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Uncoupling of the DNA topoisomerase and replication activities of an initiator protein

(DNA binding protein/site-specific mutagenesis/initiation of rolling-circle replication)

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ABSTRACT **The replication initiator proteins encoded by the pT181 and related plasmids have sequence-specific DNA binding and topoisomerase activities. These proteins create a site-specific nick in one strand of the DNA at the origin of replication that serves as a primer for the initiation of replication. To define the regions of the pT181-encoded initiator protein, RepC, that are involved in its DNA binding, topoisomerase, and replication activities, we have carried out site-directed mutagenesis of the *repC* gene. Analysis of mutant RepC proteins *in vitro* and *in vivo* has identified the amino acids that are critical for its various biochemical activities. The DNA binding domain of RepC was found to be located near its C-terminal region and was different from the domain involved in its sequence-specific topoisomerase activity. These studies also showed that the DNA topoisomerase activity of the initiator protein can be uncoupled from its tight noncovalent DNA binding and replication activities.**

Plasmid pT181 is a prototype of a family of small, multicopy staphylococcal plasmids that replicate by a rolling-circle mechanism (1, 2). The plasmids of this family include pT181, pC221, pC223, pS194, pCW7, and pUB112, range in size from 4.2 to 4.6 kilobase pairs (kb), and have extensive sequence homology in their replication regions. These plasmids encode replication initiator proteins with 76% overall amino acid sequence identity (1). The initiators encoded by pT181 and pC221 (RepC and RepD, respectively) have been purified and shown to have origin-specific DNA nicking–closing activities (3, 4). These activities resemble those of CisA protein of ϕX174, gene 2 proteins of the filamentous phages of *Escherichia coli*, and type I DNA topoisomerases (5–7). These initiator proteins nick one strand of the DNA at a specific site, and the free 3′ OH end serves as a primer replication by an asymmetric rolling-circle mechanism (2, 8).

The RepC protein consists of 314 amino acids. It binds to a 32-base-pair (bp) sequence located between positions 37 and 68 and nicks the bottom strand of the DNA between nucleotides 70 and 71 within the pT181 origin of replication (3, 9–11). Genetic studies have suggested that a divergent amino acid sequence located near the C-terminal region of the initiator proteins of the pT181 family is involved in their replication activities (1, 12–14). We have carried out site-directed mutagenesis of the repC gene in the regions encoding its predicted topoisomerase and DNA binding domains. Mutant RepC proteins were overexpressed in *E. coli* and purified, and their DNA binding, topoisomerase, and replication activities were analyzed. These studies showed that amino acids 267–270 are critical for the sequence-specific DNA binding and replication activities of RepC and the tyrosine residue at position 191 is involved in its topoisomerase activity. Our results demonstrate that the topoisomerase activity of RepC can be uncoupled from its DNA binding and replication activities. Furthermore, although nicking of the DNA by the initiator protein is required for the initiation of replication, other interactions play a critical role in this step.

MATERIALS AND METHODS

Plasmids. The *Staphylococcus aureus* plasmid pT181cop-608 is a high-copy-number mutant of pT181 and was used as the source of this DNA (15). Plasmid pT181cop-620 is a mutant of pT181 that expresses RepC at higher levels and has a 3-fold increase in copy number as compared to the wild-type plasmid (14). Plasmid pSA7540 is a cointegrate of pT181cop-620 and pSA1212, which contains the active replicon of pE5 (14). The pT181cop-620 sequence in the pSA7540 plasmid contains the *ori-11* mutation that eliminates the RepC nick site and inactivates the pT181 origin but maintains the RepC binding sequence as well as the encoded amino acid sequence of RepC (14).

