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Sequence-specific interaction between the replication initiator protein of plasmid pT181 and its origin of replication

(initiation of DNA replication/DNA–protein interactions)

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ABSTRACT **The replication of the pT181 plasmid is dependent on the plasmid-encoded initiator protein RepC. We have previously shown that RepC protein has sequence-specific endonuclease and topoisomerase-like activities. In this paper we demonstrate that this initiator protein has sequence-specific DNA-binding properties. Based on filter binding of plasmid restriction fragments, RepC protein specifically recognizes only the pT181 origin region. Using DNase I and neocarzinostatin "footprinting" techniques, we show that RepC protein specifically binds to a 32-base-pair sequence within the origin that is part of the initiator cistron. Using dimethyl sulfate as a chemical probe, we have identified the purine residues that interact with the initiator protein. The features of the DNA region that interacts with RepC protein include sequences with the potential to form Z DNA and/or hairpin structures. The specific DNA–protein interaction at the origin may be critical in the initiation of pT181 DNA replication by RepC protein in association with other host initiation proteins.**

Most bacterial plasmids, although nonessential for the host cell growth, are stably inherited and maintained at a fixed copy number. Initiation of replication of most plasmids requires plasmid-encoded initiator proteins, in addition to several host initiation proteins (1, 2). Interaction of initiator proteins with origin sequences is a key step in the initiation of DNA replication. Furthermore, the frequency and specificity of this interaction may regulate the initiation of replication.

pT181 is a completely sequenced, 4437-base-pair (bp) plasmid from *Staphylococcus aureus* that encodes tetracycline resistance (3). This plasmid has been studied in considerable detail for its replication properties. Replication of pT181 both *in vivo* and *in vitro* requires a 38-kDa plasmid-encoded initiator protein, RepC (4–7). This initiator protein has been purified and shown to trigger the *in vitro* replication of pT181 DNA (5). RepC protein has also been shown to have a sequence-specific endonuclease activity that acts at the pT181 origin (8). The origin of replication of pT181 has been located to within a 127-bp region, from which the plasmid replicates unidirectionally (9). The origin sequence is contained within the cistron of the initiator protein (3, 6). The copy number of pT181 plasmid is approximately 22, and it is regulated by two plasmid-encoded small RNAs that act by inhibiting the synthesis of the initiator protein (10, 11). The considerable information available on pT181, as outlined above, makes this plasmid a good model system for studying the initiation of DNA replication in a Gram-positive bacterium.

In this paper we show that RepC is a sequence-specific DNA-binding protein. Highly purified RepC protein binds to specific sequences within the origin of replication of pT181. Using nuclease and dimethyl sulfate "footprinting" experiments, we have identified the precise domain of DNA that interacts with the initiator protein. We also show that RepC protein cleaves the bottom strand of linear DNA (single- or double-stranded) at the same site as in the supercoiled DNA (8). The structural features of the origin sequence that may play a role in interaction with RepC during initiation of DNA replication are discussed.

MATERIALS AND METHODS

Strains, Plasmids, and Enzymes. *S. aureus* strains RN450 and RN1786 and plasmids pT181 and cop608 were obtained from R. Novick. cop608 is a high copy number mutant of pT181, which has a 180-bp deletion in the copy control region of pT181 (3, 10). Enzymes were purchased either from Bethesda Research Laboratories or New England Biolabs and used as recommended by the suppliers. Proteinase K and DNase I were obtained from Boehringer Mannheim. Neocarzinostatin (NCS) was obtained from Bristol Laboratories (Syracuse, NY).

Preparation of End-Labeled DNA Fragments. Restriction fragments were labeled at their 5′ ends under denaturing conditions using polynucleotide kinase and [γ-^{32}P]ATP, after treatment with bacterial alkaline phosphatase (12). The 3′ ends were labeled by using [α-^{32}P]dNTPs and *E. coli* DNA polymerase I large fragment. After digesting with the appropriate restriction enzyme, the singly labeled fragments were purified by polyacrylamide gel electrophoresis.

