxed CCC monomer (VI). Transesterification to subunit A generates form (V) in which subunit B remains attached and the oligonucleotide is transferred to the 5′ end of the plus strand. (**B**) Demonstration of form V. After incubation of RepC/C (control) or RepC/C* with supercoiled pT181 DNA, the plasmid DNA was purified and digested with *Pac*I. The digestion mixture was 5′-end-labeled with ^{32}P and resolved on 8% sequencing gel followed by autoradiography. Band 1 corresponds to the fragment between the *Pac*I (P) site to the left of the nick site and the nick site, on the plus strand. Band 2 corresponds to the same fragment with a nine nucleotide extension resulting from transfer of the oligonucleotide bound to RepC/C* to the plasmid. All of the other *Pac*I fragments are larger and are not shown.

initial rate of nicking by RepC/C* was 1/140 that of RepC/C at 16°C, though it was only 12-fold lower at 32°C.

Formation of the pT181 initiation complex

A further differential between RepC/C and RepC/C*, owing to the inability of RepC/C* to induce cruciform formation, can be suggested on the basis of a proposed role for the cruciform in the formation of the pT181 initiation complex or replisome. Unlike ϕX174, for which the nicked monomer with GpA attached is a substrate for replication *in vitro* (Reinberg *et al*., 1983), nicked pT181 DNA with RepC attached is not, as shown in Figure 9. In these experiments, pT181 plasmid DNA was first nicked with increasing amounts of RepC/C protein (lanes 1–5) and the mixture immediately used for *in vitro* replication. Figure 9A shows a 1% agarose gel electrophoresis pattern, in which the various forms of plasmid DNA after RepC/C treatment are resolved. In lane 5, where the highest amount of RepC/C protein was used, most of the plasmid DNA was converted into the open circular DNA species and no supercoiled plasmid DNA was detectable. When this DNA was used for *in vitro* replication, as shown in Figure 9B, no incorporation of radioactivity was detected (Figure 9B, lane 5). This result indicates that open circular pT181 plasmid DNA generated by RepC/C nicking is not a substrate for replication. It is suggested that for pT181, following cruciform extrusion and nicking by RepC/C, the DSO must be held in an open configuration during assembly of the replisome, whereas for ϕX174, unwinding by Rep helicase subsequent to nicking is apparently sufficient. An approach to the assembly of the pT181 initiation complex has been provided by a mutant strain deficient in PcrA helicase, the staphylococcal analog of Rep helicase (Iordanescu, 1993). This strain has a major defect in plasmid replication and accumulates a nicked DNA–protein complex (Iordanescu, 1991). We have analyzed this complex with $KMnO_4$ and found that certain T residues in the DSO are $KMnO_4$ sensitive, as shown in Figure 8B. In that DNA–protein complex, newly exposed T residues were observed and the sensitivity of some previously detected T residues (without cell extract) was strongly increased. These T residues would be predicted to be single-stranded in a complex formed by cruciform extrusion followed by nicking and re-alignment of the DSO sequences (Figure 8C), and the result suggests that the complex forms and the realignment occurs in the absence of the helicase. Presumably, one or more host factors are involved in stabilizing the open structure. We suggest that assembly of the pT181 replisome requires a melted region, as with theta replicons. Thus, RepC/C* cannot initiate replication for two reasons: it cannot nick the DSO efficiently and it cannot generate the required melted region.

Discussion

On the basis of the results presented here and elsewhere, we have developed a comprehensive model for the replication of T181 and probably other members of pT181 family. According to this model, which is illustrated in Figure 2, replication is initiated when a newly synthesized RepC/C dimer binds to the DSO of a randomly chosen supercoiled plasmid molecule. On binding to its recognition site (IR III) (Wang *et al*., 1992), RepC/C increases the static bend in the DSO (Koepsel *et al*., 1986) and uses the free energy of superhelix formation to melt the IR II loop region, which is assumed to result in extrusion of the IR II cruciform (Noirot *et al*., 1990), which it binds and stabilizes prior to nicking (Jin *et al*., 1996). Our results suggest that RepC/C may actively initiate melting/extrusion, rather than simply stabilizing the cruciform, once formed. Support for this idea is based on the kinetics of the nicking reaction and on the difference between RepC/C and RepC/C*: nicking of supercoiled DNA by RepC/C was essentially complete within 10 s, at either 32 or 16°C, whereas that with RepC/C* was much slower and showed a profound temperature effect—much more in keeping with what might be expected for nicking following spontaneous cruciform extrusion. It is difficult to see how stabilization, *per se*, could account for the rapidity of nicking by RepC/C or for the profound differ-