***In Vitro* Mutagenesis.** The *repC* gene from pSA7540 was cloned into the *Eco*RI site of the replicative form DNA of phage M13mp19. *In vitro* mutagenesis of the *repC* gene was carried out by the procedure of Kunkel *et al.* (16). Single-stranded, uridine-containing M13mp19 DNA carrying the *repC* gene was prepared from the *ung*$^-$ *dut*$^-$ *E. coli* strain CJ236. Oligonucleotide primers were annealed to this DNA and extended with the Klenow fragment of DNA polymerase I in the presence of T4 DNA ligase. The reaction mixtures were used to transform *E. coli* JM109. DNA was prepared from several plaques and the desired mutations in the *repC* gene were identified by sequencing the DNA by the dideoxy method (17). The sequences of the oligonucleotides used for mutagenesis of the topoisomerase and DNA binding domains of RepC are as follows: Tyr-191 → Phe: TTTATTAGAATTTTTAATAAAAAGCAA; Tyr-191 → Ser: ATTAGAATTTCTAATAAAAAG; Asn-267 → Asp: CTTCACAGAGATTCTAGAACA; Ser-268 → Ala: TCACAGAAATGCTAGAACAAA; Arg-269 → Gly: CAGAAATTCTGGAACAAAATATAAG; Thr-270 → Ala: AGAAATTCTAGAGCAAAATATAA; and ΔNSRT: AAGCTTCACAGAAAATATAAGAAT. Nucleotides altering the sequences are underlined.

Overexpression and Purification of the Initiator Proteins. Replicative form DNA was isolated from bacteria harboring M13 derivatives, and a 1.3-kb *Eco*RI fragment carrying the *repC* gene was ligated into the *Eco*RI site of a derivative of the *E. coli* expression vector pKJB825 (9). Plasmids were introduced into *E. coli* MB2, and the initiator proteins were overexpressed by shifting exponential cultures to 43°C as described (9). Most of the mutant RepC proteins were

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Abbreviations: Em, erythromycin; Cm, chloramphenicol; *tsr*, temperature-sensitive for replication; Gdn·HCl, guanidinium hydrochloride.

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purified by ammonium sulfate fractionation and heparin-agarose column chromatography as described (9). The proteins were about 95% pure as judged by SDS/PAGE and silver staining (data not shown). The Tyr-191 → Phe and Ser-268 → Ala mutant proteins were partially insoluble in aqueous solution and were kept in solution by the presence of 0.05% Triton X-100 during the purification steps. The Arg-269 → Gly, Thr-270 → Ala, and ΔNSRT mutants were insoluble upon cell lysis and precipitated from aqueous solutions. These proteins were solubilized in a buffer containing either 3 or 4.5 M guanidinium hydrochloride (Gdn·HCl) and then dialyzed against a buffer containing 2 M Gdn·HCl. No further purification of these proteins was necessary as they were >80% pure. To prevent the precipitation of the appropriate mutant proteins during various reactions, a final concentration of 0.02% Triton X-100 or 50 mM Gdn·HCl was maintained. These concentrations of Triton X-100 and Gdn·HCl did not affect the activity of wild-type RepC in DNA binding, topoisomerase, and replication assays (data not shown).

DNA Binding Assays. The binding of the initiator proteins to the pT181 origin was studied by gel mobility shift assays as described (18), except that 0.5 ng of ^{32}P-labeled DNA fragments (specific activity, 5–9 × 10^7 cpm/μg) and various amounts of poly[d(I-C)] and the initiator proteins were used. The reaction mixtures were incubated at room temperature for 10 min and electrophoresed on 5% native polyacrylamide gels at 10 V/cm for 5 hr at 4°C. The gels were dried and subjected to autoradiography.

DNA Topoisomerase Assays. DNA topoisomerase assays were carried out as described (3). One microgram of supercoiled plasmid DNA was incubated with various amounts of the initiator proteins at 32°C for 30 min and the reaction products were analyzed by electrophoresis on 0.7% agarose gels. The gels were stained with ethidium bromide and photographed under UV illumination.

***In Vitro* DNA Replication.** Plasmid DNA was replicated using *S. aureus* extracts and various amounts of purified initiator proteins as described (2). The replication products were treated with *Hpa* II restriction endonuclease to convert the various forms of pT181 DNA (supercoiled, open circular, and covalently closed relaxed circular) into one band to facilitate the estimation of the extent of replication. The DNA samples were analyzed by electrophoresis through 1.2% agarose gels (3 V/cm for 17 hr) in Tris/acetate/EDTA buffer containing 0.5 μg of ethidium bromide per ml. The gels were dried and subjected to autoradiography. To measure the extent of replication, the labeled DNA bands were excised and the radioactivity was determined by liquid scintillation spectrometry.

