

# An Oligonucleotide Inhibits Oligomerization of a Rolling Circle Initiator Protein at the pT181 Origin of Replication\*

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A large number of plasmids have been shown to replicate by a rolling circle (RC) mechanism. The initiators encoded by these plasmids have origin-specific, nicking-closing activity that is required for the initiation and termination of RC replication. Since the initiators of many RC plasmids are rate-limiting for replication, these proteins are usually inactivated after supporting one round of replication. In the case of the pT181 plasmid, inactivation of the initiator RepC protein occurs by the attachment of an oligonucleotide to its active tyrosine residue. We have generated the inactivated form of RepC, termed RepC\*, *in vitro* and investigated the effects of attachment of the oligonucleotide on its various biochemical activities. Our results demonstrate that while RepC\* is inactive in nicking-closing and replication activities due to the blockage of its active tyrosine residue, it is competent in origin DNA binding and DNA religation activities. We have investigated the oligomeric state of RepC and RepC\* and found that RepC exists as a dimer in solution and can oligomerize on the DNA. We have generated heterodimers *in vitro* between the wild-type and epitope-tagged RepC proteins. In electrophoretic mobility shift experiments, the initiator heterodimers generated a novel DNA-protein complex, demonstrating that it binds to DNA as a dimer. We have shown that a DNA binding mutant of RepC can be targeted to the origin in the presence of the wild-type protein primarily through a protein-protein interaction. Interestingly, RepC\* is defective in its ability to oligomerize on the DNA. RepC\* inhibited the DNA binding and replication activity of wild-type RepC to only a very limited extent, suggesting that it may play only a minor regulatory role in replication *in vivo*. Based on these and earlier results, we propose a model for the role of RepC during the initiation and termination of pT181 RC replication.

Bacterial plasmids provide useful model systems to study the mechanism and regulation of DNA replication. A large group of plasmids in Gram-positive bacteria and many in Gram-negative bacteria replicate by a rolling circle (RC)<sup>1</sup> mechanism (for reviews, see Refs. 1–3). Replication of RC plasmids is mecha-

nistically similar to those of single-stranded (SS) DNA bacteriophages of *Escherichia coli* (4–6). Replication of RC plasmids is initiated by the generation of a single strand nick at the origin of replication by the plasmid-encoded initiator proteins. Plasmids of the pT181 family in *Staphylococcus aureus* encode initiators that have origin-specific DNA binding and topoisomerase-like activities (7–9). The origin of replication of pT181 consists of three sets of inverted repeat (IR) elements, termed IRI, IRII, and IRIII (Ref. 10; Fig. 1). We have previously shown that the pT181 origin contains a bend that is enhanced by binding of RepC (11). The pT181 origin also contains a cruciform region centered around IRII, and RepC has been shown to enhance cruciform extrusion (12). It has also been shown that while both IRII and IRIII are required for initiation of replication, IRII is sufficient for the termination step (13, 14).

The RepC protein consists of 314 amino acids with a molecular weight of 38,000 (15, 16). Footprinting studies have shown that the initiator encoded by pT181, RepC, binds to IRIII and the proximal arm of IRII (Ref. 8; Fig. 1). The RepC nick site is located between nt 70 and 71 within the loop of IRII (7). After nicking, RepC becomes covalently attached to the 5'-phosphate of the DNA through the active site tyrosine residue at position 191 (17, 18). The sequence-specific DNA binding domain of RepC has been identified, and amino acids 267–270 in RepC were shown to be important for this activity (19).

Plasmid pT181 is maintained at an approximate copy number of 22 (20). Previous studies have shown that RepC is rate-limiting for replication and that the primary mode of regulation of pT181 copy number involves a transcription attenuation mechanism of RepC mRNA by an antisense RNA (21, 22). It has also been shown that overexpression of RepC *in vivo* can inhibit pT181 replication, probably due to the titration of the rate-limiting PcrA helicase (23–25). To prevent the reutilization of an initiator molecule that has been used for a round of replication, RepC is inactivated by the attachment of an approximately 11-nt-long oligonucleotide corresponding to pT181 positions 70–60 located immediately downstream of the initiator nick site (26, 27). This probably results from the passage of the replication fork past the RepC nick site to the end of IRII (nt 60) after one round of replication and the subsequent cleavage and religation of the DNA by RepC (3, 14). Interestingly, preparations of the initiator protein from cells containing either wild-type pT181 or its copy mutants contain an approximately 1:1 mixture of the RepC and RepC\* forms (27, 28). Based on this and other findings, it has been proposed that RepC exists as a dimer in solution and that attachment of the oligonucleotide to one subunit results in the inactivation of the other, wild-type subunit by an as yet unknown mechanism (25,

polyacrylamide gel electrophoresis; bp, base pair(s); EMSA, electrophoretic mobility shift assay.

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<sup>1</sup> The abbreviations used are: RC, rolling circle; SS, single-stranded; SC, supercoiled; IR, inverted repeat; nt, nucleotide(s); MBP, maltose-binding protein; FPLC, fast protein liquid chromatography; PAGE,

27). Recently, the RepC/RepC\* heterodimer isolated from pT181-containing cells was found to retain its DNA binding activity but was defective in its ability to induce cruciform extrusion at the origin (25, 28, 29). Since preparations of RepC\* from pT181-containing cells always contain a 1:1 mixture of RepC and RepC\* forms, we have generated RepC\* *in vitro*. This preparation consists of approximately 95% of the molecules in the RepC\* form and is suitable for investigation of its biochemical activities. We have studied the oligomeric state of RepC and found that RepC exists as a dimer in solution and can oligomerize upon binding to the DNA. We also report here the finding that while RepC\* also exists as a dimer in solution, attachment of an oligonucleotide to the initiator protein blocks its oligomerization at the origin. Our results also demonstrate that while RepC\* is defective in its DNA nicking and replication activities, it retains origin binding and DNA religation activities.

