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The replication initiator protein of plasmid pT181 has sequence-specific endonuclease and topoisomerase-like activities

(initiation of DNA replication/RepC protein/endonuclease action/origin of replication)

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ABSTRACT **Initiation of pT181 DNA replication specifically requires the plasmid-encoded RepC protein. Here we demonstrate that highly purified RepC protein has sequence-specific endonuclease and topoisomerase-like activities. A maximum sequence of 127 base pairs containing the pT181 origin of replication is required for nicking-closing by RepC protein. RepC introduces a single strand break within the pT181 origin. The nick site has been shown by DNA sequencing to lie between nucleotides 70 and 71 in the bottom strand of the DNA within the origin sequence. This nick site probably corresponds to the start site of pT181 replication. The results presented here suggest that, unlike most other plasmids, pT181 replicates by a rolling circle mechanism.**

Replication of most plasmids requires plasmid-encoded initiator proteins in addition to gene products encoded by the host chromosome (1). An important approach towards the elucidation of the mechanism of initiation of DNA replication involves purification of the initiator protein(s) followed by the determination of their physical and enzymatic properties *in vitro*. In this paper we report the enzymatic activities associated with RepC, the initiator protein of plasmid pT181.

pT181 is a 4437-base-pair (bp) plasmid from *Staphylococcus aureus*, which encodes resistance to tetracycline and has a copy number of 20–25 per chromosome (2, 3). Previously we have described a cell-free system for the replication of pT181 DNA (4). The origin of replication of pT181 has been located to within a 127-bp region, from which the plasmid replicates unidirectionally (5, 6). Initiation of pT181 replication requires the plasmid-encoded RepC protein, which acts in *trans* and is rate limiting for replication (7, 8). Replication of pT181 is negatively regulated by two small RNA species that probably interfere with the translation of RepC mRNA (9). A copy mutant of pT181, cop608, is deleted in the region encoding the two small inhibitor RNAs and has a copy number of about 800 per cell (6, 9). Recently we have reported the purification of RepC protein to homogeneity from an overproducing strain (10). Purified RepC protein was shown to be specifically required for the initiation of pT181 DNA replication *in vitro*.

In this paper we demonstrate that RepC protein has site-specific nicking-closing or DNA topoisomerase-like activity. Supercoiled pT181 DNA and heterologous plasmids containing the pT181 origin sequence were efficiently relaxed by RepC protein, whereas plasmids that lack the pT181 origin were not relaxed. The RepC protein generates a single-stranded nick in the origin. The nucleotide sequence at the nick site has been determined and the nick site has been shown to contain a blocked 5′ P end and a free 3′ OH end. This free 3′ OH end may correspond to the replication start site.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. *S. aureus* strain RN450 and plasmids pT181, cop608, pE194, and pC194 were obtained from R. Novick (6, 8, 11–13). Plasmid pSN2 was described earlier (14). *Escherichia coli* strain HB101 and plasmids pBR322 and pUC7 have been described (15–17). Plasmid pKJB825 was obtained from K. Buckley.

Preparation of Plasmid DNA, Restriction Enzyme Analysis, and Gel Electrophoresis. Plasmid DNA from *S. aureus* and *E. coli* was isolated by CsCl/ethidium bromide density gradient centrifugation (18). The conditions for restriction analysis and gel electrophoresis have been described (10).