Retention of Origin Containing Fragments on Nitrocellulose Filters. Fifteen micrograms of cop608 DNA was digested with *Hin*dIII, *Mbo* I, or *Rsa* I. The resulting fragments were 5′ end-labeled and were separated from the unreacted ATP on NACS columns (Bethesda Research Laboratories). Labeled fragments (0.3 μg) from each reaction were mixed with 0.6 μg of RepC protein in a 150-μl reaction mixture containing TEKEM buffer [10 mM Tris·HCl, pH 8.0/1 mM EDTA/100 mM KCl/10 mM $Mg(OAc)_2$/10% (vol/vol) ethylene glycol] for 10 min at 25°C. The reaction mixtures were filtered through nitrocellulose filters (HAWP, Millipore) and washed twice with 5 ml of 10 mM Tris·HCl, pH 8.0/1 mM EDTA. The DNA was eluted from the filters with 1 M NaCl/0.1% $NaDodSO_4$ at 65°C for 10 min and then at 37°C for 2 hr. The extracted DNAs were recovered by alcohol precipitation, electrophoresed on a 5% polyacrylamide gel, and autoradiographed.

DNase I Protection Experiments. DNase protection experiments were carried out according to the method of Galas and Schmitz (13). The 157-bp origin-containing *Pvu* I–*Taq* I fragment (3, 9, 10) (10–50 ng, 18,000 cpm), which was 5′ or 3′ end-labeled at the *Taq* I site, was incubated at 25°C for 15 min with 2 or 4 μg of RepC protein and then treated with DNase I (5 μg/ml) for 5 min in a 50-μl reaction volume. The

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Abbreviations: bp, base pair(s); NCS, neocarzinostatin.

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samples were electrophoresed on 8% polyacrylamide/urea sequencing gels (12). DNA size markers were produced by subjecting the same labeled restriction fragment to A+G and C+T chemical cleavage reactions as described by Maxam and Gilbert (12).

Protection from NCS Attack. NCS protection experiments were performed essentially as described for DNase protection except that after incubation of the DNA with the protein, the reaction mixtures were made up to 10 mM 2-mercaptoethanol and 1 mM EDTA, NCS (20 units/ml) was added, and the reactions were continued for 10 min.

Protection from Attack by Dimethyl Sulfate. Dimethyl sulfate protection experiments were performed by the method of Ogata and Gilbert (14). The 157-bp *Pvu* I–*Taq* I fragment (10–30 ng, 18,000 cpm), either 3′ or 5′ end-labeled at the *Taq* I site, was incubated with 2 or 4 μg of RepC protein at 25°C for 15 min followed by treatment with 1 μl of dimethyl sulfate for 2.5 min. The reaction products were electrophoresed on 8% acrylamide/urea sequencing gels and autoradiographed. The autoradiograms were scanned with a Quick Scan (Helena Laboratories, Beaumont, TX) densitometer.

Nicking of Linear DNA with RepC. Double- and single-stranded *Mbo* I D fragments from cop608 (≈5 ng), labeled at the 5′ termini, were incubated with 1 μg of RepC protein for 30 min at 0°C in a 50-μl reaction volume containing 40 mM Tris·HCl (pH 8.0), 100 mM KCl, 12 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 10% (vol/vol) ethylene glycol. After the reaction, 10 μg of tRNA was added, and the mixture was alcohol precipitated. The DNA was resuspended, denatured, and electrophoresed on a 6% strand-separating polyacrylamide gel (12).

RESULTS

Specific Binding of RepC Protein to the Origin of Replication. The ability of RepC protein to specifically bind to the origin of replication was investigated by retention of DNA–protein complexes by nitrocellulose filters. Supercoiled cop608 DNA [a derivative of pT181 that contains a 180-bp deletion in the copy control region (3, 10)] was digested with the enzymes *Mbo* I, *Rsa* I, and *Hin*dIII. The resulting fragments were ^{32}P-labeled at their 5′ ends, incubated with or without RepC protein, and filtered through nitrocellulose filters. The predominant fragments retained on the filters were those that contained the origin sequence. The retained fragments were: *Hin*dIII fragment B (Fig. 1, lane 3); *Mbo* I fragment D (Fig. 1, lane 6); and *Rsa* I fragment B (Fig. 1, lane 9). The origin of replication of pT181 is contained within all the above fragments (5, 9, 10). The smallest fragment that was retained by RepC protein was the *Mbo* I fragment D (166 bp). This fragment was earlier shown to contain the complete origin of replication of pT181 (5, 9, 10). These results indicate that RepC protein recognizes DNA sequences within the origin.