Complementation of the pT181 Origin *in Vivo*. The various mutant RepC proteins were tested for their ability to amplify, in trans, the pRN6409Δ86 plasmid containing a cloned pT181 origin at a temperature restrictive for the vector. This plasmid is a derivative of the erythromycin (Em) resistance plasmid pE194 rep-1, which is temperature-sensitive for replication (*tsr*) (19). The mutant *repC* genes were cloned into the pSK265 plasmid (a pC194 derivative) that encodes resistance to chloramphenicol (Cm) and is compatible with the pT181 and pE194 plasmids (1). Plasmid pRN6409Δ86 was introduced into the above strains by electroporation (20) and transformants were obtained by plating the cells at 32°C on GL agar supplemented with Em and Cm. The heteroplasmid strains were then grown on nonselective GL agar at permissive (32°C) or nonpermissive (43°) temperature, and the plasmid content of the various strains was determined by agarose gel electrophoresis of sheared whole-cell lysates.

RESULTS

Substitution of Tyr-191 by Phe and Ser Inactivates the Topoisomerase Activity of RepC. A tyrosine residue located at position 188 has been shown to be involved in the formation of a covalent linkage between RepD (initiator encoded by the pC221 plasmid) and the 5′ phosphate of the DNA at the nick site (4). This active tyrosine residue corresponds to position 191 in RepC, and the amino acid sequence of this region is identical in the initiators encoded by the plasmids of the pT181 family (1). The nucleotide sequence surrounding the nick site is also identical in these plasmids, and purified RepC and RepD proteins have been shown to relax supercoiled pT181, pC221, and pS194 DNA with equal efficiencies (4, 21). To confirm that Tyr-191 of RepC is involved in its DNA topoisomerase activity, we replaced this residue with phenylalanine and serine by site-directed mutagenesis. These mutant proteins were tested for their DNA binding, topoisomerase, and replication activities. As expected, the Phe-191 and Ser-191 derivatives lost the pT181-specific topoisomerase activity (shown later in Fig. 4). These mutant proteins were also inactive in pT181 replication (data not shown). However, these derivatives retained their sequence-specific DNA binding activity (Fig. 1).

Comparison of the Amino Acid Sequence of the Putative DNA Binding Domain of the Initiators and the Nucleotide Sequences of the Origins of Replication of the Plasmids of the pT181 Family. The initiator proteins of the pT181 family are homologous, except for two regions of significant divergence (1). One of these regions corresponds to the initiator binding site within the origin that encodes the N-terminal portion of these proteins and the other portion near their C-terminal ends. Previous studies with *in vivo* and *in vitro* recombinants of pT181 and pC221 plasmids encoding hybrid initiator proteins suggested that amino acids 265–276 are likely to determine the specificities of the initiators of the pT181 family in replication (12, 13). Within this region, amino acids 267–270 are completely different (with a single exception) in the initiators encoded by the pT181, pC221, and pS194 plasmids (Fig. 2*A*). Furthermore, the pS194 and pCW7 initiators, which have an identical sequence at positions 267–270 but are otherwise quite divergent in the C-terminal region, are able to drive the replication from the pCW7 and pS194 origins (1). A major part of the initiator binding sequence within the origin