#### EXPERIMENTAL PROCEDURES

**Purification of RepC and MBP-RepC Proteins**—Wild-type RepC protein was purified using an overexpression system as described earlier (16). For the production of the maltose binding protein (MBP)-RepC fusion protein (MBP-RepC), the *repC* gene was cloned in frame with the MBP coding sequence utilizing the vector pMAL-c, which contains the isopropyl-1-thio- $\beta$ -D-galactopyranoside-inducible *tac* promoter (30). This resulted in the generation of a MBP-RepC fusion protein (80-kDa) in which MBP was present at the amino-terminal end of RepC. Synthesis of the fusion protein was induced by the addition of isopropyl-1-thio- $\beta$ -D-galactopyranoside to exponentially growing cultures, and the MBP-RepC fusion protein was purified by affinity chromatography on amylose-resin columns (30). The MBP-RepC fusion protein was eluted with a buffer containing 20 mM Tris-HCl (pH 8), 200 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM maltose, and the fractions were dialyzed against RepC buffer (20 mM Tris-HCl (pH 8.0), 200 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, and 10% ethylene glycol). The RepC and MBP-RepC proteins were generally more than 95% pure as determined by SDS-PAGE and staining with Coomassie Brilliant Blue. Heterodimers between the RepC and MBP-RepC proteins were generated by denaturation *in vitro*. Equimolar amounts of the two proteins were mixed and incubated at room temperature for 1 h in RepC buffer containing 7 M urea. This was followed by successive dialysis of the protein against RepC buffer containing 3 M urea, 1 M urea, and no urea. As controls, the RepC and MBP-RepC proteins were treated individually in a similar manner.

**Preparation of RepC\***—Seventy micrograms of RepC protein were incubated with an approximately 4-fold molar excess of an oligonucleotide containing the right arm and loop of IRII (pT181 nt 75–60) at 32 °C for 15 min. Substantial amounts of the unreacted oligonucleotide were removed by centrifugation of the reaction mixture on a G-50 spin column. However, this RepC\* preparation was further purified by FPLC (described below) to completely remove the unreacted oligonucleotide. This was necessary, since we found that in DNA relaxation and *in vitro* replication experiments involving competition between RepC\* and wild-type RepC, the presence of free oligonucleotide and magnesium ions resulted in the conversion of RepC to RepC\* and gave artificial results that suggested that RepC\* severely inhibited the RepC activity (data not shown). The extent of conversion of RepC to RepC\* was determined by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Typically, RepC\* preparations contained no more than 5% of wild-type RepC. To recover RepC activity from RepC\*, the RepC\* preparation was incubated with a 20-fold molar excess of an oligonucleotide (13-mer) representing the region immediately 5' to the RepC nick site (nt 83–71) for 15 min at 32 °C in TEKEM buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM KCl, 10 mM MgCl<sub>2</sub>, and 10% ethylene glycol). The reaction product was then directly used to assay for the recovered RepC activities.

**DNA Relaxation Assays**—Nicking-closing assays were done as described previously (7). One microgram of supercoiled pT181*cop608* DNA was incubated with various amounts of the RepC or RepC\* proteins at 32 °C for 30 min in TEKEM buffer, and the reaction mixtures were subjected to electrophoresis on 0.7% agarose gels using TBE (Tris borate-EDTA) buffer containing 1  $\mu$ g/ml ethidium bromide. To study the inhibitory effect of RepC\* on the relaxation activity of RepC, the proteins were mixed together prior to the addition of DNA.

**Nicking and Religation of Oligonucleotides**—For nicking, 1 pmol of

5'-end-labeled oligonucleotides (pT181 nt 83–60 containing the IRII and nt 75–60 representing the right arm and loop of IRII (Fig. 1) were incubated with 200 ng of the RepC or RepC\* proteins for 30 min at 32 °C in TEKEM buffer in a reaction volume of 10  $\mu$ l. For religation, reaction mixtures containing 600 ng of RepC\* along with 1 pmol of labeled oligonucleotides representing the sequence immediately 5' to the RepC nick site (pT181 nt 83–71 and 75–71; Fig. 1) were incubated for 15 min at 32 °C in TEKEM buffer. The above reactions were stopped by the addition of 5  $\mu$ l of sequencing dye, and the products were resolved on 20% polyacrylamide-urea DNA sequencing gels (31).

**Electrophoretic Mobility Shift Assays**—The binding of various proteins to the pT181 origin subregions was investigated by electrophoretic mobility shift assays (EMSA). Double-stranded oligonucleotides containing various subregions of the pT181 origin were 5'-end-labeled with polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (31). Unless otherwise indicated, the 53-bp oligonucleotide (pT181 positions 37–83) was used as the probe. Reaction mixtures (20  $\mu$ l) containing the <sup>32</sup>P-labeled oligonucleotides (2–5 pmol), 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10% ethylene glycol, 100 ng of poly(dI-dC), and the indicated amounts of the initiator proteins were incubated at room temperature for 10 min and electrophoresed on native polyacrylamide gels using TBE buffer. The gels were dried and subjected to autoradiography.

**In Vitro Replication Reactions**—Plasmid DNAs were replicated *in vitro* using *S. aureus* extracts as described earlier (16, 32). Replication reactions were carried out using 200 ng of pT181*cop608* plasmid DNA, 600  $\mu$ g of protein extract, and the indicated amounts of the RepC or RepC\* proteins. The reaction products were labeled with [ $\alpha$ -<sup>32</sup>]dATP and incubated for 60 min at 32 °C. DNA was recovered by treatment with proteinase K, phenol-chloroform extraction, and alcohol-precipitation, and the samples were analyzed by electrophoresis on 0.7% agarose gels using TBE buffer containing 0.5  $\mu$ g/ml ethidium bromide. The gels were dried and subjected to autoradiography.

**FPLC of RepC and RepC\***—Approximately 500  $\mu$ g of RepC or 250  $\mu$ g of RepC\* proteins were loaded onto an Amersham Pharmacia Biotech Superose 12 FPLC column (20 ml) that had been previously equilibrated with the RepC buffer. The column was eluted with 20 ml of the RepC buffer, and 0.5-ml fractions were collected. In a separate experiment, bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) were used as molecular weight standards to generate a calibration curve.

**Sucrose Gradient Sedimentation Analysis of the RepC Protein**—RepC protein was subjected to sedimentation in a 5–20% sucrose gradient (5 ml) in a SW50.1 rotor at 40,000 rpm for 24 h at 4 °C. Ten micrograms each of bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease A along with 16.5  $\mu$ g of RepC were used. Fractions (185  $\mu$ l each) were collected from the bottom of the tubes and analyzed by SDS-PAGE and silver staining. Fractions were also assayed for the topoisomerase activity of RepC as described above.