Interaction Between RepC Protein and the Lower Strand of the Origin. To determine the exact binding site of RepC within the origin, we performed DNase I footprinting experiments. The 653-bp *Taq* I fragment containing the origin (3, 9, 10) was 5′ end-labeled and digested with *Pvu* I. The singly end-labeled 157-bp fragment containing the origin was isolated and partially digested with either DNase I or NCS with or without complexing with RepC protein. The results of the footprinting experiments are shown in Fig. 2. RepC protein protected about 32 bases of the lower strand between positions 37 and 68 from DNase I attack (Fig. 2*A*). There do not seem to be any preferred binding sites within the protected region. The protected region is bounded by residues that show enhanced cleavage. These residues correspond to nucleotides 36 and 71 on the pT181 map. Enhanced cleavages are also seen at residues 43 and 53. The results of

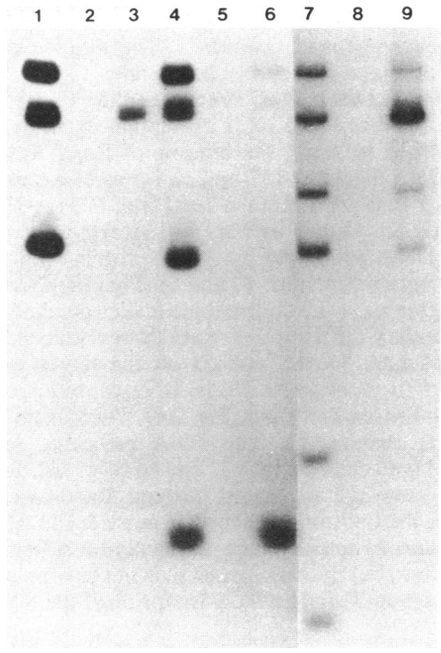

FIG. 1. Filter retention of origin containing fragments incubated with RepC. cop608 plasmid DNA was digested with *Hin*dIII, *Rsa* I, or *Mbo* I, and the fragments were 5′ end-labeled. Aliquots of the labeled DNA were incubated with RepC protein and filtered through nitrocellulose. The DNA remaining on the filters was eluted, precipitated, and electrophoresed. Lanes 1–3, *Hin*dIII-treated cop608 DNA. Lane 1, input DNA; lane 2, no protein control; lane 3, DNA retained in the presence of RepC. Lanes 4–6, *Mbo* I-treated cop608 DNA. Lane 4, input DNA; lane 5, no protein control; lane 6, DNA retained in the presence of RepC. Lanes 7–9, *Rsa* I-treated cop608 DNA. Lane 7, input DNA; lane 8, no protein control; lane 9, DNA retained in the presence of RepC.

RepC protection of DNA from cleavage by NCS are shown in Fig. 2*B*. The region of DNA that is protected is the same

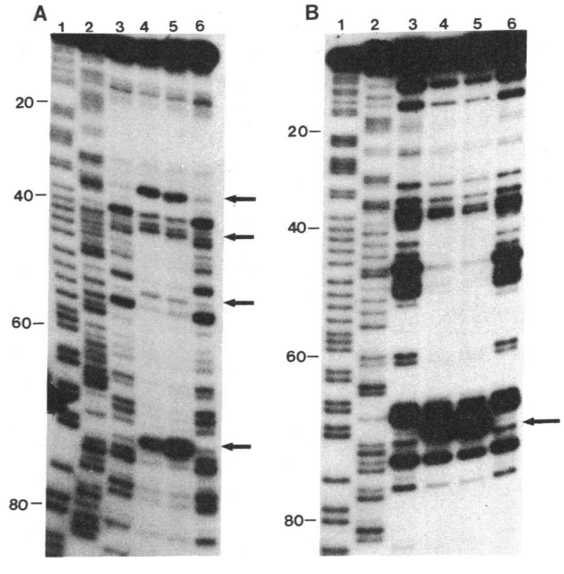

FIG. 2. DNase I and NCS footprints of the lower strand. Protection by RepC protein from attack by DNase and NCS of the 157-bp *Taq* I–*Pvu* I fragment that was 5′ end-labeled at the *Taq* I site. (*A*) DNase protection. Lanes 1 and 2, G+A and C+T sequencing markers, respectively. Lanes 3 and 6, no RepC controls. Lanes 4 and 5, 2 and 4 μg of RepC protein, respectively. (*B*) NCS protection. Lanes 1 and 2, G+A and C+T sequencing markers, respectively. Lanes 3 and 6, no RepC controls. Lanes 4 and 5, 2 and 4 μg of RepC, respectively. The arrows indicate enhanced cleavage bands.