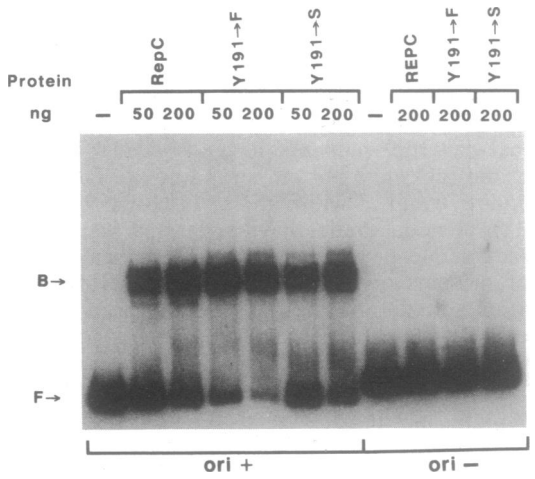

FIG. 1. Binding of the RepC topoisomerase domain mutants to the pT181 origin. Gel electrophoresis DNA binding assays were carried out using the 166-bp *Mbo* I-D fragment (*ori*$^+$) from pT181cop-608 that contains the pT181 origin and the 201-bp *Taq* I-E fragment (*ori*$^-$) from the same plasmid as a negative control. Two hundred nanograms of poly[d(I-C)] and the indicated amounts of the initiator proteins were used. F, free DNA; B, DNA bound by the protein.

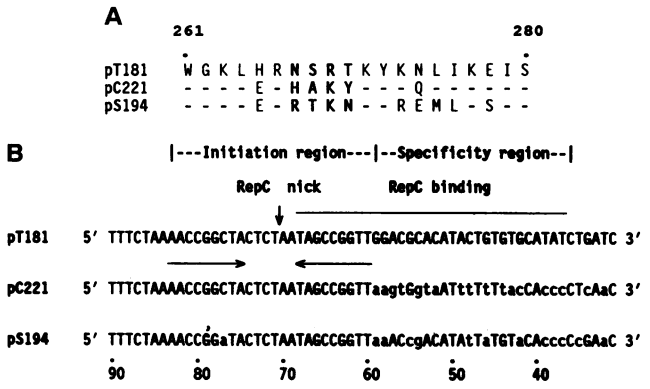

FIG. 2. Comparison of the amino acid sequences of the DNA binding domain of the various initiator proteins and the nucleotide sequences of the origins of replication. (*A*) The amino acid sequence of the DNA binding domain of the initiators encoded by the pT181, pC221, and pS194 plasmids (1). Identity of the amino acids is indicated by dashes. Numbering corresponds to the amino acids of RepC. Amino acids that are likely to be critical for origin binding are shown in boldface. (*B*) Comparison of the nucleotide sequence of the pT181, pC221, and pS194 origins of replication (1). Nucleotides in pC221 and pS194 that are identical to pT181 are shown in uppercase letters. The constant origin region of these plasmids that can form a cruciform structure (indicated by inverted arrows) and includes the initiator nick site is termed the initiation region. The variable region that includes the binding site of RepC as determined by footprint analysis is underlined and is referred to as the specificity region. The RepC nick site is indicated by a vertical arrow.

(nucleotides 37–59 in pT181) is also not conserved in plasmids of the pT181 family (except for the pS194 and pCW7 pair) (Fig. 2*B*). Based on the above results, it was previously postulated that amino acids 267–270 are likely to determine the DNA replication specificities of the initiator proteins (1). We, therefore, altered amino acids 267–270 (NSRT) of RepC individually by site-directed mutagenesis. Asn-267 was changed to aspartic acid, Ser-268 to alanine, Arg-269 to glycine, and Thr-270 to alanine. We also deleted these four amino acids from RepC. These mutant proteins were overexpressed, purified, and tested for their biochemical activities *in vitro* as described below.

RepC Mutants Are Defective in Their Sequence-Specific DNA Binding Activity. We performed gel mobility shift assays to test the ability of the various mutant initiators to bind to the pT181 origin. Although the wild-type initiator protein bound efficiently to the origin DNA, its derivatives exhibited either weak or no DNA binding activity (Fig. 3). Only the Ser-268 → Ala and Thr-270 → Ala derivatives showed detectable DNA binding activity, although it was much weaker than that of wild-type RepC (Fig. 3). These results suggested that each of the amino acids located between positions 267 and 270 is critical for the stable, noncovalent binding of RepC protein to the pT181 origin.