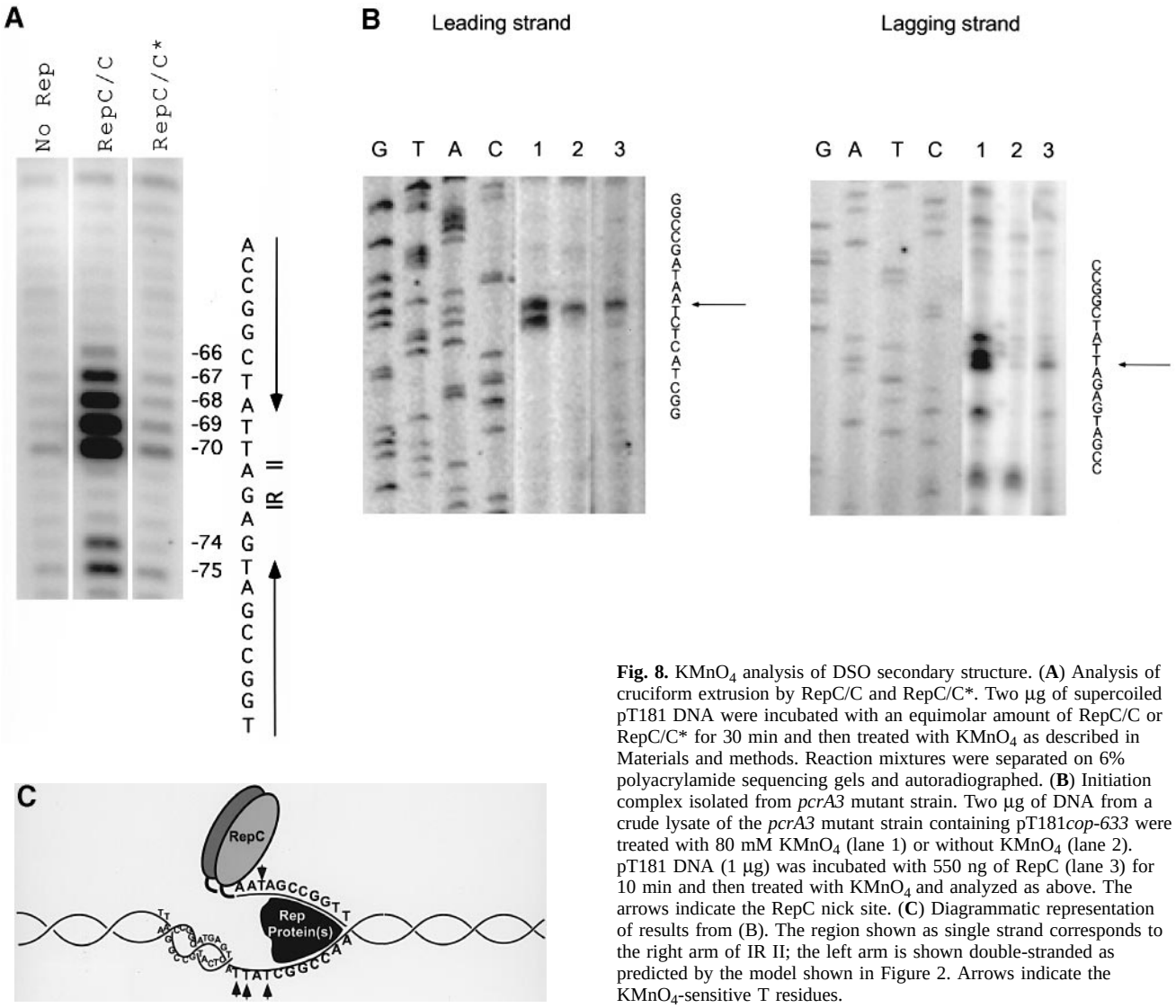

Fig. 8. $KMnO_4$ analysis of DSO secondary structure. (**A**) Analysis of cruciform extrusion by RepC/C and RepC/C*. Two μg of supercoiled pT181 DNA were incubated with an equimolar amount of RepC/C or RepC/C* for 30 min and then treated with $KMnO_4$ as described in Materials and methods. Reaction mixtures were separated on 6% polyacrylamide sequencing gels and autoradiographed. (**B**) Initiation complex isolated from *pcrA3* mutant strain. Two μg of DNA from a crude lysate of the *pcrA3* mutant strain containing pT181*cop-633* were treated with 80 mM $KMnO_4$ (lane 1) or without $KMnO_4$ (lane 2). pT181 DNA (1 μg) was incubated with 550 ng of RepC (lane 3) for 10 min and then treated with $KMnO_4$ and analyzed as above. The arrows indicate the RepC nick site. (**C**) Diagrammatic representation of results from (B). The region shown as single strand corresponds to the right arm of IR II; the left arm is shown double-stranded as predicted by the model shown in Figure 2. Arrows indicate the $KMnO_4$-sensitive T residues.

ence between the two forms. It is possible, incidentally, that at least part of the activity seen with RepC/C* represents very weak nicking activity on double-stranded B-form DNA, which has been reported for RepC/C (Koepsel *et al*., 1985) (though we have failed to demonstrate it). It must also be recognized that the sensitivity of the IR II hairpin loop to bromoacetaldehyde (Noirot *et al*., 1990) or $KMnO_4$ (Jin *et al*., 1996) proves only that the hairpin loop is single-stranded; the cruciform structure is likely on the thermodynamic grounds that once the loop region has been melted, superhelicity will drive extrusion of the stem (Lilley and Hallam, 1984; Lilley, 1985), aided in this case by binding of RepC/C to the right arm of the cruciform on the lagging strand (Jin *et al*., 1996).

