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In Vitro Inhibitory Activity of RepC/C*, the Inactivated Form of the pT181 Plasmid Initiation Protein, RepC

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pT181 is a *Staphylococcus aureus* rolling circle plasmid that regulates its replication by controlling the synthesis of its dimeric initiator protein RepC/C and by inactivating the protein following its use in replication (A. Rasooly and R. P. Novick, Science 262:1048–1050, 1993). This inactivation consists of the addition of an oligonucleotide, representing several nucleotides immediately 3′ to the initiation nick site, to the active site tyrosine of one of the two subunits, generating a heterodimer, RepC/C*. Previous results suggested that the inactive form was metabolically stable and was present at a much higher level than the active form (A. Rasooly and R. P. Novick, Science 262:1048–1050, 1993). In the present study we have measured total RepC antigen as a function of plasmid copy number and have analyzed the interaction of the two forms. We find that pT181-containing staphylococci contain approximately one RepC dimer per plasmid copy over a 50-fold range of copy numbers. This is consistent with previous measurements of the rate of RepC synthesis, which suggested that one RepC dimer is synthesized per replication event (J. Bargonetti, P.-Z. Wang and R. P. Novick, EMBO J. 12:3659–3667, 1993). The RepC/C* heterodimer, which is inactive for replication, is a competitive inhibitor of the replication and the topoisomerase-like and cruciform-enhancing activities of the native protein. These results suggest that the inactive form may have a specific regulatory role in vivo. Since the known plasmid-determined controls, which maintain a constant plasmid copy number, are designed to ensure the synthesis of one RepC/C dimer per plasmid replication event, it is difficult to envision any role for yet another negative regulator of replication. Conceivably, under conditions where the initiator is overproduced, such as in the absence of the normal antisense regulation of initiator production, RepC/C* could serve as a fail-safe means of preventing autocatalytic replication.

It is well-established that bacterial plasmids determine their own copy numbers by regulating the frequency of replication initiation. Most plasmids encode specific initiation proteins and set their initiation frequencies by maintaining the intracellular concentrations of these proteins at low, rate-limiting levels (17, 24, 26). With some plasmids, initiator synthesis is precisely coordinated with replication, and newly replicated plasmids cannot replicate again until sufficient new initiator is synthesized (5, 28). With others, there is hyperbolic negative regulation; that is, the rate of initiator synthesis is inversely proportional to the concentration of a diffusible negative regulator (19). We have surmised that this type of regulation requires that the initiator be inactivated after its use, and we have demonstrated this inactivation for rolling circle (RC) plasmids of the pT181 family (25), in which initiator (Rep) synthesis is negatively regulated by diffusible antisense RNAs and therefore is coupled only indirectly to plasmid replication (22). Inactivation of the pT181 initiator is an automatic consequence of the termination mechanism, which leaves a short oligonucleotide covalently attached to one subunit of the dimeric initiator protein. This oligonucleotide represents the DNA sequence immediately 3′ to the initiation nick site (25). A similar form has recently been described for the Rep protein of an unrelated RC plasmid, pUB110 (18).

The steady-state ratio of inactive (RepC/C*) to active (RepC/C) RepC should generally be at least 10:1 simply because plasmid replication events occur throughout the cell cycle and each lasts about 1/10 of the cell doubling time (6). In other words, if the average plasmid copy number is N, about N/10 are engaged in replication at any given time point. If one RepC/C dimer is used and inactivated per replication event, then N new RepC dimers would be made and used in each cell cycle. This means that in a steady-state culture, at least 90% of the existing RepC dimers will already have been used for replication and will be in the RepC/C* form. Therefore, since RepC/C* is metabolically stable (25), it is predicted that less than 10% of the total RepC antigen from pT181-containing cells will represent RepC/C which has been synthesized but not yet utilized. The actual level of RepC/C present in the cell will reflect the time interval between the synthesis and utilization of each molecule. This situation potentially sets up a dynamic interaction between the two forms, and in this paper we show that RepC/C* binds to the leading or double-strand replication origin (DSO) and inhibits the replication functions of RepC/C in vitro. However, it is unlikely that it has any significant inhibitory activity in vivo under normal conditions, though it may seriously interfere with normal replication when overproduced, raising the question of how the in vitro and in vivo situations differ.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used are listed in Table 1. Bacterial stocks were stored at −80°C. Inocula were grown overnight on GL agar (21) with antibiotic supplements as required. All strains were grown in CY broth (21) with vigorous aeration at 37°C. Growth was monitored turbidimetrically with a Klett-Summerson colorimeter with a green (540 nm) filter.