Construction of Recombinant Plasmids. The recombinant plasmids described below were constructed by the procedures reported previously (10). Plasmid pSK29 was constructed by ligating *Hpa* II-cleaved plasmid pSN2 DNA with 4421-bp *Hpa* II fragment from pT181, and plasmid pSK179 was obtained by ligating the 1062-bp *Rsa* I fragment containing the *repC* gene from the cop608 plasmid into the *Hin*cII site of plasmid pUC7 (10). Plasmids pSK205, pSK218, and pSK206 were obtained by ligating the *Mbo* I fragments B (1306 bp), C (529 bp), and D (166 bp) from cop608, respectively, into the *Bam*HI site of pBR322 and transforming *E. coli* HB101 (2, 19). Plasmid pSK219 was constructed by inserting the 127-bp *Mbo* I–*Taq* I origin-containing fragment from cop608 plasmid into the *Cla* I and *Bam*HI sites of pBR322 and transforming *E. coli* HB101. Plasmid pSK116 was constructed by inserting the pT181 tetracycline-resistance region (including part of the *Mbo* I A fragment) from pSK41 into the *Hpa* II site of the pSN2 plasmid and transforming *S. aureus* RN450 (11, 20).

Purification of RepC Protein. The construction of a recombinant plasmid that overproduces RepC protein and the purification of this protein has been described elsewhere (10).

Preparation of End-Labeled DNA Fragments and DNA Sequence Analysis. Restriction fragments and RepC-dependent cleavage fragments were labeled at their 5′ ends under denaturing conditions by using polynucleotide kinase and [γ-^{32}P]ATP, after treatment with bacterial alkaline phosphatase (21). The 3′ ends of DNA were labeled by using cordycepin 5′-[α-^{32}P]triphosphate and terminal transferase (21). After digestion with the appropriate restriction enzyme, the singly labeled fragments were sequenced by the method of Maxam and Gilbert (21).

Relaxation Reactions. Reaction mixtures (30 μl) contained 10 mM Tris·HCl (pH 8.0), 100 mM KCl, 10 mM $Mg(OAc)_2$, 1 mM EDTA, 10% ethylene glycol, 0.8 μg of supercoiled plasmid DNA, and 170 ng of RepC protein (unless otherwise

Abbreviations: RF, replicative form; bp, base pair(s); form I, supercoiled plasmid DNA; form I′, covalently closed relaxed circular DNA; form II, nicked open circular DNA; form III, linear double-stranded DNA.

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indicated). After incubation at 32°C for 30 min (unless otherwise indicated), reactions were stopped by the addition of 4 μl of 50% glycerol/20 mM EDTA/0.2% bromphenol blue and reaction mixtures were loaded on 1% agarose gels. The gels were electrophoresed at room temperature in a buffer consisting of 90 mM Tris borate, pH 8.3/2.5 mM EDTA for 16 hr at 3 V/cm.

RESULTS

The Replication Initiator Protein Has Nicking-Closing Activity. Initiation of replication of many plasmids and bacteriophage DNA *in vitro* requires the presence of DNA gyrase and RNA polymerase (22–28). DNA gyrase presumably plays an important role during the generation of an RNA primer by RNA polymerase. The replication of the viral DNA strand of bacteriophages φX174, fd, and fl does not require an RNA primer and proceeds via the rolling circle mechanism (29–38). The initiator proteins of these phages have nicking-closing activities similar to that of DNA topoisomerases (39, 40). Initiation of pT181 replication *in vitro* is only slightly inhibited by the presence of DNA gyrase inhibitors novobiocin and nalidixic acid and does not involve the synthesis of an RNA primer (4, 10). To determine if RepC protein has a nicking-closing activity, supercoiled (form I) cop608 (a high copy number derivative of pT181) DNA was incubated with varying amounts of highly purified RepC protein. The reaction products obtained consisted of nicked open circular DNA (form II) and a spectrum of covalently closed topoisomers (Fig. 1*A*). This distribution of products is characteristic of the action of nicking-closing enzymes (39, 40). Roughly, one or two molecules of RepC were required to relax one molecule of cop608 DNA. At the highest RepC concentrations, about 90–95% of supercoiled cop608 DNA was converted to form II and topoisomers with one to seven superhelical turns (Fig. 1*A*, lanes 8–11). The distribution of topoisomers was similar to that obtained when cop608 DNA was incubated with calf thymus DNA topoisomerase I (Fig. 1*A*, lane 12). The rate of conversion of supercoiled cop608 DNA to form II and covalently closed relaxed DNA (form I′) was visualized on a 1% agarose gel containing ethidium bromide (Fig. 1*B*). The predominant product at the earliest time was form II DNA. After longer periods of incubation, the major product was form I′ DNA. No linear (form III) plasmid DNA was produced by treatment with RepC protein. The distribution between form I′ and form II DNA was not influenced by treatment of the DNA/RepC reaction mixtures with phenol, EDTA, detergents, and proteinase K (data not shown). These experiments demonstrate that RepC protein has two activities: a single-strand endonuclease activity and a ligation activity that seals the previously nicked DNA. These two activities can be separated in the presence of Ba^{2+} ions. The sealing reaction was inhibited in the presence of Ba^{2+} ions and the reaction products primarily consisted of form II DNA (Fig. 1*B*, lane 11). The topoisomerase-like activity of RepC protein required Mg^{2+} and K^+ cations, was ATP-independent, and was not inhibited by novobiocin or nalidixic acid (data not shown).