Following introduction of the nick, the DSO DNA is maintained in an open configuration during assembly of the replisome, which, by analogy with that of ϕX174 includes Pol III holoenzyme, single-strand binding protein and PcrA helicase (Iordanescu, 1993), in addition to RepC. It is predicted that the open region would correspond to the IR II cruciform, and would be reconfigured so that one end of the nicked leading strand is reassociated with the template strand, providing the primer for DNA synthesis. Since this complex evidently can form in the absence of the helicase, we suggest that the helicase is loaded last. If the nicked molecule is allowed to relax in the absence of other proteins, the resulting product is not a substrate for replication and the nick is eventually resealed, generating a relaxed CCC molecule, which is also not a substrate for replication. According to this model, cruciform extrusion satisfies two separate initiation requirements, namely generating a single-stranded substrate for the site-specific nicking reaction and establishing the melted region in the DSO that is necessary for assembly of the replisome. Since the modified protein cannot promote cruciform extrusion, it cannot satisfy either of these requirements. An alternative possibility is that the cruciform mechanism is used to ensure that initiation occurs at the correct site rather than at any of several subsidiary sites that are cleaved when in single-stranded form (Koepsel and Khan, 1987). This seems unlikely because RepC will not nick supercoiled pT181 DNA *in vitro* with detectable frequency at any of these secondary sites, even though some of them are in AT-rich regions (Koepsel *et al*., 1986; Koepsel and Khan, 1987).

At the end of a replication cycle, the leading strand is extended for a short distance past the initiation nick site, as it is with ϕX174 (Reinberg *et al*., 1983), displacing the