as that seen with the DNase footprints. Interestingly, the only enhanced cleavage is at residue 71. The significance of this enhanced cleavage will be discussed later.

Interaction Between RepC Protein and the Upper Strand of the Origin. DNase I and NCS protection experiments were also performed to study the binding of RepC to the upper strand of DNA using the 157-bp *Taq* I–*Pvu* I fragment (3, 10), which was 3′ end-labeled at the *Taq* I site. The results of these experiments are shown in Fig. 3. The region of the upper strand of DNA protected by RepC protein from DNase I attack lies between residue 37 and 68 (Fig. 3*A*). Note that the lack of fragments in the control lane makes the determination of this boundary difficult. There are three enhanced cleavage sites at residues 36, 43, and 53 on the upper strand that correspond to those seen in the DNase protection experiments with the lower strand (Fig. 2*A*). There is no enhanced cleavage at residue 71. The DNA protected from NCS cleavage is between residues 37 and 68 (Fig. 3*B*). Since there are more cleavage fragments around the boundary near residue 71, the limit of protection is more easily determined. There appears to be an enhanced cleavage at residue 69 in the NCS reaction, but the enhancement is not as dramatic as that seen at position 71 in the NCS footprint of the lower strand (Fig. 2*B*).

Protection from Attack by Dimethyl Sulfate. Results from the DNase and NCS footprinting experiments show that RepC protein binds to both strands of the DNA at a specific region within the pT181 origin. The contact points of RepC protein with the purine residues in the origin sequence were determined by methylation protection experiments. The 157-bp *Pvu* I–*Taq* I fragment containing a 5′ or 3′ label at the *Taq* I end (for lower and upper strands, respectively) was complexed with RepC protein and then treated with dimethyl sulfate. The reaction products were electrophoresed on polyacrylamide/urea sequencing gels and autoradiographed. Fig. 4 shows the densitometric scans of the autoradiograms. The initiator protein made contact with the guanosine residues at positions 42, 44, 46, 55, and 63 of the lower strand.

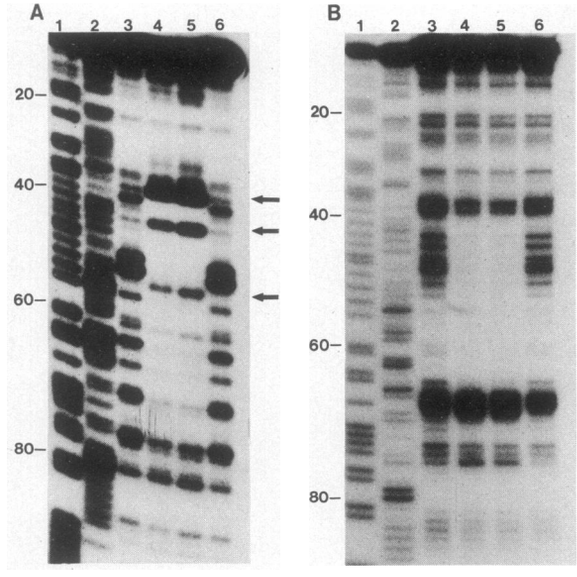

FIG. 3. DNase I and NCS footprints of the upper strand. Protection by RepC protein from attack by DNase I and NCS of the 157-bp *Taq* I–*Pvu* I fragment that was 3′ end-labeled at the *Taq* I site. (*A*) DNase I protection, lanes 1 and 2, are the G+A and C+T sequencing markers, respectively. Lanes 3 and 6, no RepC controls; lanes 4 and 5, 2 and 4 μg of RepC protein, respectively. (*B*) NCS protection. Lanes 1 and 2 are the C+A and C+T sequencing markers, respectively. Lanes 3 and 6, no RepC controls; lanes 4 and 5, 2 and 4 μg of RepC, respectively. The arrows indicate enhanced cleavage bands.