Relaxation by RepC Protein Is pT181-Specific. *In vitro* replication of pT181 (and derivative plasmids containing an intact pT181 origin) is specifically stimulated by RepC protein (4, 10). Plasmids that do not contain a pT181 origin are not replicated in the RepC-dependent *in vitro* system. Several *S. aureus* and *E. coli* plasmids were tested as substrates for relaxation by RepC protein. As shown in Fig. 2, only cop608 and recombinant plasmids containing the pT181 replication region were relaxed by RepC protein. *S. aureus* plasmids pE194 and pC194 and *E. coli* plasmids pBR322, pUC7, and pKJB825 were not relaxed by RepC protein. Plasmid pSK29, in which the pT181 origin is interrupted and has a deletion of

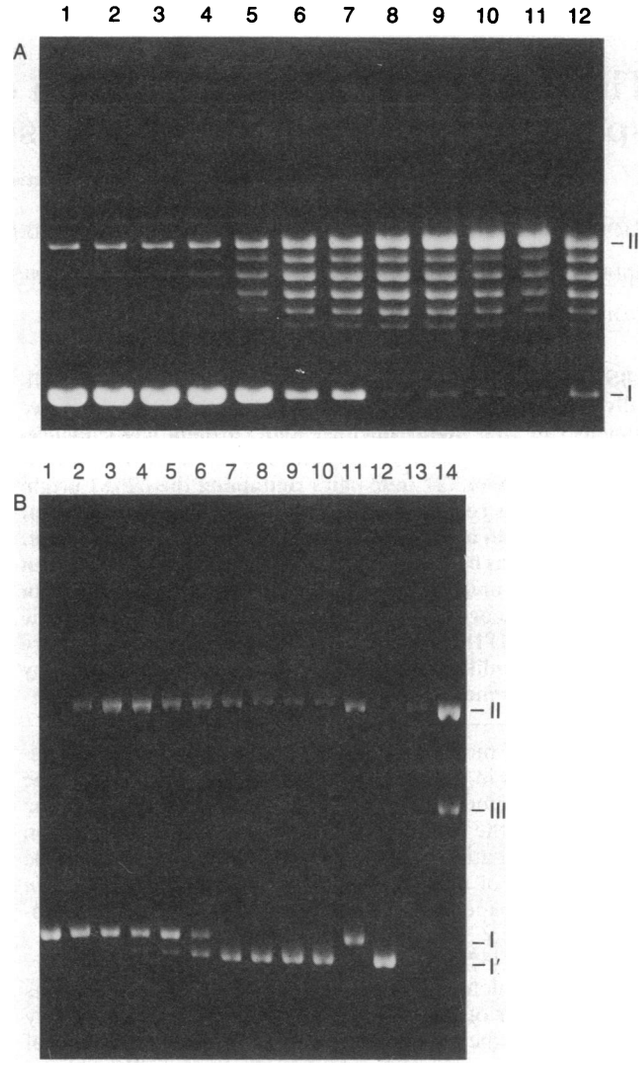

FIG. 1. Relaxation of cop608 DNA by RepC protein. (*A*) Effect of increasing RepC protein concentrations. Lane 1, no RepC; lane 2, 0.85 ng; lane 3, 1.7 ng; lane 4, 3.4 ng; lane 5, 8.5 ng; lane 6, 17 ng; lane 7, 34 ng; lane 8, 85 ng; lane 9, 170 ng; lane 10, 340 ng; lane 11, 510 ng. Lane 12 shows the relaxation of 0.8 μg of cop608 DNA with 10 units of calf thymus DNA topoisomerase I. Positions of form I and form II cop608 DNA are marked. (*B*) Time course of relaxation. Electrophoresis was carried out in the presence of 1 μg of ethidium bromide per ml. Lane 1, no RepC control. Times of incubation are as follows. Lane 2, 5 sec; lane 3, 15 sec; lane 4, 30 sec; lane 5, 1 min; lane 6, 2 min; lane 7, 