#### RESULTS

**Generation of the Inactivated RepC\* Protein in Vitro**—To investigate the biochemical activities of the modified pT181 initiator protein, we generated RepC\* *in vitro*. Treatment of an SS oligonucleotide (nt 75–60 of pT181, Fig. 1) containing the nick site with RepC generated a protein preparation that contained approximately 95% RepC\* and less than 5% wild-type RepC as determined by SDS-PAGE (Fig. 2). The above results suggested that if RepC exists as a dimer, both of its subunits must be competent in nicking of the DNA. Alternatively, RepC may exist as a monomer and can be converted to RepC\* almost quantitatively at high concentrations of the oligonucleotide. Treatment of an SS oligonucleotide containing the complete IRII (nt 83–60; Fig. 1) with RepC generated a protein preparation that contained approximately 50% each RepC and RepC\* (Fig. 2). To confirm that RepC\* generated *in vitro* was "active," we tested its religation activity utilizing SS oligonucleotides representing the region immediately 5' to the RepC nick site (Fig. 1). This experiment showed that RepC\* retained its religation activity, and while nt 83–71 ligated efficiently to RepC\*, ligation of nt 75–71 was very poor (Fig. 3). This result is consistent with the observation that more RepC\* was generated in the presence of nt 75 to 60 than with IRII (Fig. 2) due to the less efficient religation of the released oligonucleotide (nt

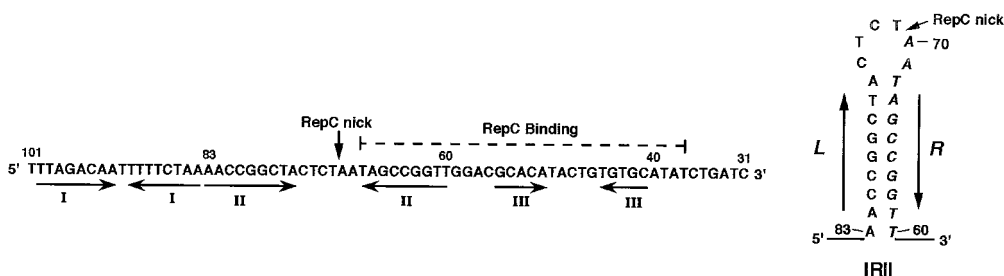


FIG. 1. Nucleotide sequence of the optimal pT181 origin and the structure of the IRII region. Only the leading strand of the DNA is shown, and replication proceeds in a rightward direction. IR sequences IRI, IRII, and IRIII are shown by horizontal arrows. The RepC binding sequence is indicated with a dotted line, and the RepC nick site is indicated by a vertical arrow. Structure of the 24-bp IRII region (nt 83–60) is shown on the right. *L* and *R* refer to the left and right arms of IRII, respectively.

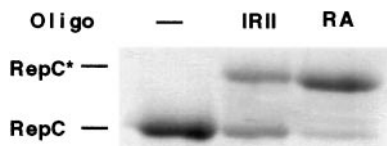


FIG. 2. SDS-PAGE analysis of the RepC and RepC\* proteins. RepC protein incubated with IRII (nt 83–60) or the right arm and loop of IRII (nt 75–60), as well as untreated RepC was subjected to SDS-PAGE on a 12% gel. The positions of RepC and RepC\* are indicated.

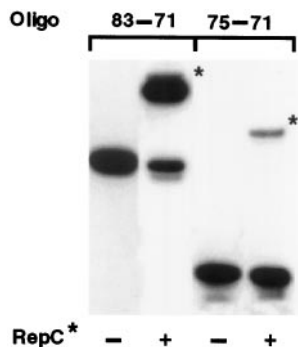


FIG. 3. Religation activity of the RepC\* protein. Purified RepC\* protein was incubated with SS oligonucleotides representing the sequence immediately 5' to the RepC nick site (nt 83–71 or nt 75–71), and the reaction products were analyzed by electrophoresis on a polyacrylamide-urea gel. The asterisks indicate the positions of the regenerated IRII (nt 83–60) or the right arm and loop of IRII (nt 75–60) oligonucleotides by the religation activity of RepC\*.

75–71) to RepC\*. The RepC\* preparation generated *in vitro* by the above procedure had an approximate molecular weight of 42,000 and is similar to that generated *in vivo* except that it contains less than 5% residual wild-type RepC as compared with a 1:1 mixture of RepC and RepC\* forms isolated from pT181-containing cells (27). The RepC\* preparation generated *in vitro* was used to investigate the various biochemical activities of the modified initiator protein.

**RepC\* Is Defective in Nicking-Closing of the DNA**—We investigated the DNA relaxation activity of RepC\* using supercoiled (SC) pT181*cop608* DNA as the substrate. While low levels of wild-type RepC efficiently relaxed the SC DNA, the RepC\* preparation was essentially inactive (Fig. 4A). The weak relaxation activity in the presence of high levels of RepC\* may be due to the residual amounts of RepC present in the preparation. To further rule out the possibility that RepC\* was inactivated during its preparation *in vitro*, we incubated RepC\* with an oligonucleotide representing the region immediately 5' to the RepC nick site (left arm and a portion of the loop of IRII, nt 83–71 in Fig. 1). This treatment was expected to result in the religation of the oligonucleotide attached to RepC\* to the substrate oligonucleotide and the concomitant regeneration of wild-type RepC. RepC\* treated in such a way was found to be