Preparation of lysates. Whole-cell minilysates were prepared by using 1.5-ml samples of exponential CY cultures at about 10^9 bacteria/ml (Klett units, 350). Cultures were centrifuged, and the pellets were washed with TE buffer (10 mM Tris HCl [pH 8], 1 mM EDTA), resuspended in 30 μl of TE with lysostaphin (100

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Reference
Strains		
S. aureus		
8325-4	Standard strain	20
RN27	8325-4(ϕ13 80α)	
SA1350	*plaC1* mutant defective in pT181 copy control	9
RN4111	RN27(pT181 *cop-623*)	4
RN3268	RN27(pT181 *cop-608*)	4
RN7805	8325-4(pRN6759, pI524)	25
RN7807	8325-4(pRN6759, pI524, pRN6366)	25
E. coli MB2	*E. coli* host for pSK184	13
Plasmids		
pT181	Naturally occurring 4.4-kb Tc^r plasmid	22
pT181 *cop-608*	Copy number mutant	4
pT181 *cop-621*	Copy number mutant	4
pT181 *cop-623*	Copy number mutant	4
pSK184	*repC* cloned under λP_L control	13

μg/ml), and incubated for 30 min at 37°C. A total of 30 μl of lysis mixture (0.25 M Tris-HCl [pH 6.8], 4% SDS, 4% glycerol, 1% β-mercaptoethanol, 0.01% bromphenol blue) was added, and the lysates were vortexed for 10 min and then subjected to three freeze-thaw cycles.

Purification of RepC protein. N-terminal histidine-tagged RepC/C protein (25a) was purified from *Staphylococcus aureus* RN8601 containing pRN6921. pRN6921 was constructed by cloning *repC-his*$_{n6}$ (6 histidine codons fused to the N terminus) to pRN5548 (23) 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 replacement of 4 nucleotides surrounding the nick site (8). RepC/C* (with a small amount of RepC/C) was purified from a strain containing pRN6921 plus pRN6397, which contains the functional pT181 DSO cloned to pE194. Cells were grown to a density of 100 Klett units in 1 liter of CY medium, 2-(2′-carboxyphenyl)benzoyl-6-aminopenicillanic acid (CBAP) was added to a final concentration of 5 μg/ml, and growth was continued for another 2 h. Cells were then harvested by centrifugation. The cell pellet was resuspended in 20 ml of buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM KCl, and 5% ethylene glycol. Lysostaphin was then added to a final concentration of 150 μg/ml, and the suspension was incubated on ice for 1 h. After two freeze-thaw cycles, the suspension was centrifuged at 38,000 rpm (Beckman SW 40 Ti) for 30 min. Streptomycin sulfate was added to the supernatant to a final concentration of 3%. The mixture was stirred on ice for 15 min and then centrifuged at 15,000 × *g* for 15 min. The pellet was dissolved in 20 ml of buffer containing 10 mM Tris-HCl (pH 8.0), 1 M KCl, and 5% ethylene glycol. Two milliliters of Ni^{+}-NTA-agarose suspension (Qiagen) was added, and the mixture was incubated at 4°C for 1 h with gentle shaking. The agarose beads were washed with the above buffer four times, and proteins were then eluted with a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM KCl, 5% ethylene glycol, 250 mM imidazole, and 0.5 mM dithiothreitol. The eluate was subjected to ammonium sulfate fractionation, and the 30 to 40% saturation fraction was pelleted by centrifugation at 15,000 × *g* and then dissolved in 10 mM Tris-HCl (pH 8.0)–150 mM KCl–0.1 mM EDTA–0.5 mM dithiothreitol–5% ethylene glycol. This material was usually >95% pure RepC antigen.

Gel mobility shift assay. Complementary strands of synthetic single-stranded oligonucleotides representing the pT181 replication origin were radiolabeled with kinase and [γ-^{32}P]ATP. They were annealed to each other to form a DSO fragment. Equimolar amounts of protein (RepC/C or RepC/C*) and DNA fragment (final volume, 15 μl) were incubated at room temperature for 10 min in a buffer containing 10 mM Tris-HCl, 150 mM KCl, and 5% ethylene glycol. The reaction mixtures were resolved on a 5% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer. Gels were dried and autoradiographed.

Potassium permanganate probing of RepC/C-DNA and RepC/C*-DNA complexes. Two micrograms of supercoiled pT181 plasmid DNA was incubated with different amounts 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^{++} 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 mixture for 1 min at 37°C. The reaction was stopped by the addition of 2.5 μl of β-mercaptoethanol, and plasmids were recovered by using Qiaprep spin plasmid miniprep 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 with ^{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 formamide-dye mixture and heated for 2 min at 90°C prior to denaturing gel electrophoresis.

TABLE 2. Forms of monomeric and dimeric RepC

Term	Meaning
RepC	Total RepC antigen or native monomer
RepC/C	Active homodimer
RepC/C*	Inactive heterodimer derived by addition in vivo of oligonucleotide to the active site tyrosine (Y191) of one of the subunits
RepC*	Modified monomeric subunit of RepC/C*
RepC/C** (N)	Heterodimer derived by in vitro cleavage of an oligonucleotide leaving the 3′ moiety, consisting of N nucleotides, attached to Y191
RepC** (N)	Modified monomeric subunit of RepC/C** (N)

SDS-PAGE and immunoblotting. Ten microliter samples of whole-cell minilysates were fractionated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) (16). Samples were heated at 90°C for 2 min immediately before being loaded. The gels were run at 150 V for 2 h, and were then Western immunoblotted (2) with rabbit anti-RepC antibodies (25) by using goat anti-rabbit alkaline phosphatase conjugate. For experiments run with purified RepC, gels were stained with Coomassie brilliant blue and the bands were quantitated by videodensitometry.