5 min; lane 8, 10 min; lane 9, 20 min; lane 10, 30 min. Lane 11, cop608 DNA incubated for 30 min with RepC protein in the presence of 50 mM $BaCl_2$ in place of $Mg(OAc)_2$; lane 12, cop608 DNA incubated with 10 units of calf thymus DNA topoisomerase I for 2 min at 32°C; lane 13, linear cop608 DNA ligated with T4 DNA ligase; lane 14, cop608 DNA nicked with limiting amount of DNase I. Positions of form I, I′, II, and III cop608 DNA are shown.

a 16-bp *Hpa* II fragment (10), was not relaxed by RepC protein. This suggested that the integrity of the pT181 origin was necessary for the nicking-closing activity of RepC protein. In a control experiment, all of the above plasmids were shown to be relaxed by calf thymus DNA topoisomerase I (data not shown).

Nicking-Closing Activity of RepC Protein Is Origin-Specific. To identify pT181 sequences that are required for nicking-closing by RepC protein, restriction fragments of cop608 were cloned into the pBR322 plasmid. Recombinant plasmids carrying different regions of cop608 were incubated with or

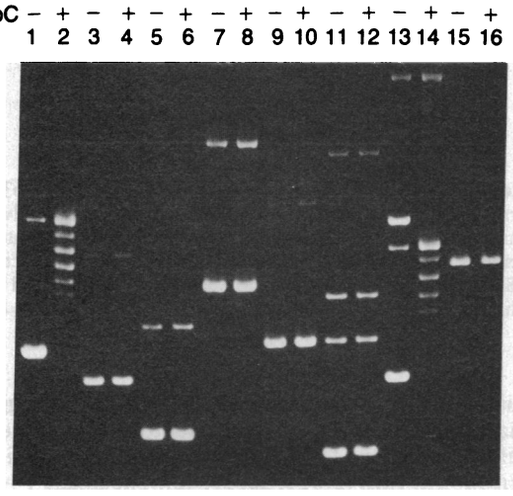

FIG. 2. Substrate specificity of RepC protein. Various supercoiled plasmid DNAs (0.8 μg) were incubated with (+) or without (−) RepC protein (170 ng). Lanes 1 and 2, cop608 DNA; lanes 3 and 4, pE194 DNA; lanes 5 and 6, pC194 DNA; lanes 7 and 8, pSK29 DNA; lanes 9 and 10, pBR322 DNA; lanes 11 and 12, pUC7 DNA; lanes 13 and 14, pSK179 DNA; lanes 15 and 16, pKJB825 DNA.