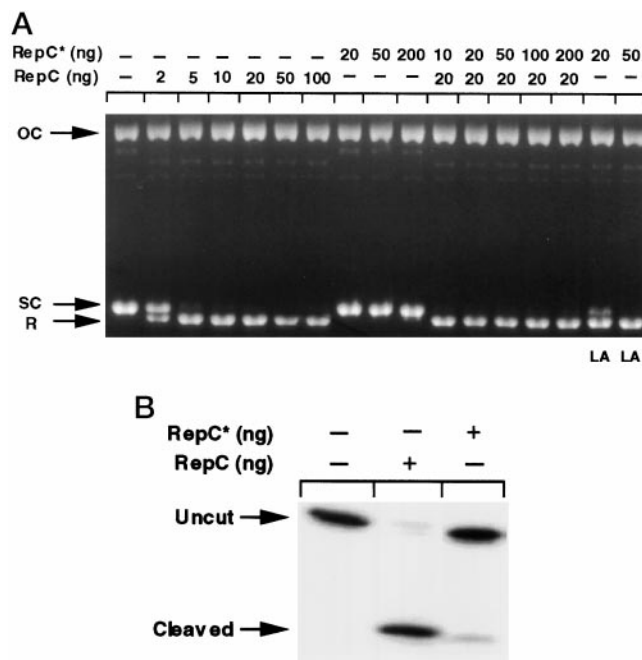


FIG. 4. Analysis of the DNA relaxation and SS DNA cleavage activities of RepC\*. A, supercoiled pT181*cop608* DNA was treated with the indicated amounts of either RepC or RepC\* or both. In the lanes labeled *LA* at the bottom, RepC\* was preincubated with a 13-nt-long oligonucleotide (positions 83–71) containing the left arm and a portion of the loop of IRII to regenerate RepC prior to its addition to the relaxation reactions. The positions of the SC, open circular (OC), and covalently closed relaxed circular (R) DNA are indicated. B, a <sup>32</sup>P-labeled oligonucleotide representing the right arm and loop of IRII (nt 75–60) was treated with 200 ng of RepC or RepC\*, and the cleavage products were analyzed on a 20% polyacrylamide-urea DNA sequencing gel. The positions of the uncut and cleaved oligonucleotides are indicated.

active in DNA relaxation (Fig. 4A). These results showed that RepC\* was inactive in nicking-closing due to the attachment of the oligonucleotide to its active tyrosine residue. RepC\* did not detectably inhibit the relaxation activity of wild-type RepC in competition experiments (Fig. 4A). We also tested the cleavage of SS DNA by RepC\*. As expected, while RepC efficiently cleaved the SS oligonucleotide (nt 75–60), RepC\* had very low cleavage activity (Fig. 4B). This low level activity of RepC\* may be due to the residual levels of RepC present in the RepC\* preparation. The above results indicate that RepC\* is defective in SS DNA cleavage and DNA relaxation activities and does not significantly inhibit the relaxation activity of RepC.

**RepC\* Is Inactive in *In Vitro* Replication**—Since RepC\* lacked DNA nicking-closing activity, it was expected to be inactive in replication. We wished to determine if this was the case and if RepC\* played a regulatory role in DNA replication by inhibiting the activity of wild-type RepC. As shown in Fig. 5,

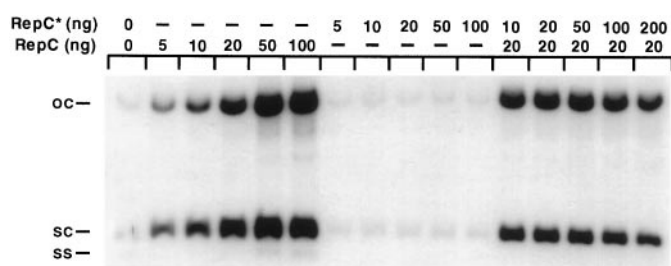


FIG. 5. Analysis of the *in vitro* replication activity of RepC and RepC\*. Plasmid pT181cop608 DNA was replicated *in vitro* in the presence of the indicated amounts of the RepC and RepC\* proteins. The reaction products were analyzed by agarose gel electrophoresis and autoradiography. SS, single-stranded DNA; RI, replicative intermediates; SC, supercoiled DNA; OC, open circular DNA.

wild-type RepC was active in *in vitro* replication even at low levels, while RepC\* was totally inactive in replication. In competition experiments, RepC\* inhibited the replication activity of wild-type RepC only to a limited extent (Fig. 5), with an approximately 2-fold inhibition at a 10:1 molar ratio of RepC\* to RepC as determined by quantifying the radioactivity using an Ambis 100 radioanalytic detector.

**DNA Binding and Oligomerization of RepC and RepC\***—We have previously shown by footprinting studies that RepC binds to double-stranded pT181 origin DNA (nt 37–68) that includes IRIII and the right arm of IRII (8). The DNA binding activity of RepC has also been shown to be critical for its replication activity (17, 19). We wished to determine whether RepC\* generated *in vitro* is able to bind to the pT181 origin of replication. EMSA were carried out using the following four synthetic, double-stranded oligonucleotides representing the various subregions of the pT181 origin: a 53-bp DNA (containing pT181 nt 37–83; Fig. 1) that included both IRIII and IRII; a 46-bp fragment (pT181 nt 37–60); a 30-bp fragment containing only IRIII (pT181 nt 37–60); and a 26-bp fragment containing only IRII (pT181 nt 60–83). The sizes of the various oligonucleotides used were slightly larger than the respective pT181 sequence they contained due to the presence of linker sequences at their ends. With the 53- and 46-bp oligonucleotides and low levels of RepC, the C1 DNA-protein complex was present at much higher levels than the larger C2 complex, and the levels of the C2 complex increased substantially at higher levels of RepC (Fig. 6A). Interestingly, only a single DNA-protein complex (C1) was observed, with the 30-bp region containing only the IRIII region even at high levels of RepC (Fig. 6A). Similarly, only a single complex was observed with the 26-bp IRII region, although the levels of this complex were much lower than those seen with all the other origin regions (Fig. 6A). It should be noted that while the sizes of the four DNA probes used were different, the C1 and C2 complexes migrated to the same position regardless of the probe used. This may be due to the small sizes of the probes used where the electrophoretic mobility was predominantly determined by the size, shape, and charge of the RepC protein. The above results suggested that two RepC-DNA complexes were formed at the origin, one due to the binding of RepC to IRIII, and the other upon binding to both the IRIII and IRII regions. The RepC\* protein retained significant DNA binding activity as tested by EMSA using the 53-bp fragment as the probe (Fig. 6B). Interestingly, in the presence of RepC\* only the C1 complex was obtained even at high concentrations of the protein. The C1 complex observed with RepC\* (RC\*-C1) migrated slightly slower than the C1 complex observed with RepC, consistent with the fact that the molecular weight of monomeric form of RepC\* is approximately 42,000 as compared with 38,000 for RepC (26). RepC\* competed with RepC for binding to the DNA to a limited extent when present at higher