RepC and RepC* quantitation. RepC purified to homogeneity from *Escherichia coli* MB2 (pSK184) and quantitated by the dye binding method (3) was used as a standard. RepC and RepC* were then quantitated in *S. aureus* by comparing the intensity of the RepC and RepC* bands (from a whole-cell minilysate) on a Western blot to that of the standard RepC by using a Shimadzu densitometer or an Agfa densitometer.

Determination of RepC activities. Replication, topoisomerase, and nicking activities of RepC preparations and their inhibition by RepC/C* were determined in vitro by standard procedures, as described by Khan and coworkers (11, 13, 15). Enhancement of inverted repeats II (IRII) cruciform extrusion of pT181 double-strand origin was determined by treatment with $KMnO_4$.

Nomenclature. Because there are several different forms of monomeric and dimeric RepC, an outline of our nomenclature is provided in Table 2 to help avoid confusion.

RESULTS

Quantitation of RepC. We have previously estimated the rate of RepC production in vivo by measuring the rate of synthesis of β-lactamase translationally coupled to *repC* (1). These measurements suggested that approximately one RepC dimer is produced per replication event (1, 29), consistent with the utilization by an RC replicon of a single initiator molecule for each replication cycle. Measurements of total RepC antigen were undertaken to refine and strengthen the earlier results.

To measure intracellular RepC and RepC*, we prepared whole-cell lysostaphin lysates from RN3268 (RN27 containing pT181 *cop-608*). The samples were separated on SDS-PAGE gels, and RepC antigen was detected by immunoblotting and quantitated by densitometric comparison to a RepC standard. Total RepC antigen was estimated in lysates prepared from measured samples (corresponding to 10^8 to 10^9 total cells) of the *cop-608* (RN3268) strain. A typical example (Fig. 1, compare lane 4 to lane 6) gave a value of approximately 7 ng of RepC antigen per 10^8 starting cells by densitometry. Since the molecular mass of the RepC monomer is 38 kDa, 7 ng corresponds to approximately 600 dimers per cell. The copy number of *cop-608* has been estimated at 800 to 1,000 (4). Thus, there appears to be a little less than one RepC/C* heterodimer molecule per plasmid per cell. Similar determinations with other copy mutants gave similar results, as shown in Fig. 2. Given that determinations of copy number and cell numbers are somewhat imprecise, we consider these results to be consistent with our earlier results on RepC production and suggest that approximately one RepC dimer is produced per replication event and that there is approximately one dimer per plasmid copy, over a wide range of plasmid copy numbers.

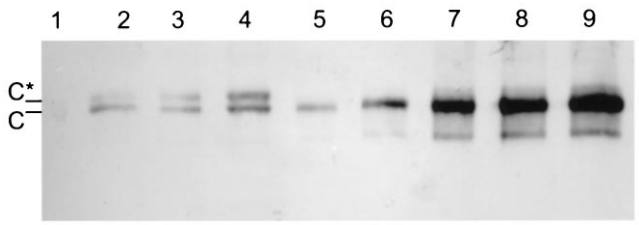

FIG. 1. Quantitation of RepC antigen by immunoblotting. Samples containing different amounts of RepC purified from *E. coli* MB2(pSK184) or of RepC and RepC* in a whole-cell minilysate of *S. aureus* RN3268 (RN27 containing pT181 *cop-608*) were separated by SDS-PAGE, blotted to nitrocellulose, treated with rabbit anti-RepC antiserum, and developed by treating with goat anti-rabbit alkaline phosphatase conjugate and staining for alkaline phosphatase. Lanes 1 to 4: RepC/C* samples in microliters of *S. aureus* lysate; lanes 5 to 9: RepC/C samples in nanograms of purified RepC. Lane 1, 0.5 μl; lane 2, 1 μl; lane 3, 2 μl; lane 4, 4 μl; lane 5, 2.5 ng; lane 6, 5 ng; lane 7, 25 ng; lane 8, 50 ng; lane 9, 100 ng.