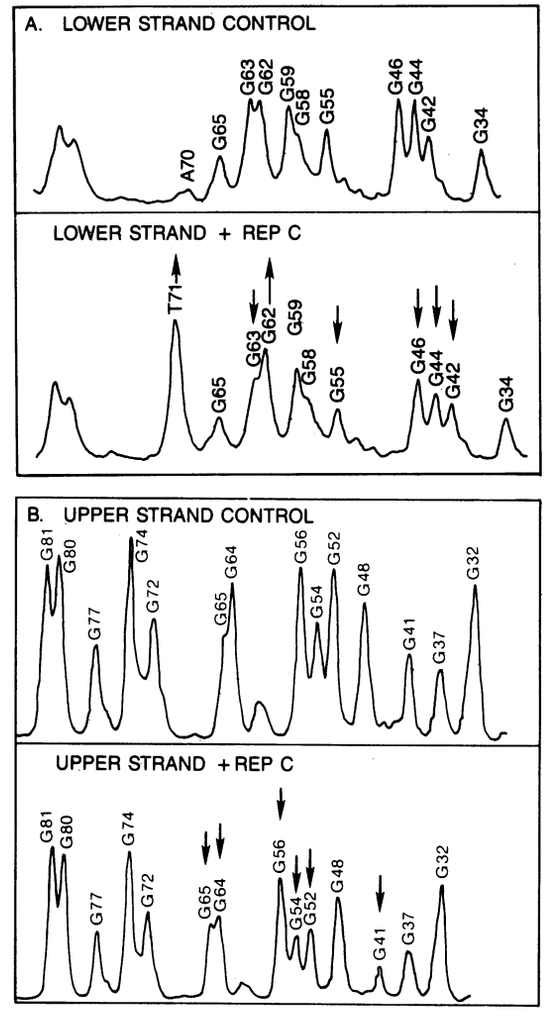

FIG. 4. Densitometric scans of protection from methylation by dimethyl sulfate. The 157-bp *Taq* I–*Pvu* I fragment labeled at the 5′ and 3′ positions at the *Taq* I site was incubated with and without RepC protein (2 μg) in the presence of dimethyl sulfate. After electrophoresis and autoradiography, the films were scanned, and the peak areas were calculated. (*A*) Scans of the lower strand. (*B*) Scans of the upper strand. ↓, Protection from dimethyl sulfate attack. ↑, Enhancement of dimethyl sulfate attack.

The guanosine residue at position 62 of the lower strand showed a slightly enhanced cleavage after dimethyl sulfate treatment. The initiator contact points with the guanosine residues on the upper strand were located at positions 41, 52, 54, 56, 64, and 65. As seen with the nuclease protection experiments, the initiator protein made contact with both strands of DNA. Since the cleavage by dimethyl sulfate at the adenosine residues was weak, no specific protection of the adenosine residues in the origin was observed in these experiments. The enhanced cleavage seen at thymidine-71 of the lower strand will be discussed below.

Nicking of Linear DNA by RepC Protein. The *Mbo* I D fragment of cop608 DNA was shown earlier to contain the nick site of the RepC protein in supercoiled DNA between positions 70 and 71 (8). An experiment was performed to determine whether nicking of the DNA by RepC protein was responsible for the enhanced cleavage bands seen at position 70 in the footprinting experiments with the lower strand of DNA. The 166-bp *Mbo* I D fragment (3, 10) was labeled at its 5′ end, the strands were separated, and the two single strands of DNA were isolated. The double-stranded and single-stranded fragments were incubated with or without RepC and electrophoresed on a 6% strand-separating polyacrylamide

gel. Fig. 5 shows the results of a typical experiment. The lower strand of the DNA was nicked by RepC protein when the DNA is either in the double-stranded or single-stranded form (Fig. 5, lanes 3 and 5) while the upper strand was affected only to a limited extent by treatment with RepC protein. A few faint bands that are visible upon treatment of the upper strand with RepC do not correspond to the known cleavage site of this protein and may represent weak, secondary cleavage sites. This experiment showed that the initiator protein can also cleave the lower strand of the DNA when the substrate is linear (either double- or single-stranded). That the RepC cleavage site is the same as that identified for the supercoiled DNA was shown by the enhanced cleavage bands at thymidine-71 in the lower strand footprinting experiments.