DNA Topoisomerase Activity of the Various RepC Mutants. Supercoiled pT181cop-608 DNA was incubated with the various RepC mutants to determine whether they were active in DNA relaxation. With the exception of the deletion mutant ΔNSRT and mutants lacking the active Tyr-191 residue, all RepC derivatives retained their pT181-specific topoisomerase activity to a significant extent (Fig. 4). These derivatives did not relax the pSA7540 DNA carrying the *ori-11* mutation in the pT181 origin (data not shown). A residual amount of DNA was resistant to relaxation even when the initiators were present in excess (Fig. 4, lanes 4, 7, 10, 13, and 16). Approximately two molecules of RepC were calculated to be required for the complete relaxation of one molecule of the DNA. The topoisomerase activity of Ser-268 → Ala mutant was about 2- to 3-fold better than that of wild type RepC (Fig.

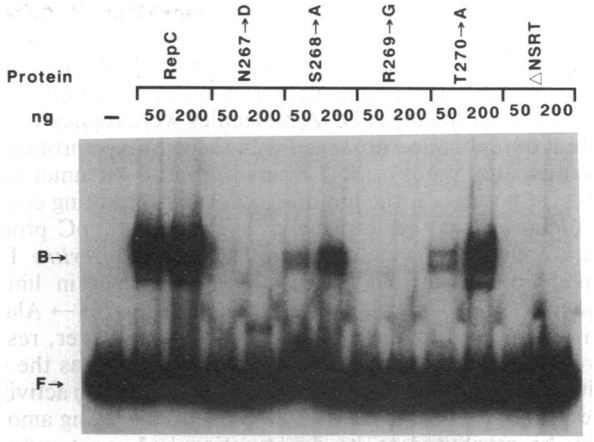

FIG. 3. Comparison of the DNA binding activity of RepC and its mutants. A ^{32}P-labeled pT181 origin DNA fragment was incubated with the indicated amounts of RepC or its derivatives in the presence of 100 ng of poly[d(I-C)]. The DNA–protein complexes were electrophoresed through a native polyacrylamide gel. F, free DNA fragment; B, DNA bound by the protein.

4, lanes 2 and 8), whereas the activities of the Asn-267 → Asp, Arg-269 → Gly, and Thr-270 → Ala mutant proteins were approximately 3-, 10-, and 5-fold lower, respectively, as compared to the wild-type protein (Fig. 4, lanes 5, 11, and 14). However, the topoisomerase activity of the Arg-269 → Gly mutant was only 2-fold lower and that of the Thr-270 → Ala derivative was comparable to that of wild-type RepC that was denatured with Gdn·HCl and subsequently renatured. This treatment resulted in a 3- to 5-fold reduction in the topoisomerase activity of RepC when it was present in limiting amounts (10 ng/μg of template DNA) but it relaxed the DNA completely when present in higher amounts (50 ng or more of initiator per μg of template DNA) (data not shown). The various RepC mutants relaxed the pT181 DNA completely when present in higher amounts (Fig. 4, lanes 7, 10, 13, and 16). However, the ΔNSRT, Tyr-191 → Phe, and Tyr-191 → Ser mutants failed to relax pT181 DNA even when present in excess (Fig. 4). It is possible that the deletion in the ΔNSRT mutant results in a change in the conformation of the initiator such that it is unable to carry out proper protein–DNA interactions required for this activity.

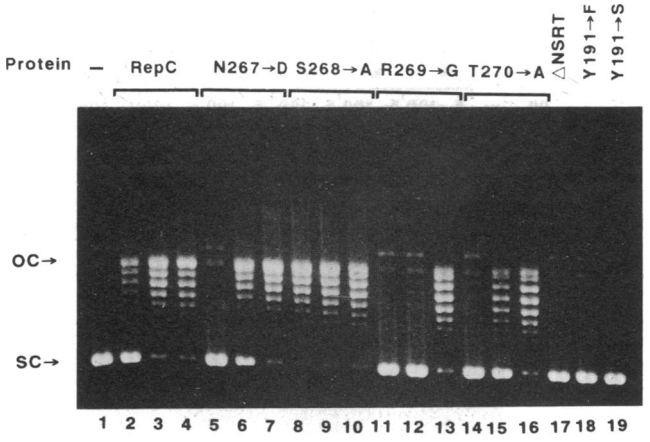

FIG. 4. Topoisomerase activities of the mutant RepC proteins. One microgram of supercoiled pT181cop-608 DNA was treated with RepC or its derivatives, as indicated, and the reaction products were analyzed by agarose gel electrophoresis. The amounts of proteins used in the reactions were as follows: lane 1, no protein; lanes 2, 5, 8, 11, and 14, 10 ng; lanes 3, 6, 9, 12, and 15, 50 ng; lanes 4, 7, 10, 13, and 16–19, 200 ng. Positions of supercoiled plasmid DNA (SC) and open circular DNA (OC) are shown.