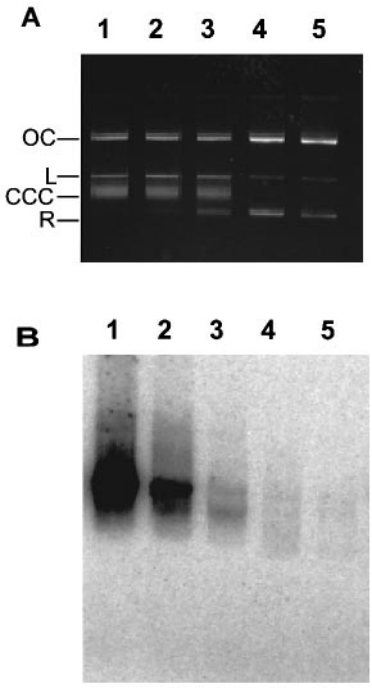

Fig. 9. Effect of prior nicking by RepC/C on replication of pT181 DNA in a cell-free extract. Samples of pT181 plasmid DNA were incubated with increasing amounts of purified RepC/C for 5 min, half of each sample was loaded onto an agarose gel and the other half added to a reaction mixture capable of supporting replication *in vitro* and containing [α-^{32}P]dATP. Additional RepC/C was added to each sample so that the final RepC/C concentration was constant. After 30 min at 30°C, replication samples were separated on agarose and the gel analyzed in the phosphorimager (Molecular Dynamics). (**A**) 1% agarose gel (with 1 μg/ml ethidium bromide) electrophoresis of pT181 plasmid DNA after RepC/C treatment for 5 min. Lanes 1–5: 0, 25, 50, 100 and 200 ng of RepC/C respectively. (**B**) 1% agarose gel electrophoresis of samples from *in vitro* DNA replication. The DNA substrates used for replication in lanes 1–5 correspond to the plasmid DNA in (A) from lanes 1–5 respectively.

junction between the nascent and old leading strands and thus generating the necessary single-stranded substrate for cleavage by the free subunit of the bound initiator dimer. This cleavage would be followed by a transesterification that would recircularize the displaced leading strand. A second cleavage–transesterification would circularize the nascent leading strand, releasing the initiator with the observed oligonucleotide attached to one subunit.

Initiation and termination of RC replication are mechanistically closely related because the same protein catalyzes both. A necessary consequence of this close relationship for pT181 is that the modified initiator generated during termination, which is metabolically stable and present in great excess, must be prevented from interfering with initiation. This is accomplished by a rather sophisticated initiation mechanism involving cruciform extrusion. A price of this mechanism, however, is that the system cannot tolerate major changes in the concentrations of the interacting macromolecules: thus it recently has been reported that gross overproduction of RepC inhibits plasmid replication (Iordanescu, 1995). We have found that, under these conditions, the plasmid is degraded and there is a vast accumulation of RepC/C* (unpublished data). We have observed a similar overaccumulation of RepC/C* with a pT181 high copy mutant (pT181*cop-633*) maintained in the helicase-deficient mutant, *pcrA3* (Iordanescu, 1993) (unpublished data). We suggest that in the absence of helicase activity, leading strand extension stops at or near the end of the melted region, triggering a strand exchange which aborts the replication cycle and releases the RepC/C* derivative, and that overproduction of this material makes matters worse: the remaining supercoiled molecules would be nicked and would either be relaxed or converted to the non-replicatable form V (Figure 8C). The similarity of these two situations suggests that PcrA helicase may be the rate-limiting factor when RepC is grossly and rapidly overproduced.

Given that under normal conditions, approximately one RepC/C dimer is synthesized per plasmid replication event, it seems odd, in view of the ability of RepC/C* to nick supercoiled DNA, that the 90–95% of non-replicating plasmid molecules are supercoiled *in vivo* despite the large excess of RepC/C*. Perhaps the level of unrestrained superhelicity *in vivo* (Pettijohn and Pfenninger, 1980) is too low to support spontaneous extrusion of the IR II cruciform at a frequency sufficient for nicking by RepC/C*, except when the protein is grossly overproduced.

Comparative analysis of RepC/C and RepC/C* suggests that attachment of an oligonucleotide to one subunit of the protein induces an allosteric shift in structure that determines the differences in properties. Thus RepC/C* binds considerably less strongly to the DSO, induces a considerably weaker bend, has a considerably less extensive footprint on supercoiled DNA (Jin *et al*., 1996) and is unable to promote extrusion of the IR II cruciform; subtle differences have also been observed for cleavage of oligonucleotide substrates. Significantly, the two forms have indistinguishable footprints on linear DNA (Jin *et al*., 1996). Differential protease sensitivity of the corresponding forms of the closely related RepD protein has been observed recently by C.Thomas and co-workers (personal communication).