Although we have estimated on the basis of theoretical considerations that there could be as much as 10% RepC/C in preparations of RepC antigen from pT181-containing cells, densitometric measurements of Western blots and stained SDS-polyacrylamide gels suggest that the ratio is closer to 1.0 than to the predicted 1.2 for plasmids that encode and utilize RepC for replication. These results suggest that there is very little free RepC/C in growing, pT181-containing bacteria. One possible interpretation is that the time interval between the synthesis and utilization of a RepC/C dimer is much shorter

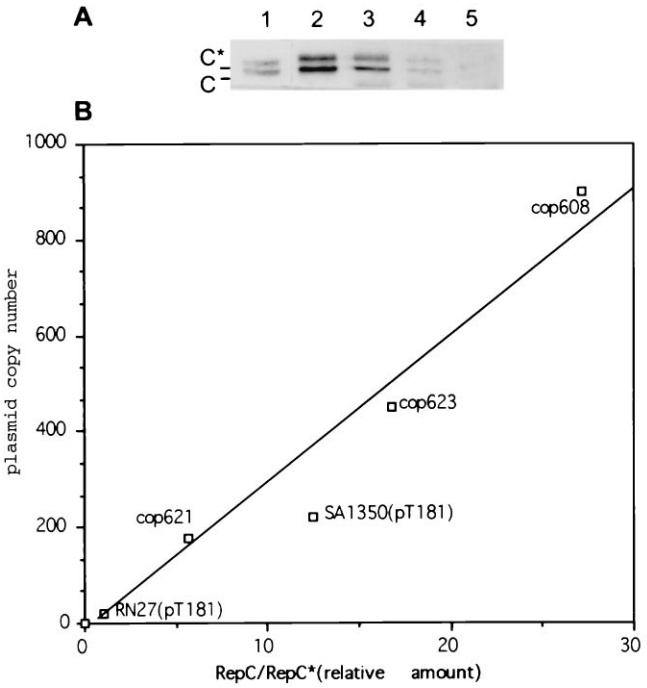

FIG. 2. Total RepC antigen as a function of copy number. (A) Whole-cell minilysates were separated by SDS-PAGE and Western blotted with anti-RepC as described in the legend for Fig. 1. Preparations from the following strains were analyzed: lane 1, pT181 in SA1350 (220 copies); lane 2, pT181-*cop608* in RN27 (800 to 1,000 copies); lane 3, pT181-*cop623* in RN27 (400 to 500 copies); lane 4, pT181-*cop621* in RN27 (30 to 160 copies); lane 5, pT181 wild type in RN27 (20 to 25 copies). (B) Plot of total RepC antigen versus plasmid copy number. RepC and RepC* were quantitated by measuring the intensity of the RepC and RepC* bands from the Western blot shown in panel A by using a Shimadzu densitometer.

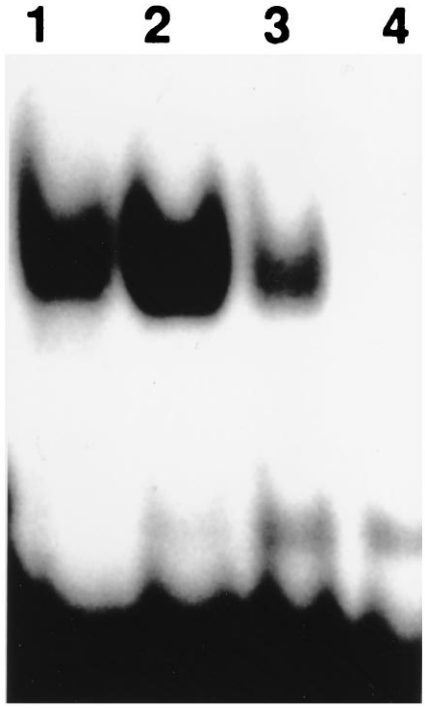

FIG. 3. Retardation of pT181 DSO gel mobility by RepC/C and RepC/C*. Different amounts of RepC/C and RepC/C* were incubated with a radiolabeled synthetic double-stranded oligonucleotide (63-mer) containing the pT181 DSO (10 pmol), and the mixture was resolved on 5% PAGE. Lane 1, 5 pmol of RepC/C; lane 2, 10 pmol of RepC/C; lane 3, 10 pmol of RepC/C*; lane 4, no Rep protein.

than the average interplasmid replication time of 4 min (6), upon which the 10% estimate was based. We have observed that when RepC/C is provided in *trans* the ratio of RepC/C to RepC/C* may be slightly higher (data not shown), possibly indicating a slight *cis* effect for utilization of the protein.

DNA binding activity of RepC/C and RepC/C*. It has been shown by gel shift and DNA footprinting (14) that RepC/C is able to bind specifically to the pT181 DSO. In order to determine the DNA binding ability of RepC/C*, we performed a gel mobility shift assay as shown in Fig. 3. A radiolabeled sample of a DSO-containing double-stranded synthetic oligonucleotide was used as the substrate. Purified RepC/C and RepC/C* were incubated with the DNA fragment and the mixture was resolved by polyacrylamide gel electrophoresis. The results indicated that RepC/C* has significant DSO binding activity, though the affinity is considerably weaker than that of RepC/C. Thus, lanes 2 and 3 in Fig. 3 represent equimolar ratios of RepC/C and RepC/C* to DNA, respectively, and lane 1 has half the amount of RepC/C. As can be seen, the retarded RepC/C* band in lane 3 is much weaker than the RepC/C-DNA band in lane 1, indicating that the affinity of RepC/C* for DNA is less than 50% of that of RepC/C. We have calculated that the binding constants of the two forms actually differ by a factor of about 4 (10).