***In Vitro* Replication Activity of Mutant RepC Proteins.** *In vitro* replication experiments were carried out using limiting and saturating amounts of the initiator proteins. In the presence of saturating concentrations of wild-type RepC, about 10% of the input DNA molecules were replicated. The extent of replication observed with the wild-type protein was 1060 and 139 pmol of dNTPs incorporated per nmol of the initiator per min at the limiting (5 ng) and saturating concentrations (100 ng), respectively. The mutant RepC proteins were found to be defective in supporting *in vitro* DNA replication to various extents. When present in limiting quantities, the replication activities of the Ser-268 → Ala and Thr-270 → Ala mutants were 3- and 40-fold lower, respectively, than the wild-type protein (Fig. 5). As was the case with its topoisomerase activity, the low replication activity of the Thr-270 → Ala mutant when present in limiting amounts may be attributed to its denaturation and renaturation in Gdn·HCl. However, the replication activities of the Ser-268 → Ala and Thr-270 → Ala derivatives were comparable to that of wild-type RepC when present in saturating amounts (Fig. 5). The replication activity of the Asn-267 → Asp and Arg-269 → Gly derivatives was >100-fold lower than that of the wild-type protein even at saturating concentrations (Fig. 5). The deletion derivative ΔNSRT failed to replicate pT181 DNA, consistent with its lack of topoisomerase activity. The pSA7540 DNA lacking the RepC nick site was inactive as a template in the *in vitro* system with all of the proteins (data not shown).

Complementation of the pT181 Origin *in Vivo* by Mutant Initiator Proteins. The ability of plasmids encoding the mutant initiator proteins to drive *in vivo* replication of the coresident *tsr* plasmid pRN6409Δ86 containing a cloned pT181 origin was tested at the restrictive temperature. It should be noted that the pT181 origin is contained within the *repC* gene (15). However, the *repC* gene used in these experiments contained the *ori-11* and *cop-620* mutations that allowed expression of the initiators at moderately high levels and determination of their activity in trans in the absence of a competing pT181 origin. Plasmid pSK265 derivatives encoding the wild-type, Ser-268 → Ala, and Thr-270 → Ala proteins supported the maintenance of plasmid pRN6409Δ86 at 43°C (Fig. 6). However, the copy number of the pRN6409Δ86 plasmid was severalfold lower at 43°C in host strains expressing the Ser-268 → Ala mutant as compared to those containing wild-type RepC or its Thr-270 → Ala derivative. The Asn-267 → Asp and Arg-269 → Gly derivatives were unable to drive replication from the pT181 origin at the restrictive temperature (Fig. 6). To rule out the possibility that the RepC derivatives that showed either reduced or no replication activity were thermolabile, another approach was taken. This utilized testing the ability of *S. aureus* strains expressing the various RepC derivatives to allow the establishment of a plasmid carrying the cloned pT181 origin but no active replication system. The Em resistance gene of pE194 (1) was cloned into the *E. coli* plasmid pSK463 that contains the leading and lagging strand origins of pT181 (22). The resulting plasmid, pSK769, was introduced into *S. aureus* strains carrying pSK265 derivatives encoding the various RepC mutants with selection for Em and Cm at 32°C. The presence of the RepC donor and pT181 origin-containing target plasmids in the transformants was confirmed by agarose gel electrophoresis and was indicative of a functional initiator protein. Although pSK769 could be established in *S. aureus* strains expressing the wild-type, Ser-268 → Ala, and Thr-270 → Ala initiators, no transformants were obtained with hosts expressing the Asn-267 → Asp, Arg-269 → Gly, ΔNSRT, and Tyr-191 → Phe derivatives (data not shown). Additionally, the Ser-268 → Ala mutant replicated the pSK769 plasmid to a much lower copy number than the wild-type or Thr-270 → Ala proteins (not shown).