A comparison of RC phage (ϕX174, for example) and plasmid replication strategies suggests that the latter is the more highly developed form owing to its more complicated regulatory requirements. One critical difference is that the phage requires only superhelix-driven melting of an AT-rich region containing its nicking site, whereas the plasmid appears to extrude a GC-rich cruciform to generate the necessary single-stranded target. This cruciform is only a subsidiary site of nuclease S1 sensitivity in supercoiled pT181 (Noirot *et al*., 1990), indicating that it is disfavored for spontaneous extrusion. A related important difference is that the phage can assemble its replisome on a nicked circular template, probably using Rep helicase to unwind the DNA for recruitment of the replication proteins, whereas the plasmid requires a melted template, generated by a reconfiguration of the extruded cruciform using host replication proteins, and not requiring PcrA helicase.

We note that the M13 DSO nick site is also located at the tip of a potential cruciform and that melting of the putative loop region is promoted by the binding of the initiator (here a tetramer) to a supercoiled substrate. Deletion of one arm of the IR in this case does not affect nicking (Higashitani *et al*., 1994); however, it profoundly affects replication (Dotto *et al*., 1982) as our model would predict. Since M13 has a stable existence as a plasmid it

may represent an intermediate between RC plasmids and phages. A general prediction that follows from these considerations is that the IR II cruciform is required for efficient replication. Tests of this prediction are currently in progress.

Finally, ϕX174 terminates replication by a single cleavage–transesterification, corresponding to the first stage of termination for the plasmid (Figure 2C–E), in which the second active site tyrosine is attached to the 5′ end of the nascent leading strand and the displaced leading strand is released as a single-stranded circular monomer. Successive phage replication cycles then follow automatically, alternating the two active site tyrosines. The plasmid avoids this by a second cleavage–transesterification, releasing the modified initiator which cannot re-initiate replication. Thus, a second critical difference between plasmid and phage is the nature of the signal that stops progress of the replication fork. A third remaining unknown is how the plasmid reconfigures the extended leading strand for cleavage. One among several possibilities is that the stopping signal for the plasmid is strong enough to allow the 3′ end of the nascent leading strand to displace the homologous 3′ extension, allowing the latter to be cleaved by the bound initiator.

Materials and methods

Bacterial strains, plasmids and growth conditions

Staphylococcus aureus strains used were derivatives of NTCC8325. Plasmids pRN5548, pRN6921 and pRN6397 were from this laboratory (Novick *et al*., 1993; Jin *et al*., 1996). pRN6921 was constructed by cloning *repC-his*$_{n6}$ (six histidine codons fused to the N-terminus) to pRN5548 (Novick *et al*., 1993) so that its expression is driven by the β-lactamase promoter. In this construct, the pT181 DSO, which is located within *repC*, was inactivated by a synonymous substitution of four nucleotides surrounding the nick site (Iordanescu, 1989). pRN6397 contains the functional pT181 DSO cloned to pE194. SA2342, a mutant defective in PcrA helicase (Iordanescu and Bargonetti, 1989) and containing plasmid pT181*cop-633* was kindly provided by S.Iordanescu. All strains were grown in CY broth (Novick and Brodsky, 1972) or BHI broth (Difco) with vigorous aeration at different temperatures. Growth was monitored turbidimetrically using a Klett-Sumersen colorimeter with a green (540 nm) filter.

Purification and analysis of RepC protein

N-terminal histidine-tagged RepC/C protein was purified from *S.aureus* strain RN8601 containing pRN6921, and RepC/C* was purified from a strain containing pRN6921 plus pRN6397 as previously described (Jin *et al*., 1996). RepC/C and its heterodimeric derivatives were analyzed by quantitative SDS–PAGE and in some cases by quantitative Western immunoblot analysis. Gels and blots were analyzed by scanning densitometry using an Alpha-Innotech videoimager.