Does pT181 Replication Initiate by Extension Synthesis of the Leading Strand? An experiment was carried out to determine if the replication of pT181 initiates by the covalent extension of the lower strand of DNA that is nicked by RepC protein within the origin. Cop608 DNA was replicated *in vitro* in the presence of 400 μM dideoxy TTP, digested with *Mbo* I, and the intensely labeled, origin-containing D fragment (166 bp) was isolated by polyacrylamide gel electrophoresis as described (5). The pT181 origin contains an *Mbo* I site 40 nucleotides downstream from the RepC nick site (3, 8, 9). If replication initiates by extension synthesis, the lower strand is expected to be labeled between nucleotides 70 (RepC nick site) and the *Mbo* I site at position 31. In addition, replication should result in the displacement of the parental lower strand beyond this *Mbo* I site. When the replicated *Mbo* I D fragment was denatured and electrophoresed on a polyacrylamide gel, the lower strand of the *Mbo* I D fragment was found to be labeled, while the upper strand was not labeled (Fig. 6, lane 3). The involvement of ribonucleotides in the initiation of pT181 replication was ruled out since treatment of the *Mbo* I D fragment with alkali had no effect on the mobility of the lower strand (Fig. 6, lane 4). These results show that pT181 replication initiates by covalent extension of the lower strand of DNA, presumably starting from the RepC nick site.

DISCUSSION

The results presented in this paper demonstrate that the plasmid pT181-encoded replication initiator protein is a sequence-specific DNA-binding protein. The filter-binding

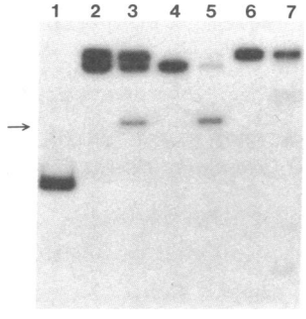

FIG. 5. Nicking of linear and single-stranded DNA by RepC protein. The 166-bp *Mbo* I D fragment from cop608 was 5′ end-labeled, and a portion of the labeled fragments was separated into single strands. The double-stranded and each single-stranded fragment were incubated with RepC protein, electrophoresed on an 8% strand-separating polyacrylamide gel, and autoradiographed. Lane 1, double-stranded fragment, not denatured; lane 2, double-stranded fragment denatured; lane 3, double-stranded fragment + RepC; lane 4, lower strand; lane 5, lower strand + RepC; lane 6, upper strand; lane 7, upper strand + RepC. Arrow indicates the band produced by the action of RepC.

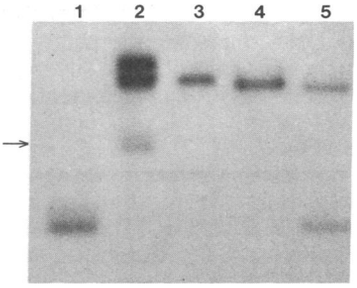

FIG. 6. Replication of the lower strand of the *Mbo* I D fragment by extension synthesis. Lanes 1 and 2, control *Mbo* I D fragment, nondenatured and denatured, respectively; lane 3, *Mbo* I D fragment replicated in the presence of dideoxy TTP, denatured; lane 4, same as lane 3, except treated with 0.1 M KOH for 1 hr; lane 5, same as lane 3, but partially denatured. Arrow indicates the position of a control band produced by the cleavage of the lower strand of the *Mbo* I D fragment by Rep C.

results demonstrate that RepC protein binds specifically to pT181 restriction fragments that contain the origin of replication. A summary of the RepC-origin interactions as analyzed by footprinting techniques is shown in Fig. 7. The initiator protein binds to a 32-nucleotide sequence on both the upper and lower strands of the DNA in the origin. This sequence also encodes a portion of the amino-terminal region of the initiator protein. The RepC protein makes specific contacts with five guanosine residues on the lower strand and six on the upper strand.