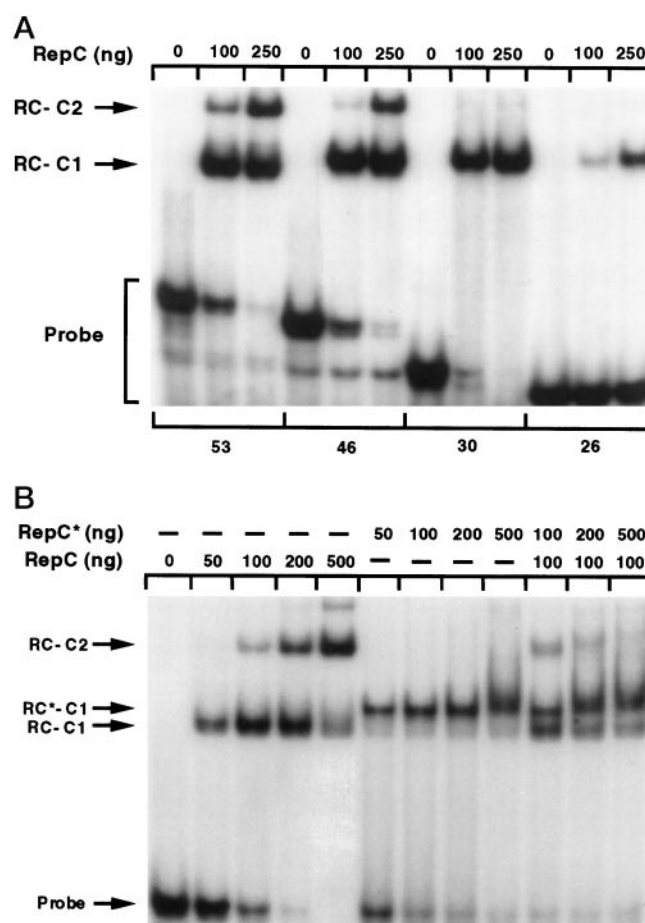


FIG. 6. Binding of the RepC and RepC\* proteins to the pT181 origin.  $^{32}$ P-labeled synthetic oligonucleotides containing various portions of the pT181 origin were incubated with the indicated amounts of the RepC or RepC\* proteins in the presence of 100 ng of poly[d(I-C)]. The DNA-protein complexes were electrophoresed through 6% native polyacrylamide gels. A, binding of the RepC protein to the various origin regions. RC-C1 and RC-C2 indicate the two RepC-DNA complexes. The probes used were 53 bp (nt 37–83); 46 bp (nt 46–83); 30 bp (nt 37–60), and 23 bp (nt 60–83). B, binding of the 53-bp oligonucleotide to RepC and RepC\* or both. RC\*-C1 indicates the complex formed between RepC\* and DNA. Other complexes are as shown in A.

levels (Fig. 6B). RepC\* also generated a single complex with the 46-, 38-, and 23-bp oligonucleotides (data not shown). The above results showed that RepC\* retains significant DNA binding activity and that while RepC is capable of oligomerization at the origin, RepC\* is inactive in this function.

We further investigated oligomerization of RepC and RepC\* when bound to the DNA utilizing a MBP-RepC fusion. As seen with RepC, the MBP-RepC protein generated two DNA-protein complexes when the 53-bp oligonucleotide was used as the probe (Fig. 7). The binding of MBP-RepC to the DNA was weaker than that of unfused RepC, indicating that the epitope tag may interfere with the DNA binding activity of RepC to a moderate degree. The migration of the C1 and C2 complexes obtained with MBP-RepC (MBRC-C1 and MBRC-C2) was consistent with its monomeric molecular weight of 80,000, which is slightly more than twice that of wild-type RepC. When RepC and MBP-RepC were incubated together with the DNA, their respective C1 and C2 complexes were obtained (Fig. 7). Interestingly, a novel intermediate complex (IC) was also observed (Fig. 7). The position of the intermediate complex (between the MBRC-C1 and MBRC-C2 complexes) is consistent with it containing one molecule each of RepC and MBP-RepC, further suggesting that RepC may oligomerize on the DNA. Interest-



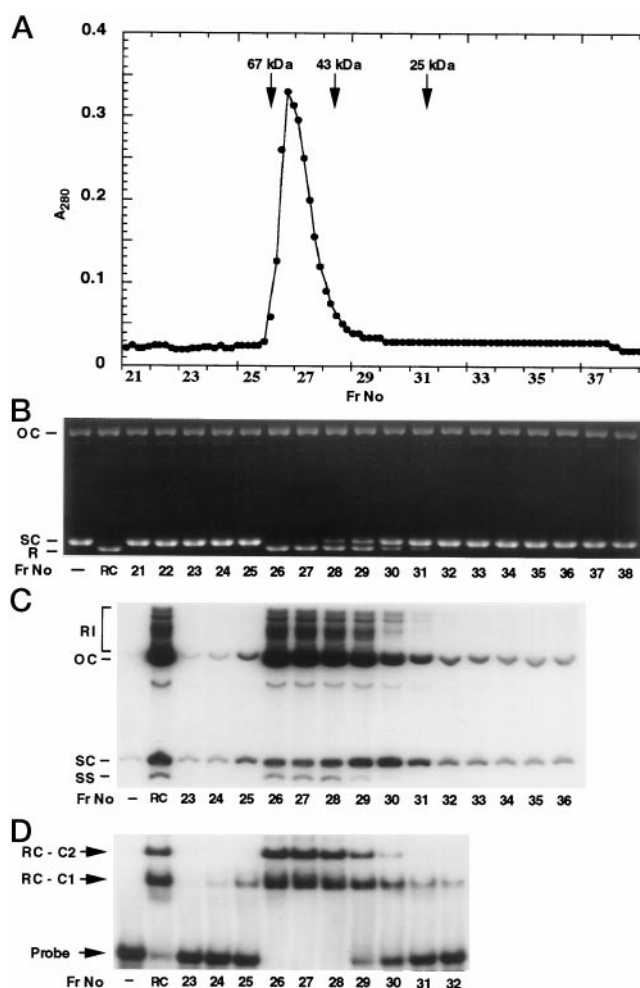


FIG. 8. FPLC analysis of the RepC protein. A, elution profile of the RepC protein on a Superose 12 FPLC column. The positions of the standard protein markers are shown in A. The DNA relaxation (B), *in vitro* replication (C), and DNA binding (D) activities of the column fractions are shown. Abbreviations are as shown in the legends for Fig. 4A, 5, and 6A. RC indicates wild-type RepC used as a control in these experiments.

ing to binding by RepC homodimer and RepC/MBP-RepC heterodimer were present, only low levels of the MBRC-C1 complex corresponding to binding of the MBP-RepC homodimer were observed (Fig. 10). This may be due to the fact that the MBP-RepC protein does not bind as well to the DNA as the RepC homodimer (and possibly the RepC/MBP-RepC heterodimer). This prediction is consistent with data shown in Fig. 7. Faint bands corresponding to the RC-MBRC complex were also visible in urea-treated MBP-RepC samples (Fig. 10). This band was also observed with the untreated MBP-RepC sample upon overexposure of the autoradiogram. This weak band may be generated by the presence of trace amounts of free RepC in MBP-RepC preparation due to cleavage at the fusion point followed by the generation of RepC/MBP-RepC heterodimers. The low-level cleavage of MBP epitope from MBP-RepC protein may increase slightly during the urea denaturation-renaturation steps. In any event, the presence of such a band is consistent with a dimeric state of RepC in solution.