Interference by RepC/C* with RepC/C activities in vitro. Given that the intracellular RepC/C* concentration is ordinarily in excess of 10 times that of RepC/C and that RepC/C* binds the DSO, the question is raised of whether the inactive material affects the activity of the RepC/C homodimers, and, if so, whether such an effect could have any role in the regulation of replication. Accordingly, we tested RepC/C* for its ability to inhibit the several, easily measurable activities of RepC/C,

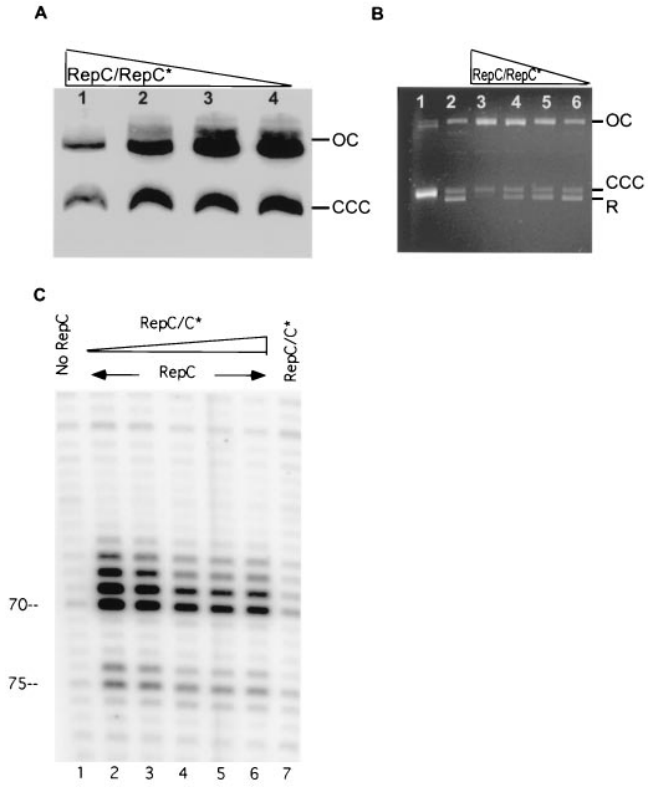

FIG. 4. Effect of RepC/C* on RepC/C activity. (A) Inhibition of in vitro replication. Reactions were done in a final total volume of 50 μl with 40 mM Tris-HCl (pH 8.0); 100 mM KCl; 12 mM $Mg(OAC)_2$; 50 mM (each) dGTP, dCTP, and dTTP; 5 mM dATP; 2 mM ATP; 0.5 mM (each) rGTP, rUTP, rCTP, and rGTP; 0.05 mM NAD; 0.05 mM cyclic AMP; 1 μg of pT181 covalently closed circular plasmid DNA; and 5 μCi of [α-^{32}P]dATP. Different amounts of premixed RepC/C and RepC/C* were added to the reaction mixture followed by the addition of 10 μl of cell extract. The reactions were allowed to continue at 30°C for 1 h and were stopped by addition of 50 μl of 2% SDS and tracking dye. Ten microliters of the mixture was subjected to electrophoresis on a 1% agarose gel with TBE buffer. The gel was dried and autoradiographed. Lanes 1 to 4, 0.5 μg of RepC/C; lanes 1 to 3, 4, 2, and 0.5 μg of RepC/C*, respectively. (B) Inhibition of topoisomerase activity. Reactions were done in a final total volume of 50 μl with 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 12 mM $Mg(OAC)_2$, and 200 ng of supercoiled pT181 *cop-623* DNA. Different amounts of RepC/C* were premixed with 100 ng of RepC/C and added to the 200 ng of *cop-623* DNA. The reaction mixtures were incubated at 32°C for 30 min. The mixtures were electrophoresed on a 1% agarose gel (containing ethidium bromide) with TBE buffer. Lane 1, supercoiled DNA alone; lanes 2 to 6, 100 ng of RepC/C; lanes 3 to 6, RepC/C*, 2 μg, 600 ng, 200 ng, and 60 ng, respectively. (C) Inhibition of cruciform extrusion. Cruciform extrusion was assayed by determining $KMnO_4$ sensitivity of nucleotides predicted to be unpaired in the extruded configuration. Molecular mass markers in kilodaltons are on the left. Lane 1, no RepC antigen; lanes 2 to 6, 50 ng of RepC/C; lanes 2 to 7, 50, 200, 400, 800, and 50 ng of RepC/C*, respectively. OC, open circular; CCC, covalently closed circular. R, relaxed.