A striking feature of the origins of replication of plasmids F, R6K, RK2, pSC101, and P1, the simian virus 40, phage λ, and the *E. coli* chromosomal origin, *oriC*, is the presence of repeated sequences (15–26). The corresponding initiator proteins have been shown to specifically interact with these sequences that are either within or vicinal to the origins of replication. The binding of a few of these initiator proteins to the origin is cooperative (17, 18, 23, 27). It is likely that multiple copies of a directly repeated sequence are critical for initiator binding and for successful initiation of DNA replication. The synthesis of several initiator proteins that interact with directly repeated sequences in the origin is autoregulated (28–32). In contrast to the above systems, the replication origin of pT181 does not contain repeated sequences (3, 9), and the synthesis of RepC protein is controlled by two small, plasmid-encoded RNAs (10, 11). However, the initiator of pT181, RepC, is capable of highly specific interaction with the origin sequence that results in the initiation of rounds of DNA replication. There are several striking features of the pT181 origin sequence that are worth noting. The origin region contains a sequence that can potentially form a hairpin structure in the supercoiled DNA (Fig. 7). The binding by RepC is asymmetric, since it binds only to one side of the self-complementary stem region. Most origins of replication

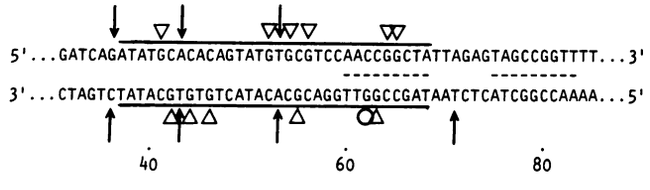

FIG. 7. Summary of the protection data. The sequence of the pT181 origin in the region that is protected by RepC is shown. The bases protected from attack by DNase I and NCS are indicated by the lines above and below the sequence. ↓ or ↑, Enhanced cleavages by DNase (on top and bottom strands, respectively). ▽ or △, Protection from attack by dimethyl sulfate. ○, Enhanced cleavage by dimethyl sulfate. The dotted lines indicate sequences that may be involved in the formation of a hairpin structure.

in Gram-negative bacteria have been found to be A+T rich (33). *S. aureus* DNA, including the pT181 plasmid, is extremely A+T rich, consisting of about 70% A·T and 30% G·C bp (3). The RepC binding site and the potential hairpin region in the origin are relatively G+C rich, consisting of 50% G·C bp (nucleotides 37–83, Fig. 7). A G+C-rich sequence containing a possible hairpin structure in an A+T-rich DNA would be a very distinctive feature, and binding of RepC protein to this region could facilitate the initiation of DNA replication. Another striking feature of the pT181 origin sequence is a 21-bp stretch of alternating pyrimidine/purine residues from position 37 to 57, with only one nucleotide out of alternation (Fig. 7). It has been shown earlier that a region containing a 14-bp stretch of alternating purine/pyrimidine residues in the supercoiled pBR322 plasmid can be present in the left-handed Z form (34). Several other studies have also shown that regions of DNA containing alternating purine/pyrimidine residues can exist in the Z form (35). It is tempting to speculate that the possible Z structure in the pT181 origin may act as a recognition signal for RepC during the initiation of DNA replication. It is interesting to note that the binding site of RepC to the origin includes the complete segment (nucleotides 37–57) that may exist in the Z form in negatively supercoiled DNA (Fig. 7).

Previously we have shown that RepC protein introduces a nick in the lower strand of DNA within the origin in the supercoiled plasmid DNA (8). Here we show that the initiator protein can cleave the lower strand of DNA when incubated with linear single- or double-stranded DNA containing the origin sequence. This nicking occurs between nucleotides 70 and 71 and results in the appearance of an enhanced cleavage band in the DNase I, NCS, and dimethyl sulfate footprints of the lower strand of the DNA. This nick probably serves as the start site of pT181 replication by a rolling circle mechanism, since only the lower strand of the DNA in the origin region was replicated in the presence of dideoxy TTP (Fig. 6).

The sequence-specific binding of RepC protein to the origin may provide the specificity required for the formation of an initiation complex involving RepC and the host-encoded proteins. This complex could then trigger the initiation of a round of DNA replication. The frequency of RepC–origin interaction may, therefore, regulate the frequency of initiation.

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