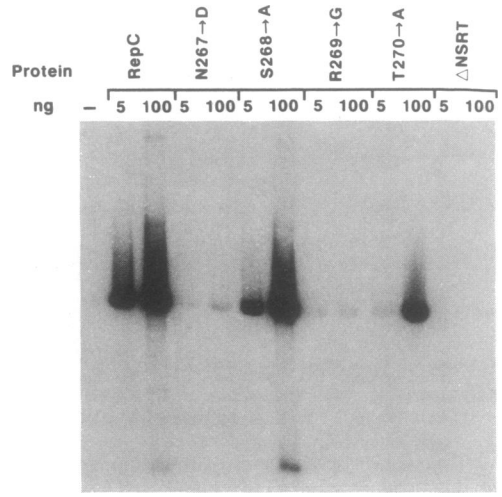

FIG. 5. *In vitro* replication activities of the mutant initiators. Indicated amounts of RepC or mutant initiators were incubated with pT181cop-608 DNA (500 ng) in standard *in vitro* replication assays. The replication products were linearized with *Hpa* II and resolved by agarose gel electrophoresis.

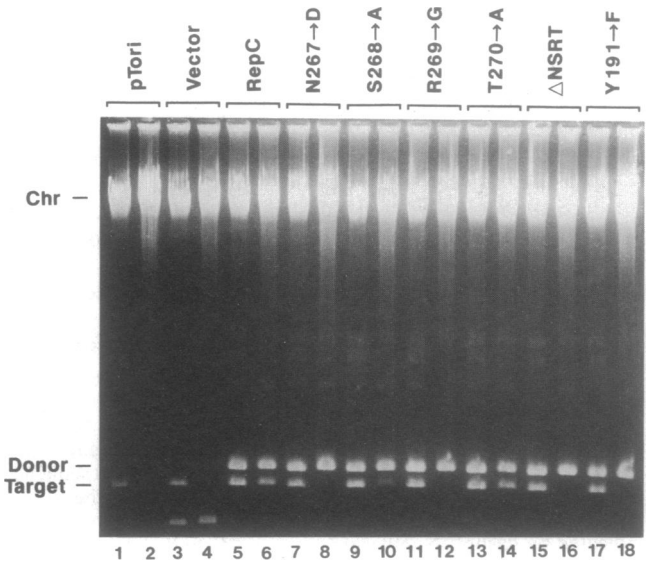

FIG. 6. Complementation of the pT181 origin *in vivo* by the various RepC mutants. *S. aureus* strains were grown at 32°C (lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17) or 43°C (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18) and their plasmid content was analyzed by agarose gel electrophoresis. Lanes 1 and 2 represent a host strain carrying only the pRN6409Δ86 plasmid (target), whereas the other lanes represent strains carrying the target and either the pSK265 plasmid (vector) or its derivatives (donor) encoding the indicated initiator proteins. Chr, chromosomal DNA.

DISCUSSION

We have mutagenized the *repC* gene and identified the amino acids that are critical for its sequence-specific topoisomerase, DNA binding, and replication activities. The Tyr-191 → Phe and Tyr-191 → Ser mutants of RepC lost their pT181-specific topoisomerase and replication activities (Fig. 4 and data not shown). However, they retained their DNA binding activity (Fig. 1), suggesting that these changes did not cause a gross conformational change in the structure of the protein. These results demonstrate that Tyr-191 is involved in the topoisomerase activity of RepC but is not critical for its noncovalent DNA binding activity. It is also clear that origin-specific

nicking of the DNA by the initiator protein is absolutely essential for the initiation of replication.

