Cleavage of single-stranded DNA by plasmid pT181-encoded RepC protein

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ABSTRACT

RepC protein encoded by plasmid pT181 has single-stranded endonuclease and topoisomerase-like activities. These activities may be involved in the initiation (and termination) of pT181 replication by a rolling circle mechanism. RepC protein cleaves the bottom strand of DNA within the origin of replication at a single, specific site when the DNA is in the supercoiled or linear (double or single-stranded) form. We have found that RepC protein will also cleave single-stranded DNA at sites other than the origin of replication. We have mapped the secondary cleavage sites on pT181 DNA. When the DNA is in the supercoiled, or linear, double-stranded form, only the primary site within the origin is cleaved. However, when the DNA is present in the single-stranded form, several strong and weak cleavage sites are observed. The DNA sequence at these cleavage sites shows a strong similarity with the primary cleavage site. The presence of *Escherichia coli* SSB protein inhibited cleavage at all of the secondary nick sites while the primary nick site remained susceptible to cleavage.

INTRODUCTION

Replication of the *Staphylococcus aureus* plasmid pT181 both *in vivo* and *in vitro* requires the plasmid-encoded replication initiator protein, RepC (1,2). RepC protein initiates pT181 replication by introducing a nick in the lower strand of supercoiled DNA at the unique origin site (3). The cleavage generates a free 3'-OH end that is likely to serve as a primer for synthesis of the leading strand by a rolling circle mechanism (3,4). The RepC cleavage site which serves as the start-site of pT181 replication was determined *in vitro* and shown to lie between nucleotides 70 and 71 on the pT181 map (3,5). RepC protein may play a role in the termination of pT181 replication by cleaving the single-stranded, displaced leading strand as the replication fork passes the origin and by the subsequent circularization at the origin at the end of each round of replication. Support for the rolling circle mode of pT181 replication also comes from the observation that a small percentage of pT181 DNA synthesized *in vivo* (6,7) and *in vitro* (unpublished data) is single-stranded circular DNA. The observation that a single-stranded

intermediate is formed in the replication of pT181 is reminiscent of the replication of the bacteriophages ϕ X174, ϕ 1 and ϕ d (8,9). This single stranded intermediate in replication is unique among the plasmids studied to date. In this paper we have investigated the ability of RepC protein to cleave single-stranded DNA. We have identified strong and weak RepC cleavage sites in pT181 DNA. From analysis of the major cleavage sites we have determined that RepC cleaves pyrimidine rich sequences which contain an absolutely conserved AA dinucleotide. The strong cleavage sites were used to determine a consensus sequence for RepC cleavage. This sequence is: 5' TPYNTGPyAAN 3'. The secondary sites were cleaved by RepC protein only when DNA was present in the single-stranded form, but were not cleaved when the DNA was in linear double-stranded form. This suggests that RepC cleavage at secondary sites requires the presence of at least partially single-stranded regions. When single-stranded DNA was incubated with RepC protein in the presence of *E. coli* single-stranded DNA binding protein (SSB), the secondary cleavage sites were protected from cleavage by RepC. The cleavage of single-stranded pT181 DNA by RepC at the primary cleavage site was not appreciably affected by SSB. These findings suggest that the origin of replication of pT181 has unique features which are recognized by RepC protein in double- or single-stranded forms even in the presence of proteins which mask other potential RepC cleavage sites.

MATERIALS AND METHODS

Enzymes

Bacterial alkaline phosphatase, T4 polynucleotide kinase and all restriction endonucleases were purchased from Bethesda Research Laboratories and used as recommended by the supplier. RepC was prepared according to the published procedure (2).

Isolation of DNA fragments and end-labeling

Plasmid DNA from the pT181 copy mutant, cop608, was prepared by CsCl-ethidium bromide density gradient centrifugation as described previously (10). Supercoiled cop608 DNA was cleaved with MboI or TaqI and the fragments separated by polyacrylamide gel electrophoresis (11). The MboI D and TaqI E fragments were isolated from the gel by crushing and elution (11). The DNA fragments were treated with bacterial alkaline phosphatase and labeled at their 5' ends with [32 P] γ -ATP and T4 polynucleotide kinase as described (11).