including topoisomerase-like activity, nicking activity, cruciform extrusion, and in vitro replication. In each of these tests, RepC/C* inhibited the activity of RepC/C, as shown in Fig. 4. Figure 4A presents the results of an experiment demonstrating inhibition of RepC/C-induced replication in vitro by RepC/C*. The inhibition was approximately 50% at a 4:1 ratio of RepC/C* to RepC/C, consistent with the relative binding affinities of the two species. Note that extracts of this type do not support significant repair replication with supercoiled substrates and that the incorporation is RepC/C specific (11, 12). Figure 4B shows the inhibition of topoisomerase-like activity. In this assay, the lowest RepC/C* concentration was equimolar with that of RepC/C and the highest represented about a 20-fold excess, which essentially eliminated the topoisomerase-like activity of the native protein. It appears from this photograph, however, that RepC/C* retains some nicking activity (see below). Note that the apparent reduction in relaxation activity cannot be due simply to the removal of substrate by nicking, since there is a greater reduction in the relaxation band than in the supercoiled band. Figure 4C shows the effect of RepC/C* on cruciform extrusion by RepC/C. Again, there is significant inhibition, and as one would expect, the inhibition was not complete at a fourfold excess of the inhibitor. In this case, the RepC/C* preparation had no detectable cruciform extrusion activity as measured in the assay shown, and so the conclusion is that the activity seen with a fourfold excess of RepC/C* cannot be due to any activity of RepC/C* itself, but must represent incomplete inhibition of RepC activity by RepC/C*.

In view of the above results one might expect a significant inhibitory effect of RepC/C* on the functions of RepC/C in vivo. This expectation does not fit with the kinetics of plasmid replication observed with pT181 and with the various copy mutants discussed above. Since there appears to be approximately one molecule of RepC/C synthesized per plasmid replication event, any significant inhibition would be incompatible with the observed plasmid copy numbers.

Nicking activity of RepC/C*. Given that dimeric RepC bound to the 5′ end of the displaced leading strand is responsible for the termination of replication, it follows that the free subunit must be responsible for the first of the two required strand transfer reactions. Therefore, it is not surprising that RepC/C* has detectable nicking activity, even though it inhibits the replication and relaxation activities of RepC/C. This nicking activity suggests that RepC/C* may be biochemically analogous to the bound RepC dimer, and therefore that it should be able to cleave a single-stranded oligonucleotide containing its recognition site. Figure 5B shows the results of an experiment in which RepC/C was incubated with a 38-mer containing the nick site at position 18 (from the 3′ end) (CCGG TAAAACCGGCTACTCTAATAGCCGGTTGGATGCA). As seen in lane 2, RepC/C was converted to a mixture of RepC and RepC** (18). Figure 5C shows the results of a similar experiment in which RepC/C* was incubated with the above 38-mer. As shown in lane 2, RepC/C* was converted to a mixture of RepC, RepC*, and RepC/C** (18), and the amount of RepC decreased whereas the amount of RepC* was not affected. It is concluded, therefore, that RepC/C* can cleave the oligomer and that the free subunit is responsible. Since the RepC* concentration was unaffected, this result suggests that molecules of the form RepC*/C** (18) were produced. Molecules of this configuration would be analogous to the product of the initial cleavage during termination. This product is expected to be attacked immediately by the newly generated free 3′ OH group of the nascent leading strand and therefore to be very short lived during the termination of replication. We have shown elsewhere (10a) that the second stage of this strand transfer occurs in vitro but that it is very slow and does not result in significant net reduction of RepC*. The reason for this difference in the presumed in vivo and in vitro reaction rates is not yet clear; it may, in any case, enable us to analyze the termination reaction in detail. The relation of the reaction shown in Fig. 5 to the nicking activity of RepC/C* on the intact supercoiled plasmid molecule (see Fig. 4) could be that in the latter case, the substrate is the rare spontaneous cruciforms that form in supercoiled DNA (see below).