without RepC protein and subsequently analyzed by agarose gel electrophoresis. cop608 DNA was converted to form II and several topoisomers when incubated with RepC protein (Fig. 3, lanes 1 and 2), whereas this protein had no effect on pBR322 form I DNA (Fig. 3, lanes 3 and 4). Plasmids pSK205 and pSK218, which contain the *Mbo* I B and C fragments, respectively, from pT181, were not affected by RepC protein (Fig. 3, lanes 5–8). In addition, plasmid pSK116, which contains the tetracycline-resistance region from pT181 (this includes part of the *Mbo* I A fragment), was not relaxed by RepC (Fig. 3, lanes 13 and 14). Plasmids pSK206 (Fig. 3, lanes 9 and 10) and pSK219 (Fig. 3, lanes 11 and 12) were relaxed by RepC protein. These plasmids carry the 166-bp *Mbo* I D fragment from cop608 and the 127-bp *Mbo* I–*Taq* I fragment from pT181, respectively. The pT181 origin of replication has been shown previously to be located within the above region

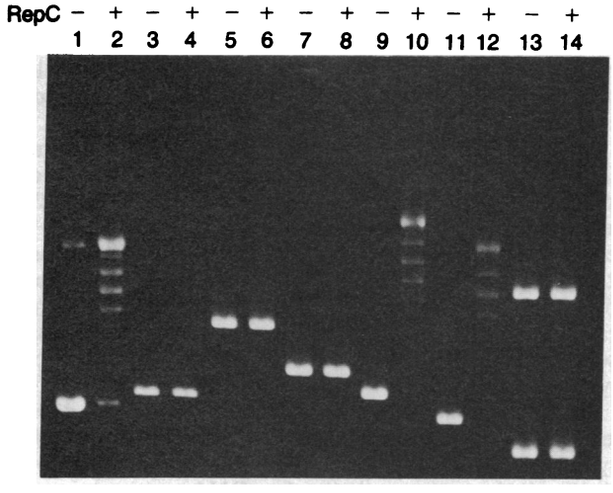

FIG. 3. Relaxation by RepC protein is specific for the pT181 origin. Relaxation by RepC protein was assayed by using pBR322 or derivative plasmids containing different regions of the cop608 plasmid. DNA substrates are as follows. Lanes 1 and 2, cop608; lanes 3 and 4, pBR322; lanes 5 and 6, pSK205; lanes 7 and 8, pSK218; lanes 9 and 10, pSK206; lanes 11 and 12, pSK219; lanes 13 and 14, pSK116. Presence (+) or absence (−) of RepC protein is indicated in the top margin.

[between the *Mbo* I site at position 31 and the *Taq* I site at position 158 (5, 6)]. We conclude from the above data that nicking-closing by RepC protein requires the presence of an intact pT181 origin of replication.

Site of Cleavage by RepC Protein. Since the pT181 origin sequence was required for relaxation by RepC protein, we tested the ability of this protein to cleave cop608 DNA within the origin. cop608 DNA was incubated with RepC protein and after various treatments, the DNA was digested with *Mbo* I and electrophoresed through a polyacrylamide gel. Digestion of untreated cop608 DNA with *Mbo* I generates six fragments, the four largest of which are shown in Fig. 4*A*, lane 1. The *Mbo* I D fragment has been shown earlier to contain the pT181 origin of replication (5). Treatment of cop608/RepC reaction mixtures with proteinase K followed by phenol extraction and *Mbo* I digestion resulted in the appearance of a new fragment, D′ (Fig. 4*A*, lanes 3 and 4). When proteinase treatment was omitted the D′ band was not detected (Fig. 4*A*, lanes 2 and 5). These experiments show that the appearance of the D′ band requires treatment of the reaction mixture with proteinase K prior to phenol extraction. As demonstrated below, the D′ band results from a site-specific cleavage by RepC protein in the bottom strand of the *Mbo* I D fragment and the probable attachment of RepC protein at the 5′ side of the nick. The migration of the D′ fragment may be retarded relative to the fragment D because even rigorous proteinase treatment may leave some amino acid residues of RepC protein attached to the DNA.