Isolation of single-stranded DNA fragments

A portion of the 5' end labeled fragments was denatured by heating at 90°C for 2 min in 30% DMSO containing 0.05% each of xylene cyanol and

bromphenol blue. The DNA was quickly chilled and electrophoresed on 6% polyacrylamide gels using Tris-borate-EDTA buffer as described by Maxam and Gilbert (11). The gels were exposed to X-ray films and the ^{32}P -labeled single strands of DNA were eluted as described earlier (11).

Synthesis of oligonucleotides

Deoxyribooligonucleotides of defined sequences were synthesized by using an applied Biosystems DNA Synthesizer. The oligonucleotides were 5' end-labeled as described above.

Cleavage of DNA by RepC protein

^{32}P -labeled single-stranded or double-stranded DNA fragments (about 2 ng) were incubated with RepC protein (20 ng) in a 10 μl reaction mixture containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM KCl, 10 mM magnesium acetate and 10% ethylene glycol. After 30 min at 32°C, the DNA was recovered by alcohol-precipitation, resuspended in formamide buffer and electrophoresed on polyacrylamide-urea sequencing gels (11). Sequencing markers were also run on the gels by subjecting the same single-stranded fragments to A + G and C + T chemical cleavage reactions as described by Maxam and Gilbert (11). The reaction products were visualized by autoradiography. To study the effect of SSB protein on RepC cleavage various concentrations of *E. coli* SSB protein were included in the reaction mixtures.

Treatment with Phosphodiesterase

Fifteen pMoles of the 16 mer oligonucleotide (Table 1) labeled with ^{32}P at the 5' end was treated with 1×10^{-6} units of snake venom phosphodiesterase (Boehringer-Mannheim) in a 100 μl reaction. Ten μl aliquots were taken at 0, 1, 2, 3, 4, 5, 7, 10 and 15 minutes and mixed with 100 μl phenol. The reaction was extracted 2 times with phenol, 3 times with ether, and the DNA was precipitated with ethanol. The pellet was resuspended in 30 μl distilled H_2O and 5 μl was incubated with RepC as described above.

RESULTS

Cleavage of single-stranded DNA by RepC protein

We have previously shown that RepC protein nicks both single-stranded and double-stranded (supercoiled as well as linear) pT181 DNA at a specific site within the origin of replication (3). Furthermore, nicking was found to be most efficient when DNA was present in the supercoiled form. We wished to determine if RepC protein has additional cleavage sites on single-stranded DNA. Restriction fragments were isolated from the pT181 high copy number derivative, cop608. Two DNA fragments, the *Mbo*I D fragment containing the

Figure 1. Nicking of cop608 MboI D fragment by RepC protein. The double-stranded MboI D fragment was labeled at its 5' end. The strands were separated and incubated with RepC protein. Lanes 1 and 2, G + A and C + T sequencing reactions of the upper strand, respectively; lane 3, upper strand, untreated; lane 4, upper strand incubated with RepC protein; lane 5, double-stranded MboI D fragment, untreated; lane 6, double-stranded fragment incubated with RepC protein; lane 7, lower strand, untreated, lane 8, lower strand incubated with RepC protein; lanes 9 and 10, G + A and C + T sequencing reactions of the lower strand, respectively. Arrow indicates the band generated by cleavage by RepC protein at the primary nick site.

pT181 origin of replication and the TaqI E non-origin fragment were isolated. These fragments were 5' end-labeled, strand separated, and the isolated single-stranded DNA fragments were incubated with RepC protein. The reaction mixtures were electrophoresed on polyacrylamide-urea gels and autoradiographed. To identify the sequence at the cleavage sites, the fragments were also subjected to Maxam-Gilbert chemical sequencing reactions and run on the same gel. The results of experiments with the MboI D fragment are shown in Figure 1. As observed earlier (3), the origin-containing, double-stranded MboI D fragment is cleaved by RepC protein in the lower strand at a single, specific site (the primary nick site) (Figure 1, lanes 5 and 6). When the

Figure 2. Cleavage of single-stranded cop608 TaqI E fragment by RepC protein. The cop608 TaqI E fragment was 5' end-labeled, strand separated, and used for cleavage analysis. Lanes 1 and 2, G + A and C + T sequencing reactions of the upper strand, respectively; lane 3, upper strand, untreated; lane 4, upper strand incubated with RepC protein; lane 5, lower strand, untreated; lane 6, lower strand incubated with RepC protein; lanes 7 and 8, G + A and C + T sequencing reactions of the lower strand, respectively.