#### DISCUSSION

Previous studies have shown that in pT181-containing cells, RepC and RepC\* are present in an approximately 1:1 ratio regardless of the plasmid copy number (27, 28). Using partially purified initiator preparations from such cells, RepC\* was found to be defective in its ability to extrude cruciform at the

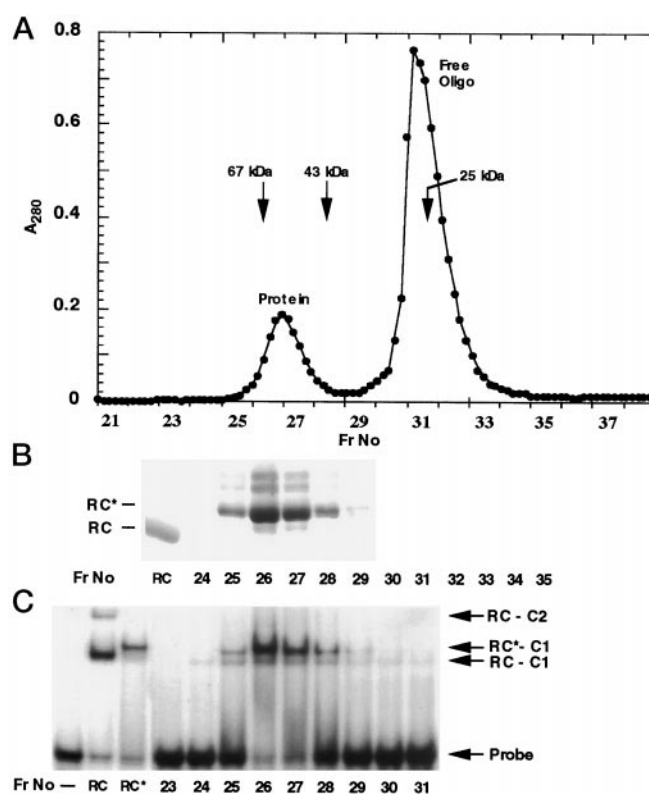


FIG. 9. FPLC analysis of the RepC\* protein. A, elution profile of the RepC\* protein on a Superose 12 FPLC column. The positions of the standard protein markers and the excess oligonucleotide present in the RepC\* preparation are shown in A. B, SDS-PAGE analysis; C, EMSA of column fractions. RC and RC\* indicate RepC and RepC\* proteins used as positive controls in these assays. Other abbreviations are as defined in the legend for Fig. 6B.

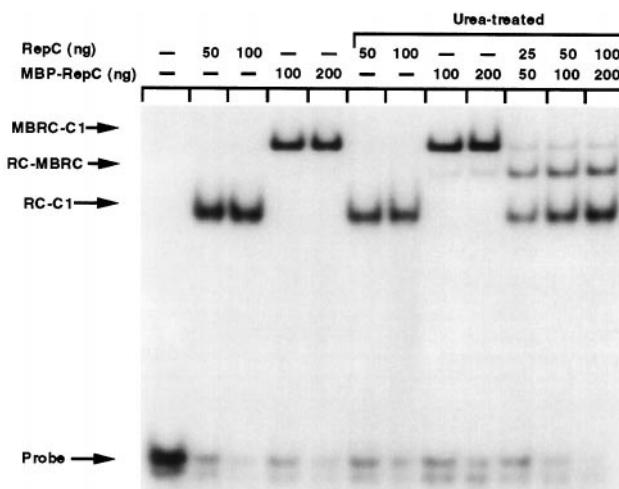


FIG. 10. DNA binding activity of urea-treated RepC and MBP-RepC proteins. RepC and MBP-RepC proteins were denatured either individually or together in urea followed by renaturation. EMSA were then carried out with a probe containing pT181 nt 37–60 that includes the IRIII region. RC-MBRC, DNA-protein complex formed in the presence of the RepC/MBP-RepC heterodimer. Other abbreviations are as defined in the legend for Fig. 7.

pT181 origin and therefore defective in its replication activity *in vitro* (25, 28). RepC\* was also found to inhibit the replication and nicking-closing activities of wild-type RepC (25, 28). Previous studies have also shown that the RepC/RepC\* heterodimer is present in great excess over RepC *in vivo* but does not appear to have any regulatory role in replication (28). Thus, some of the above *in vitro* results are not consistent with the *in*