We changed each of the amino acids located at positions 267–270 of RepC by site-directed mutagenesis. The mutant proteins carrying single amino acid substitutions were found to be defective in their noncovalent DNA binding activity (Fig. 3). Replacement of Asn-267 with a negatively charged residue (aspartic acid) and Arg-269 with a small, noncharged amino acid (glycine) abolished the DNA binding activity of the initiator protein. The deletion mutant, ΔNSRT, also lost its DNA binding activity. However, the Ser-268 → Ala and Thr-270 → Ala derivatives showed significant DNA binding activity when present in high concentrations. In the gel retardation assays usually two closely migrating DNA–protein complexes were observed (Fig. 3). Since the pT181 origin contains a bend (11), two complexes may be observed if only a portion of the DNA molecules is bent under the experimental conditions. A correlation was observed between the origin binding and *in vitro* replication activities of the various mutant proteins. The substitution of Asn-267 with Asp and Arg-269 with Gly resulted in proteins that had very low replication activities even when present in excess (Fig. 5). However, the Ser-268 → Ala and Thr-270 → Ala derivatives showed substantial replication activities. The *in vitro* replication results are generally consistent with the *in vivo* results. The Ser-268 → Ala and Thr-270 → Ala mutants were able to drive the *in vivo* replication of a *tsr* plasmid carrying the pT181 origin at the restrictive temperature, whereas the Asn-267 → Asp and Arg-269 → Gly mutants were inactive (Fig. 6). Our results show that each of the four amino acids (NSRT) present at positions 267–270 is important for the DNA binding and replication activities of the initiator protein, and the Ser-268 → Ala and Thr-270 → Ala mutants are affected to a smaller degree than the other mutant proteins.

As shown in this study, the topoisomerase activity of the initiator proteins is absolutely essential for the initiation of DNA replication. However, our results demonstrate that nicking at the origin is not sufficient for optimal DNA replication. This conclusion is supported by the results obtained with the various mutant RepC proteins, especially the Asn-267 → Asp and Arg-269 → Gly derivatives. These mutant initiators replicated pT181 DNA very poorly even when present at high concentrations (Fig. 5). At these molar ratios of protein to the DNA, these initiators relaxed the DNA to the same extent as the wild-type protein (Fig. 4). Additionally, the topoisomerase activity of the Ser-268 → Ala mutant was more efficient than that of wild-type RepC, but it showed lower replication activity *in vivo* and when present in limiting amounts *in vitro*. Although the topoisomerase activity of some RepC mutants was lower than that of the wild-type protein, such mutants were affected to a much greater extent in their DNA binding and replication activities. The above results suggest that the topoisomerase and replication activities of the initiator proteins can be functionally uncoupled, and both the topoisomerase and DNA binding domains of RepC protein are required for its replication activity.

The pT181 nucleotides 60–83 present within the origin (the initiation region) have been shown to form a cruciform structure *in vivo* and *in vitro* (23). These nucleotides are conserved in the plasmids of the pT181 family, whereas nucleotides 37–59 (the specificity region) are significantly divergent (Fig. 2*B*). Based on our data, we suggest the following steps during the initiation of pT181 DNA replication. RepC protein recognizes and binds stably to nucleotides located between positions 37 and 59 within the pT181 origin. Through protein–protein interactions, other host enzymes such as DNA polymerase and helicase are recruited to the pT181 origin. RepC then nicks the DNA between nucleotides 70 and 71 and becomes covalently attached to the 5′ phosphate end. This nicked DNA–RepC complex is stabilized by the presence of other host initiation proteins, and the 3′ OH end at the nick is then used as a primer for DNA replication. In reactions containing only the template DNA, the initiator protein is able to nick and religate the DNA, and this event requires transient attachment of RepC to the conserved portion of the origin sequence (nucleotides 60–83 of pT181). The above hypothesis is supported by the observation that synthetic oligonucleotides corresponding to positions 64–79 are cleaved by RepC protein at the proper site (24). The above model explains the results obtained with various RepC mutants. Since the mutant initiators do not bind stably to the DNA, their association with the origin will have a much shorter half-life and most of the initiator–DNA complexes will dissociate from the origin before the assembly of an initiation complex. Any relaxation of the DNA will be nonproductive and will not result in the initiation of DNA replication. At high initiator concentrations, more initiator–DNA complexes will be present, resulting in an increase in replication.

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