lower strand of the MboI fragment is incubated with RepC protein, it is also cleaved predominantly at the primary nick site (Figure 1, lanes 7 and 8). However, the lower strand was also cleaved at a few additional secondary sites. There is a striking difference between the intensity of bands resulting from secondary cleavages. The lower strand contains four major secondary cleavage sites and two or three minor cleavage sites. The upper strand of DNA contains seven major secondary cleavage sites (five are shown in Figure 1, lane 4) and three minor sites. It should be noted that due to the partial nature of the cleavage reactions, almost all the possible cleavage sites can be deduced by treating 5' end-labeled fragments with RepC protein. We have earlier shown that the upper strand of DNA is not cleaved and the lower strand is cleaved only at the primary nick site when double-stranded MboI D fragment is treated with RepC protein (3,4).

We wished to determine if RepC protein has secondary cleavage sites in a region of pT181 DNA that is not a part of the origin of replication. The TaqI E fragment was used for this purpose. When the single-stranded upper and lower strands were treated with RepC protein, both showed the presence of major as well as minor secondary cleavage sites (Figure 2, lanes 3-6). When double-stranded TaqI E fragment was treated with RepC protein, the upper and

Secondary Cleavage Sites of Rep C Protein

pT181 Map Location (Strand)	Sequence
86 (lower)	5' T T T T C T A A A A C G G 3'
111 (lower)	C A T T T T G G A A A A T C A
122 (lower)	A T C A T T C A A A T C A T
131 (lower)	A T C A T G C A A A T C A T
59 (upper)	T G C G T C C A A C C G G A
98 (upper)	A T T G T C T A A A T C G T
113 (upper)	A T T T T C C A A A T G A T
114 (upper)	T T T T C C A A A T G A T T
168 (upper)	A C T C C T T A A T C T A A
173 (upper)	T A A T C T C A A T T T C G
371 (upper)	T T G G T C T A A T C A
3872 (lower)	A G A T T G G A A A A C T A
3882 (lower)	A T C T T G C A A C C A G A
3874 (upper)	G T T T T C C A A A T C T G G
3894 (upper)	G A T A T G T A A A T C A C
3918 (upper)	T T C C A G T A A T C C A C

Consensus Sequence

5' T P_y N T $\begin{smallmatrix} G \\ C \end{smallmatrix}$ P_y A A N 3'

Primary Nick Site

5' T A C T C T A A T 3'

Figure 3. Secondary cleavage sites of RepC protein. The sequence at 16 major secondary sites cleaved by RepC protein is shown. The consensus sequence derived from these sequences is indicated as is the sequence of the primary nick site.

lower strands of DNA were not cleaved (data not shown).

Figure 3 shows the sequences surrounding the secondary cleavage sites. These sites are aligned at the cleavage site which is indicated by an arrow. From the analysis of the 16 major secondary cleavage sites shown in Figure 3, we have deduced a consensus sequence 5' TP_yNT $\begin{smallmatrix} G \\ C \end{smallmatrix}$ PyAA_N 3'. When the sequences were aligned at the cleavage sites, the frequency of each nucleotide at each position was calculated. The probability that the frequencies were generated by chance was calculated (20) and those frequencies which had a probability of less than 0.01 were taken as significant. The most striking feature of the primary nick site as well as all the sequences at the secondary cleavage sites is the presence of an AA dinucleotide in every case at the site of the phosphodiester bond cleavage. In addition, a T residue is present 3 nucleotides upstream of the AA dinucleotides in 76% of the cases (the frequency of a pyrimidine residue at this position is 95%) and another T residue is present 6 nucleotides upstream of the AA dinucleotide in 61% of the cases. When the primary nick site is compared to the consensus sequence (Figure 3), the most highly conserved residues are the T at the 5' end and the

Figure 4. Cleavage of the lower strands of MboI D and TaqI E lower strand fragments by RepC protein in the presence of SSB protein. A) Lane 1, MboI D fragment lower strand, untreated; lane 2, lower strand incubated with RepC protein; lanes 3 to 5, lower strand incubated with RepC protein in the presence of 0.5, 1.0, and 1.5 μ g of SSB protein, respectively; B) Lane 1, TaqI E fragment lower strand, untreated; lane 2, lower strand incubated with RepC protein; lanes 3 to 5, lower strand incubated with RepC protein in the presence of 0.5, 1.0, and 1.5 μ g of SSB protein, respectively. Arrow indicates the band generated by RepC protein cleavage at the primary nick-site. Horizontal bars (-) indicate secondary cleavages.