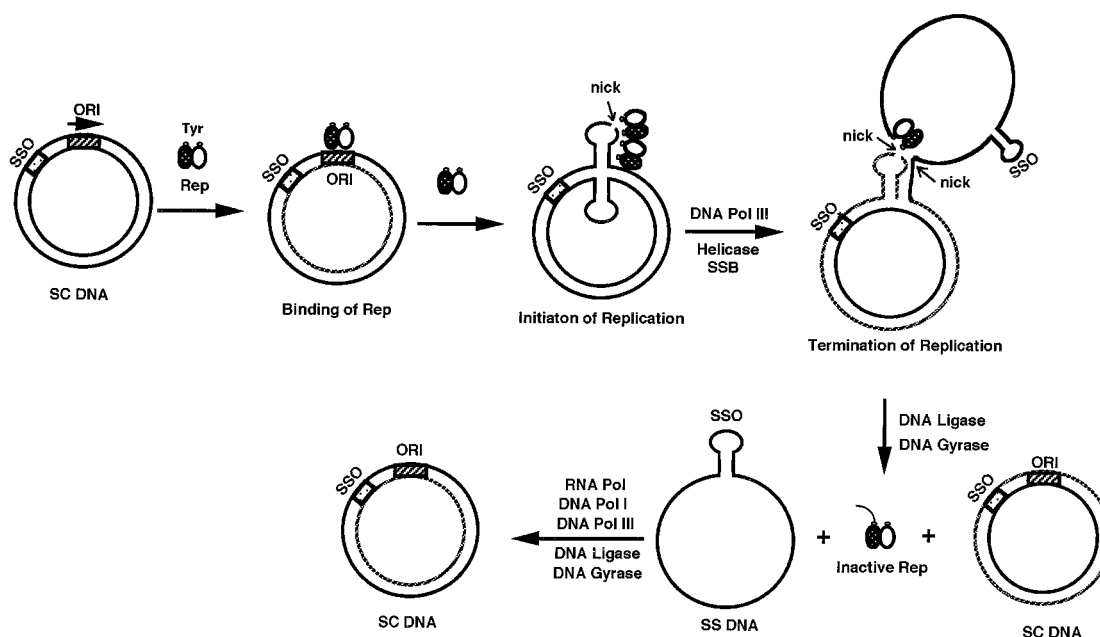


FIG. 11. A model for the role of RepC protein during the initiation and termination of rolling circle replication of plasmid pT181. Details are described under "Discussion." ORI, origin of replication; SSO, single strand origin; SSB, SS DNA-binding protein.

*in vivo* results. Also, since these protein preparations contained an equal amount of RepC and RepC\*, it was not possible to unequivocally determine the various biochemical activities of the modified RepC\* protein.

We have used a different approach to study the biochemical activities of RepC\* and its possible role in the regulation of pT181 replication. We have generated RepC\* *in vitro* by incubating purified RepC protein with an excess of an SS oligonucleotide corresponding to the right arm and a portion of the IRIII loop (Fig. 1). This treatment generated approximately 95% RepC\*, with less than 5% of residual unmodified RepC. RepC\* showed very little or no DNA relaxation activity (Fig. 3A). This was expected, since the active tyrosine residue of RepC\* is blocked due to the attachment of an oligonucleotide. Similarly, RepC\* lacked any *in vitro* replication activity and inhibited the DNA replication activity of wild-type RepC to only a very limited extent (Fig. 5). *In vivo* studies have shown that the inactive initiator is present in the form of the RepC/RepC\* heterodimer (26). The limited replication inhibitory activity of dimeric RepC\* in our experiments may result from binding of RepC\* to the DNA, which may inhibit RepC binding and/or oligomerization at the origin. Similarly, it is possible that RepC\* present *in vivo* may have a minor role in the regulation of replication. These results are consistent with the *in vivo* results, where very little inhibition of replication by the RepC\* protein is observed (28).

Electrophoretic mobility shift assays demonstrated that RepC forms two complexes at the origin containing one (C1) or two (C2) molecules of RepC, respectively. The C1 complex is likely to be due to the binding of RepC to IRIII. This assumption is based on the observations that much higher levels of the C1 complex are observed with the 30-bp IRIII oligonucleotide than with the 26-bp IRII oligonucleotide (Fig. 6A). Interestingly, RepC\* generates only the C1 complex with both the 53-bp (IRII and IRIII) and 30-bp (IRIII) regions and does not bind to the 26-bp IRII region (Figs. 6B and 7 and data not shown). The C1 complex generated by RepC\* migrates more slowly than the C1 complex observed with RepC (Fig. 6B). This is consistent with RepC\* having a molecular weight of 42,000 as compared with 38,000 for RepC (26). When RepC/RepC\* isolated from pT181-containing cells was used in EMSA exper-

iments, it generated a single DNA-protein complex that comigrated with the complex obtained with wild-type RepC (28). Therefore, it was not possible to distinguish between the binding of RepC and RepC\* to the DNA in those studies. We have also found that while an intermediate complex is formed when RepC and MBP-RepC are incubated together with the 53-bp origin region, such a complex is not observed in the presence of the RepC\* and MBP-RepC proteins (Fig. 7). These results demonstrate that the oligonucleotide blocks oligomerization of RepC\* molecules at the origin and also interferes with the assembly of an oligomer on the DNA involving one dimer each of RepC\* and wild-type RepC (or MBP-RepC) that lacks the attached oligonucleotide. Furthermore, the Tyr<sup>191</sup> → Ser topoisomerase mutant was able to dimerize on the DNA (Fig. 7). The above results are consistent with the previous observation that RepC binds to both the IRIII and IRII regions in supercoiled DNA, whereas RepC/RepC\* binds only to the IRIII region (29). The inability of RepC/RepC\* to bind to IRII and oligomerize on the DNA may result in a lack of melting of the pT181 origin involving IRII, which is critical for nicking by the initiator protein. How does the attached oligonucleotide interfere with oligomerization of RepC? The location of the RepC domain(s) involved in its dimerization or oligomerization on the DNA is not known. It is possible that this domain is located near the active tyrosine residue and that the oligonucleotide may inhibit this interaction. Alternatively, it is possible that the attached oligonucleotide interferes with the interaction of RepC\* with the IRII DNA and destabilizes the C2 complex. The Asn<sup>267</sup> → Asp DNA binding mutant generated the C2 complex in the presence of MBP-RepC (Fig. 7), suggesting that oligomerization of RepC on the DNA occurs primarily through a protein-protein interaction. This prediction is consistent with the observation that RepC binds efficiently to IRIII but only weakly to IRII (Fig. 6A).

FPLC analysis of RepC on a Superose 12 column and sedimentation analysis in a sucrose gradient showed that RepC has an approximate molecular weight of 68,400 (Fig. 8 and data not shown). RepC\* showed a pattern similar to that of RepC upon FPLC analysis (Fig. 9). The calculated molecular weight of RepC based on SDS-PAGE and DNA sequence analysis of the *repC* gene is 38,000 (15, 16). Our results confirm the previous

observations that RepC exists as a dimer in solution (9, 27) and further indicate that the RepC\* derivative is also dimeric in nature. An experiment in which RepC was first denatured in 8 M urea and then diluted to a final concentration of 1 M urea, followed by FPLC in the presence of 1 M urea, gave a pattern identical to that obtained with untreated RepC (data not shown). We have also observed that RepC retains its nicking-closing activity in the presence of 1 M urea in reactions (not shown). The above results suggest that the two monomers of RepC are tightly associated in a dimeric molecule and are resistant to dissociation under mild denaturing conditions. We have also generated initiator heterodimers *in vitro* by mixing RepC and MBP-RepC, followed by denaturation in urea and renaturation by dialysis in the absence of urea. The above protein preparations generated an intermediate DNA-protein complex migrating between the C1 complexes observed with RepC and MBP-RepC (Fig. 10). These studies further support our conclusion that RepC binds to the origin as a dimer and subsequently oligomerizes on the DNA.