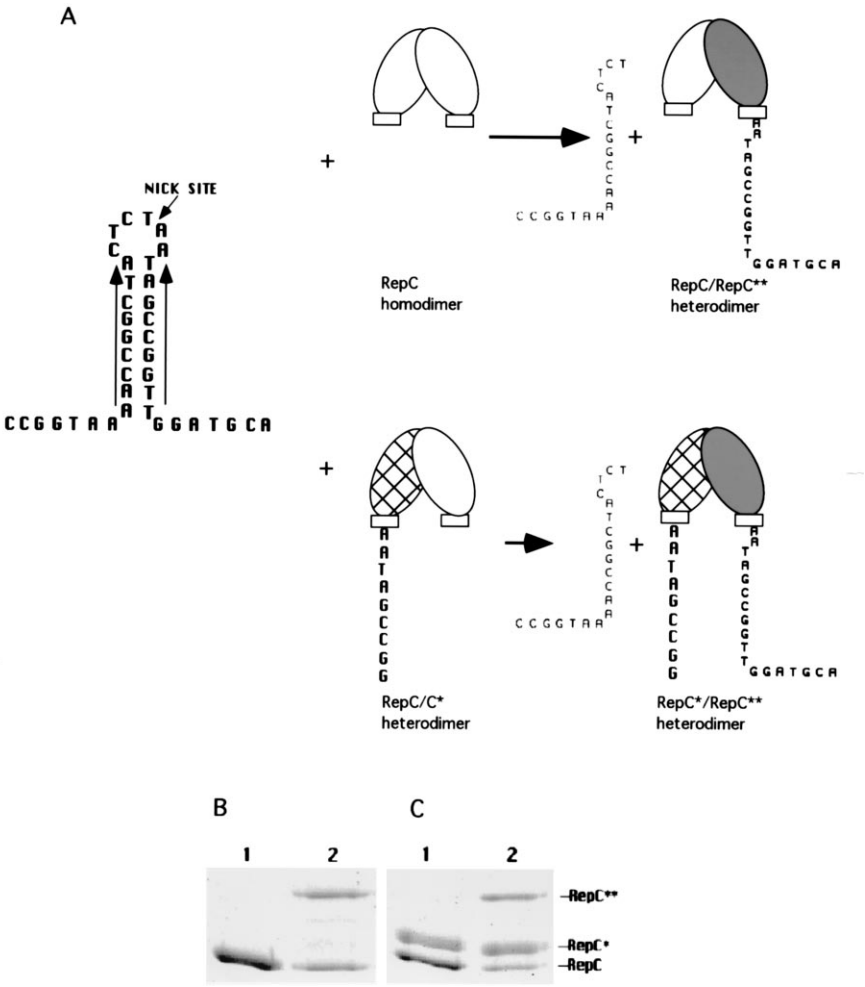

FIG. 5. Nicking reaction with a synthetic oligonucleotide in vitro. (A) Diagrammatic representation of the reaction. At left is shown the 38-mer substrate in the predicted hairpin configuration. At top is shown the predicted cleavage by RepC/C with this substrate, generating a free 20-mer representing nucleotides 5′ to the nick site and a RepC/C** heterodimer containing an 18-mer corresponding to nucleotides 3′ to the nick site attached to the active site tyrosine of one of its two subunits. Below is shown the parallel reaction in which the same synthetic oligonucleotide is cleaved by RepC/C* isolated from pT181-containing cells. Note that the oligonucleotide attached to the starting material, also representing nucleotides 3′ to the nicking site, is shown as a nonomer. This is the most frequent length of the oligonucleotide in the in vivo heterodimer (10a). In this reaction, generation of a doubly-derivatized heterodimer is predicted. (B) Nicking reaction with RepC antigen purified from a staphylococcal strain containing an Ori$^-$ mutant derivative of pT181 *cop-623*. Lane 1, 1 μg of RepC/C; lane 2, 1 μg of RepC/C after incubation with 200 pmol of the 38-mer. (C) Nicking of synthetic oligonucleotide by RepC/C* in vitro. Lane 1, 1 μg of RepC/C*; lane 2, 1 μg of RepC/C* after incubation with 200 pmol of the 38-mer described in the text.

DISCUSSION

In this report, measurements of the intracellular level of total RepC antigen suggest that there is about one dimer per plasmid copy over a wide range of copy numbers. We consider these measurements to be consistent with previous results suggesting that one dimer is made and utilized per replication event, which is very much in keeping with the biology of pT181 replication and its regulation.

Evidence presented here and elsewhere supports an overall scheme in which RepC is synthesized at the rate of approximately one dimer (RepC/C) per plasmid replication event, is used for replication, and is converted to an inactive heterodimer by the replication-specific addition of a short oligonucleotide to the active site tyrosine of one of the two subunits. The inactive form (RepC/C*) is not detectably reactivated. It is metabolically stable and is present at a level that we had assumed on theoretical grounds to represent about 90% of the total RepC antigen in exponentially growing cells. Most or all of the remaining 10% was presumed to represent active RepC homodimers that had not been used for replication. Accordingly, the cellular content of active dimers was presumed to determine the initiation frequency and to be linearly proportional to the plasmid copy number. This was tested by direct measurements of the active fraction of RepC by quantitative Western blotting or SDS-PAGE analysis. The results of many such experiments, of which an example is described here, suggest that during normal exponential growth, RepC/C is at the borderline of detectability (probably <5% of the total RepC protein). Although these measurements suffer from the inaccuracy of quantitative Western blots and from the uncertainty arising from the subtraction of two similar numbers (RepC and RepC* subunits), they are probably good enough to mean that our theoretical prediction of the RepC/C fraction, based on the assumption that the lifetime of the molecule corresponds to the plasmid's interreplication time, is incorrect. Rather, it appears that RepC/C molecules are used very rapidly following

synthesis and do not represent a significant fraction of the total. It is likely, therefore, that the plasmid replication rate (copy number) is determined by the rate of RepC/C synthesis rather than by the concentration of the protein. A general implication is that when a copy mutation occurs, there is an immediate increase in RepC synthesis followed by an increase in plasmid replication frequency. Eventually, a new equilibrium is established at an elevated copy number in which the antisense RNA concentration has been adjusted so as to reestablish the situation where one RepC dimer is produced and immediately utilized for each plasmid replication event. It is also very likely that RepC/C-induced initiation of replication is indifferent in vivo to the very great excess of RepC/C* that is present, despite the ability of the latter to inhibit initiation in vitro at concentration ratios that are probably much lower than those in vivo. The nature of the difference between the in vivo and in vitro situations in this case remains to be determined; it is suggested, in any case, that RepC/C* does not have a significant regulatory role in vivo during normal exponential growth of pT181-containing cultures.