To determine if there is a RepC-dependent nick in the origin sequence, fragments D and D′ from the proteinase K-treated sample (Fig. 4*A*, lane 4) were eluted from the gel. These fragments were dephosphorylated with bacterial alkaline phosphatase and 5′ end-labeled under denaturing conditions with T4 polynucleotide kinase (21). In a separate experiment, the D and D′ fragments were 3′ end-labeled with terminal transferase and cordycepin 5′-triphosphate. The labeled DNA fragments were denatured and analyzed by polyacrylamide gel electrophoresis (Fig. 4*B*). The 5′ end-labeled D′ fragment contained a top strand that was similar to the top strand from fragment D, but the bottom strand obtained from D′ was markedly smaller than the one obtained from the D fragment (Fig. 4*B*, lanes 1–4). The 3′ end-labeled D′ fragment separated into three bands (Fig. 4*B*, lane 5). The top band corresponds to the top strand from fragment D (Fig. 4*B*, lanes 2 and 4). The two lower bands in lane 5 correspond to the cleavage fragments of the bottom strand of fragment D. The larger fragments of the bottom strand obtained with 3′ and 5′ end-labeling of the D′ fragment are identical (Fig. 4*B*, lanes 4 and 5). The smaller fragment of the bottom strand could be labeled at its 3′ end but not at the 5′ end (Fig. 4*B*, lanes 4 and 5). These results showed that the nick site can be labeled at its 3′ end but not at its 5′ end. We conclude from the above data that RepC protein cleaves the bottom strand of DNA within the pT181 origin and this cleavage generates a free 3′ OH but a blocked 5′ phosphate end.

DNA sequencing experiments were performed to determine the sequence of the 5′ and 3′ ends at the nick. The large and small single-stranded fragments of the D′ bottom strand containing ^{32}P label at their 5′ and 3′ ends, respectively, were eluted from the polyacrylamide gel and sequenced. As a control, the intact bottom strand of untreated fragment D and the top strands of D and D′ fragments were also sequenced. These results are summarized in Fig. 5. The RepC-dependent cleavage was shown to occur between nucleotides 70 and 71 on the bottom strand of the DNA. No nucleotides were removed from the nick site by the RepC cleavage. The nucleotide sequence around the cleavage site can potentially form a hairpin structure with the nick site occurring within the unpaired loop (Fig. 5).