Based on our results as well as those published by other laboratories, we propose the following model for the assembly and role of RepC during rolling-circle replication of pT181 (Fig. 11). A RepC dimer binds to the IRIII region of the pT181 origin due to a strong sequence-specific interaction. This promotes targeting of another RepC dimer to IRII primarily through a protein-protein interaction that results in the assembly of an initiator oligomer at the pT181 origin. The RepC oligomer then bends the DNA and melts the origin that exposes the RepC nick site in IRII in an SS form. One monomer of a RepC dimer nicks the DNA at the origin and becomes covalently attached to the 5'-end of the nick through its Tyr<sup>191</sup> residue. Following this event, one dimer of the oligomerized RepC may be released from the origin after replication has initiated. After extension synthesis utilizing host replication proteins such as DNA helicase, single-stranded DNA-binding protein, and DNA polymerase III, the replication fork proceeds around the circle to approximately 10 nucleotides beyond the RepC nick site such that the hairpin structure surrounding the nick site is regenerated. At this stage, interaction of the RepC protein with the regenerated origin sequence presumably stalls the replication fork. This step could involve a termination protein that may facilitate pausing of the replication fork. The RepC protein then cleaves at the regenerated nick site, and a series of concerted cleavage/joining reactions occur that result in the release of a circular, SS leading strand DNA and a nicked open circular DNA containing the newly replicated leading strand. The nick is then sealed by the host DNA ligase, and the DNA is subsequently converted to the supercoiled form by DNA gyrase. The newly replicated SC DNA can then rejoin the plasmid pool undergoing replication. The RepC protein is inactivated by the attachment of an approximately 10-nt oligonucleotide located

immediately downstream of the RepC nick site to the active tyrosine residue of one RepC monomer. The inactivated protein may then be released as an inactive RepC/RepC\* heterodimer. The leading strand DNA released by displacement synthesis is converted to the double-stranded form utilizing the single-strand origin. This step involves synthesis of RNA primers by the host RNA polymerase followed by short extension synthesis by DNA polymerase I and subsequent replication by DNA polymerase III. The double-stranded DNA is then sealed by DNA ligase and supercoiled by DNA gyrase.

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## REFERENCES

- Gruss, A., and Ehrlich, S. D. (1989) *Microbiol. Rev.* **53**, 231–241
- del Solar, G. H., Moscoso, M., and Espinosa, M. (1993) *Mol. Microbiol.* **8**, 789–796
- Khan, S. A. (1997) *Microbiol. Mol. Biol. Rev.* **61**, 442–455
- Baas, P. D., and Jansz, H. S. (1988) *Curr. Top. Microbiol. Immunol.* **136**, 31–70
- Reinberg, D., Zipurski, S. L., Weisbeek, P., Brown, D., and Hurwitz, J. (1983) *J. Biol. Chem.* **258**, 529–537
- Zinder, N. D., and Horiuchi, K. (1985) *Microbiol. Rev.* **49**, 101–106
- Koepsel, R. R., Murray, R. W., Rosenblum, W. D., and Khan, S. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 6845–6849
- Koepsel, R. R., Murray, R. W., and Khan, S. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5484–5488
- Thomas, C. D., Balson, D. F., and Shaw, W. V. (1990) *J. Biol. Chem.* **265**, 5519–5530
- Wang, P.-Z., Projan, S. J., Henriquez, V., and Novick, R. P. (1993) *EMBO J.* **12**, 45–52
- Koepsel, R. R., and Khan, S. A. (1986) *Science* **233**, 1316–1318
- Noirot, P., Bargonetti, J., and Novick, R. P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8560–8564
- Zhao, A. C., and Khan, S. A. (1996) *J. Bacteriol.* **178**, 5222–5228
- Zhao, A. C., and Khan, S. A. (1997) *Mol. Microbiol.* **24**, 535–544
- Khan, S. A., and Novick, R. P. (1983) *Plasmid* **10**, 251–259
- Koepsel, R. R., Murray, R. W., Rosenblum, W. D., and Khan, S. A. (1985) *J. Biol. Chem.* **260**, 8571–8577
- Dempsey, L. A., Birch, P., and Khan, S. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3083–3087
- Thomas, C. D., Balson, D. F., and Shaw, W. V. (1988) *Biochem. Soc. Trans.* **16**, 758–759
- Dempsey, L. A., Birch, P., and Khan, S. A. (1992) *J. Biol. Chem.* **267**, 24538–24543
- Carleton, S., Projan, S. J., Highlander, S. K., Moghazeh, S. M., and Novick, R. P. (1984) *EMBO J.* **3**, 2407–2414
- Bargonetti, J., Wang, P.-Z., and Novick, R. P. (1993) *EMBO J.* **12**, 3659–3667
- Novick, R. P., Iordanescu, S., Projan, S. J., Kornblum, J., and Edelman, I. (1989) *Cell* **59**, 395–404
- Iordanescu, S. (1993) *Mol. Gen. Genet.* **241**, 185–192
- Iordanescu, S. (1995) *Mol. Microbiol.* **16**, 477–484
- Jin, R., Fernandez-Beros, M.-E., and Novick, R. P. (1997) *EMBO J.* **16**, 4456–4466
- Rasooly, A., and Novick, R. P. (1993) *Science* **262**, 1048–1050
- Rasooly, A., Wang, P.-Z., and Novick, R. P. (1994) *EMBO J.* **13**, 5245–5251
- Jin, R., Rasooly, A., and Novick, R. P. (1997) *J. Bacteriol.* **179**, 141–147
- Jin, R., Zhou, X., and Novick, R. P. (1996) *J. Biol. Chem.* **271**, 31086–31091
- Guan, C., Li, P., Riggs, P. D., and Inoué, H. (1987) *Gene (Amst.)* **67**, 21–30
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Murray, R. W., Koepsel, R. R., and Khan, S. A. (1989) *J. Biol. Chem.* **264**, 1051–1057



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**NUCLEIC ACIDS, PROTEIN  
SYNTHESIS, AND MOLECULAR  
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**An Oligonucleotide Inhibits  
Oligomerization of a Rolling Circle  
Initiator Protein at the pT181 Origin of  
Replication**

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