A remaining question is the level of intrinsic activity of RepC/C*. It has been known for many years that active RepC/C can be purified from pT181-containing cells (11), and we have observed that such preparations have about 5 to 10% of the specific replication and relaxation activities of pure RepC/C (25). These activities have been attributed to the RepC/C that is necessarily present in pT181-containing cells, and for reasons noted above, 5 to 10% was a reasonable expectation. Though RepC/C* is almost certainly unable to initiate replication, there are strong grounds for expecting it to have nicking activity on single-stranded DNA. Additionally Thomas and Jennings (27) have recently reported that a purified preparation of the closely related RepD/D* has about 3% of the relaxation activity seen with RepD/D. Similarly, we have not succeeded, using preparations such as those obtained in the experiments shown in Fig. 4, in eliminating all relaxation activity of the protein. In fact, nicking activity of RepC/C* could represent an essential feature of the plasmid replication cycle, if RepC/C* is biochemically similar to the RepC dimer that is attached to the displaced leading strand during replication: the attached dimer must nick at the displaced new-old leading strand junction to initiate termination of replication. And it almost certainly uses the free tyrosine OH of its second subunit to do this.

If RepC/C* can nick, why can it not initiate replication? Since RepC/C* neither nicks linear double-stranded DNA (unpublished data) nor promotes cruciform extrusion, any nicking activity on supercoiled DNA is likely to involve spontaneous IRII cruciforms. The product of this reaction would be a nicked molecule with a RepC dimer attached to the 5′ side of the nick and the oligonucleotide attached to the 3′ terminus. Such a structure would probably not be replicable but reversal of the reaction would generate a relaxed CCC molecule, which could account for the relaxation activity seen by Thomas and Jennings (27).

The existence of two forms of the pT181 initiator protein adumbrates the situation with the Rep proteins of several of the iteron-bearing plasmids, F, P1, and R6K, each of which exists in an active and an inactive form. With the iteron-bearing plasmids, the two forms represent monomers and dimers which are interconvertible, and the interconversion is involved in the regulation of replication. For example, with P1 RepA, the dimer is inactive, the monomer is active, and the two forms are interconvertible (30). With pT181, although there is no evidence for any monomer-dimer interconversion, the inactive form binds the origin and inhibits the replication functions of the active form in vitro. As noted above, however, it is difficult to envision any normal regulatory function involving significant in vivo inhibition by RepC/C*, given the apparent stoichiometry of initiator synthesis and concentration and given a regulatory system that is fully able to account for the observable behavior of the plasmid copy number under normal circumstances. Thus, the finding of replication inhibition in vitro by RepC/C* is problematical: if inhibition in vivo were at the same level as inhibition in vitro, the frequency of initiation would be about fourfold too low to account for the observed plasmid copy numbers in relation to the observed rate of RepC synthesis (1) and the intracellular RepC levels (see above). Although the binding of RepC/C* to the pT181 DSO is considerably weaker than that of RepC/C, one must nevertheless postulate one or more in vivo factors that block inhibition by RepC/C* to account for the observed plasmid replication frequencies in the presence of a greater than 10-fold excess of the inactive species. One can, in any case, envision circumstances where there may be inhibition by RepC/C*. One such situation is represented by copy mutants such as *cop-608*, in which the regulatory countertranscripts are absent yet the plasmid establishes and maintains a stable (though very high) copy number (4). Another circumstance is that subsequent to the entry of the plasmid into a new cell. Here, the plasmid initially overproduces RepC and replicates exponentially with a doubling time of 4 min, overshooting its normal copy number by two- to threefold. Replication then stops abruptly and the plasmid copy number undergoes oscillations before finally stabilizing at its normal level (6). Although overproduction of the inhibitory countertranscripts occurs in this situation, this cannot fully account for the abrupt shutoff of replication that is observed (a threefold excess of the countertranscript RNAs would not stop replication but would reduce the replication frequency by two-thirds). Thus another inhibitory factor would seem to be needed, perhaps RepC/C*.

A third situation possibly involving inhibition by RepC/C* is with constructs in which *repC* transcription is under the control of the strong β-lactamase promoter. Here, induction of the promoter, resulting in the gross overproduction of RepC, causes an initial increase followed by a dramatic reduction in copy number (7) corresponding to degradation of the plasmid. Under these conditions, there is a vast overaccumulation of RepC/C* (10b), possibly as a result of abortive initiation. The nicking-relaxing activity of RepC/C* may be at least partly responsible for the observed degradation. Although overproduction of the P1 Rep protein effectively blocks replication of the plasmid (30), degradation does not occur. Whether RepC/C* is responsible for the effects of RepC overproduction is not yet clear and experiments are currently in progress to determine whether RepC/C* is or is not inhibitory in vivo.

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