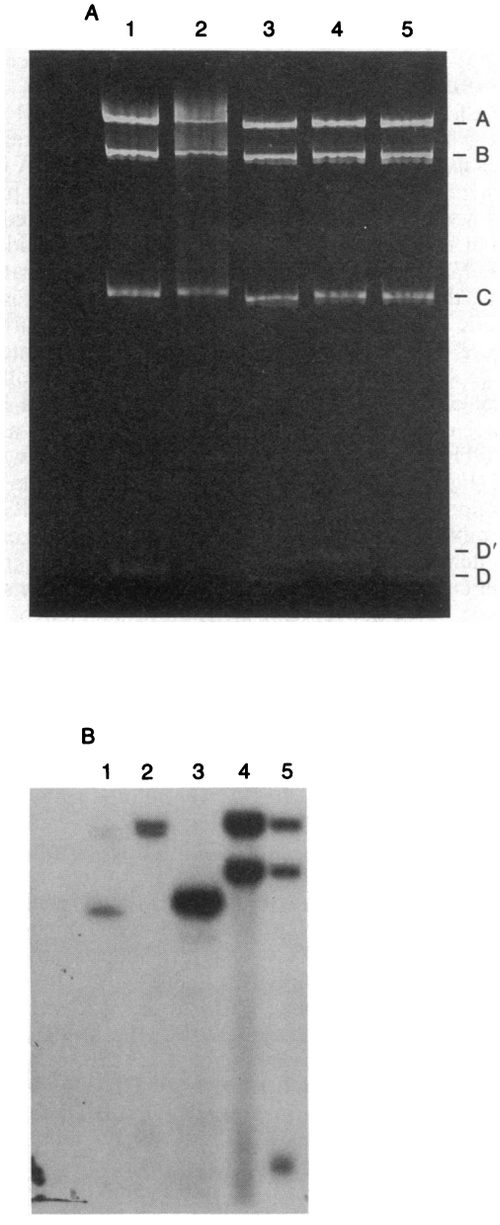

FIG. 4. RepC-dependent cleavage of cop608 DNA. (*A*) Plasmid cop608 DNA was allowed to react with RepC protein at 0°C for 30 min under standard conditions as described under *Materials and Methods*, except that 1 μg of cop608 DNA and 200 ng of RepC protein were used. The reaction mixtures were treated as described below, fractionated on a 5% polyacrylamide gel, stained with ethidium bromide, and photographed under UV light. Lane 1, cop608 DNA not treated with RepC, digested with *Mbo* I; lane 2, reaction mixture was precipitated with alcohol, resuspended, and digested with *Mbo* I; lane 3, reaction mixture was precipitated with alcohol, treated with proteinase K in the presence of $NaDodSO_4$, extracted with phenol, and digested with *Mbo* I; lane 4, reaction mixture was treated with proteinase K in the presence of $NaDodSO_4$, extracted with phenol, and digested with *Mbo* I; lane 5, reaction mixture was treated with 0.2% $NaDodSO_4$, extracted with phenol, and digested with *Mbo* I. (*B*) The D and D' fragments from the above gel were extracted and labeled at their 5' or 3' ends. The labeled fragments were then analyzed on a 8% polyacrylamide gel with or without heat denaturation and autoradiographed (21). Lane 1, 5' end-labeled D fragment, nondenatured; lane 2, 5' end-labeled D fragment, denatured; lane 3, 5' end-labeled D' fragment, nondenatured; lane 4, 5' end-labeled D' fragment, denatured; lane 5, 3' end-labeled D' fragment, denatured. In a control experiment, 5' end-labeled D fragment (lanes 1 and 2) was shown to be identical to the 5' end-labeled *Mbo* I D fragment from untreated cop608 DNA (not shown).

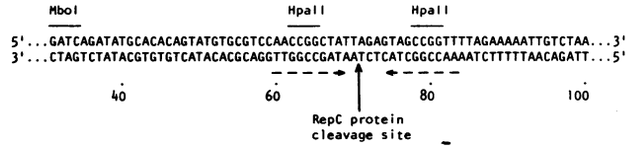

FIG. 5. DNA sequence of the origin of replication of pT181. The interrupted arrows indicate sequences that may be involved in the formation of intrastrand hairpin structures. The RepC protein cleavage site is located within the sequence shown earlier to contain the pT181 origin (5). The nucleotide sequence is numbered as in the published sequence of pT181 (2).

DISCUSSION

Initiation of DNA replication is thought to be triggered by initiator proteins (41). Several initiator proteins have been purified and shown to be required for the initiation of DNA replication *in vitro* (27–29, 34, 37, 42). The phage T4-encoded 39–52–60 protein complex that is required for the initiation of T4 DNA replication has a type II topoisomerase activity (43). The phage φX174 CisA protein and the related gene 2 protein of filamentous phages have single-stranded endonuclease and topoisomerase-like activities (29–35). The simian virus 40 T antigen, which is required for the initiation of viral DNA replication, has a DNA-dependent ATPase activity (44).