Preparation of pcrA3 extracts

*SA*2342 (*pcrA3*) cells were grown in 500 ml of BHI broth to a density of 120–130 Klett units, collected by centrifugation at 4°C, then resuspended in 3–4 ml of 20 mM Tris–HCl, pH 8.0, 10 mM EDTA, 50 mM NaCl, 20% sucrose. Cells were digested for 1 h at 4°C with 150 μg/ml lysostaphin, and then lysed by freeze–thawing. Cell debris and the chromosomal DNA were pelleted by centrifugation at 14 000 r.p.m. for 30 min at 4°C in a Sorvall SS34 rotor. The supernatant, containing the initiation complex, was stored at –80°C.

Preparation of end-labeled oligonucleotides

Oligonucleotides were obtained from Integrated DNA Technology (IDT) and purified by polyacrylamide gel electrophoresis as required. Oligonucleotides were 5′-end-labeled by polynucleotide kinase and [γ-^{32}P]ATP. Reactions contained 20 pmol of single-stranded oligonucleotide, 70 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 20 μCi of [γ-^{32}P]ATP and 20 U of T4 polynucleotide kinase. Mixtures were incubated at 37°C for 30 min and reactions stopped by heating at 65°C for 20 min.

Re-ligation reactions

Reaction mixtures (50 μl) contained 10 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM EDTA, 5 μg of RepC/C or RepC/C* and 1 pmol of ^{32}P-5′-end-labeled oligonucleotide. After incubation at 32°C for 30 min, reactions were stopped by the addition of 20 μl of 80% formamide and heated at 95°C for 2 min before loading on 8% urea–denaturing polyacrylamide gels.

Specific plasmid DNA relaxation assay

Reaction mixtures (200 μl) contained 10 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM $Mg(OAc)_2$, 5% ethylene glycol, 1 mM EDTA, equimolar amounts of Rep protein (RepC/C or RepC/C*) and pT181*cop-623* (Carleton *et al*., 1984) supercoiled plasmid DNA. The mixtures were incubated at 32°C, 20 μl aliquots were taken at different time points and reactions stopped by the addition of 2 μl of 500 mM EDTA. Samples were resolved on 0.8% agarose gels containing 1 μg/ml ethidium bromide in TBE buffer. Gels were analyzed by scanning densitometry using an Alpha-Innotech videoimager.

Cleavage of synthetic DNA substrates

Reaction mixtures (20 μl) contained 10 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM $Mg(OAc)_2$, 1 mM EDTA, 0.5 μg of RepC/C and 10 pmol of DNA substrate. Reactions were incubated at 32°C for 30 min, stopped by the addition of 5 μl of 5× SDS loading buffer, and heated at 95°C for 2 min before loading on 12% SDS–PAGE. Western blotting was performed using anti-RepC polyclonal antibodies and alkaline phosphatase-conjugated anti-rabbit monoclonal antibody.

In vitro DNA replication

Extracts of a plasmid-negative strain were prepared and assayed as described by Khan *et al.* (1981). Reaction mixtures contained various amounts of RepC/C, 1 μg of supercoiled pT181*cop-623* DNA, 5 μCi of [α-^{32}P]dATP and 50 μl of cell extract. Reaction mixtures were incubated at 30°C for 30 min then electrophoresed on a 1% agarose gel, autoradiographed and analyzed with a Molecular Dynamics phosphoimager.

$KMnO_4$ analysis

Two μg of pT181 plasmid DNA was incubated with 10 pmol of RepC/C or RepC/C* protein in binding buffer (10 mM Tris–HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA) without Mg^{2+} in a total volume of 50 μl at 37°C. After 30 min, 2.5 μl of 80 mM $KMnO_4$ was added to the reaction for 1 min at 37°C. The reaction was stopped by the addition of 2.5 μl of β-mercaptoethanol and plasmids recovered by using Qiaprep miniprep spin columns. NaOH (200 mM) was used to break the backbone of DNA at the sites of $KMnO_4$ attack and to denature the double-stranded plasmid DNA. Primer extension reactions were carried out using ^{32}P-5′-end-labeled primers hybridized to either of the plasmid strands and 3 U of Sequenase for 15 min at 43°C. The reactions were stopped by the addition of a formamide–dye mixture and heated at 90°C for 2 min prior to denaturing gel electrophoresis.

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