Initiation of pT181 DNA replication requires the plasmid-encoded RepC protein (4, 7, 10). We have found that RepC protein has a strand- and sequence-specific endonuclease and ligation activity that acts at the pT181 origin of replication. Plasmids containing the pT181 origin sequence are nicked and relaxed by RepC protein, whereas plasmids lacking the pT181 origin are not affected. Since the form II DNA was the predominant product early in the reaction (Fig. 1*B*), it is likely that the nicking activity of RepC protein is more efficient than its ligation activity. A maximum region of 127 bp is required for the nicking-closing activity of RepC protein. This 127-bp sequence also contains the pT181 origin and is required for the initiation of replication (5). The RepC protein binds tightly and specifically to the pT181 origin (Fig. 4 and data not shown). After nicking by RepC, the form II DNA has a free 3' OH end and a blocked 5' P end. This is consistent with the possibility that RepC protein becomes covalently attached to the DNA through a 5' phosphate bond. This postulated protein–DNA linkage may provide the energy for the sealing reaction, since nicking-closing by RepC protein occurs in the absence of an external energy source. This process would be similar to the reaction catalyzed by the φX174 CisA protein and by DNA topoisomerases (29, 30, 34, 35, 39, 40).

RepC protein cleaves the bottom strand of DNA within the pT181 origin of replication. DNA sequencing has revealed the position of the nick in the sequence 5' ACTCT↓AAT 3' between positions 70 and 71 in the pT181 sequence. This region contains the origin of replication of the pT181 plasmid (2, 5). The nick lies in a nucleotide sequence with a potential for intrastrand base pairing (Fig. 5). It is possible that a transient hairpin structure may exist in negatively supertwisted pT181 DNA and act as a recognition signal for nicking by RepC protein.

Leading strand synthesis of pT181 DNA, *in vitro*, does not require RNA synthesis and is only inhibited to a small extent by the DNA gyrase inhibitors novobiocin and nalidixic acid (4, 10). We have shown that no free RNA species is involved in the initiation of pT181 replication *in vitro* (4, 10). It is likely that the origin-specific nicking activity of RepC protein is similar to that of phage φX174 CisA protein and gene 2 proteins of fd and fl (29–36). The CisA and gene 2 proteins relax the phage replicative form (RF) DNA and create a site-specific nick in the viral strand at the origin of replication. The nick contains a free 3' OH end that is used as a

primer for replication by extension synthesis via the rolling circle mechanism (38). The CisA protein attaches covalently to the 5′ P end and is used for ligating the DNA after a round of replication, releasing single-stranded covalently closed circular viral DNA (29, 30, 34, 35). The gene 2 proteins of fd and fl phages also act in a similar fashion, although they do not attach covalently to the 5′ P end of the DNA (31–33, 36). The cleavage site of CisA protein is located within the *A* cistron (30). Interestingly, the nick generated by RepC protein is also located on the coding strand within the *repC* gene (2, 7). We suggest that the free 3′ OH end generated by RepC protein is used as a primer for the synthesis of pT181 leading strand by a rolling circle mechanism (38). The RepC protein is probably covalently attached to the 5′ end and may ligate the newly synthesized DNA strand in a fashion similar to the φX174 CisA protein. This mode of replication is consistent with our earlier results showing the absence of replication bubble-like structures in the *in vitro* replication experiments carried out in the presence of dideoxytriphosphates (5, 10). In addition, it has been shown that the replication of pT181 is unidirectional and proceeds in the counterclockwise direction on the pT181 map (5, 10). This supports the assumption that the 3′ OH end formed by RepC protein serves as a primer for the leading strand synthesis, since the extension of the nick in the bottom strand will result in a unidirectional and counterclockwise replication.

The replication start site of the ColE1 plasmid, which replicates by a θ-type mechanism, has been identified (24). Results presented in this paper tentatively identify the replication start site of the pT181 plasmid at nucleotide 70 of the pT181 sequence (2, 5). It is interesting to note that initiation of replication by extension of a nick at the origin has been found only for the replication of the RF DNA of several circular, single-stranded bacteriophages. Our results suggest a mechanistic similarity between the replication of RF DNA of φX174, fd, and fl phages and the pT181 plasmid. Thus, the mechanism of pT181 replication seems likely to be more closely related to the replication of circular RF DNA of bacteriophages than to many well-studied plasmids (22–26).

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