pentanucleotide sequence from positions 4 to 8 on the consensus sequence. It is interesting to note that a hexanucleotide sequence, TCTAAT, is present in the upper strand of the MboI D fragment at position 377. This sequence is

Table 1
Sequence of the synthetic oligonucleotides

			4	8	12	16	
				↓			
1.	5'	d-GGCTACTCTAATAGCC					3'
2.	5'	d-GGCTACTCAATAGCC					3'
3.	5'	d-GGCTACTCTTAGCC					3'

↓ indicates the position of RepC cleavage

identical to a hexanucleotide sequence present at the primary nick site (Figure 3). However, the cleavage at position 377 is much weaker than at the primary nick site. This could either be due to some secondary or altered structure around the primary nick site or a sequence longer than the above hexanucleotide may constitute the proper RepC recognition site. The second possibility may be more likely since we have earlier shown that RepC protein binding site includes 32 basepairs near the primary nick site (4). In general, the closer the sequence is to that of the primary nick site, the better is the cleavage. Analysis of the sequence at the minor cleavage sites shows that these sites contain one or more deviations from the consensus at the highly conserved sites. All of the minor sites, however, contained the AA dinucleotide.

Effect of single-stranded DNA binding protein on RepC cleavage

Since the results obtained above suggested that single-stranded DNA was required for cleavage by RepC protein at the secondary sites, we tested the effect of SSB protein on the ability of RepC protein to cleave single-stranded DNA (Figure 4). SSB protein severely inhibited the cleavage of the lower strand of the MboI D fragment by RepC at the secondary sites, whereas the cleavage at the primary nick site was unaffected (Figure 4A, lanes 1-5). When single-stranded DNAs other than origin containing fragments were incubated with RepC in the presence of SSB, the cleavage by RepC protein was also inhibited. An example of this is shown for the lower strand of the TaqI E fragment (Figure 4B).

Cleavage of synthetic oligonucleotides by RepC protein

Since RepC protein cleaved single-stranded DNA at a set of closely related sequences, we carried out experiments to determine if RepC would cleave small synthetic oligonucleotides carrying the primary nick site or oligonucleotides carrying changes at conserved nucleotides. Three

Figure 5. Cleavage of synthetic oligonucleotides by RepC protein. The oligonucleotides listed in Table 1 were 5' end-labeled, incubated with RepC protein, and the reaction products were electrophoresed on a 20% polyacrylamide-urea sequencing gel. A) Lanes 1 to 4, G, G + A, C + T and C specific chemical cleavage reactions of oligonucleotide 1 (Table 1), respectively; lanes 5 and 6, oligonucleotide 1, untreated and incubated with RepC protein, respectively; lanes 7 and 8, oligonucleotide 2, untreated and incubated with RepC protein, respectively; lanes 9 and 10, oligonucleotide 3, untreated and incubated with RepC, respectively. Arrow indicates the band generated by RepC protein cleavage. B) Mapping the cleavage site by comparison to the fragments generated by phosphodiesterase cleavage. Lane 1, 16-mer (oligonucleotide 1); lane 2, 16 mer cleaved by RepC; lane 3, 16 mer cleaved by phosphodiesterase; lane 4, DNA from lane 3 cleaved by RepC (fragment sizes are indicated at the right of the figure).

oligonucleotides of defined sequences were used for this purpose (Table 1). The oligonucleotide number 1 (16-mer) contained the wild-type primary nick site sequence. Oligonucleotide 2 is deleted for the conserved T residue at position 7 and oligonucleotide 3 is deleted for the conserved AA dinucleotide of the RepC cleavage consensus sequence (Figure 3 and Table 1). ^{32}P -labeled oligonucleotides were treated with RepC protein and the reaction products were separated on a polyacrylamide-urea sequencing gel (Figure 5). The 16-mer carrying the wild-type primary nick site was also subjected to Maxam-Gilbert sequencing to generate size markers. The hexadecanucleotide containing the wild-type sequence at the primary nick site was cleaved efficiently (Figure 5A, lanes 5-6) whereas, the other two oligonucleotides were not cleaved (Figure 5A, lanes 7-10). These results demonstrate that the recognition site for cleavage of single stranded DNA by RepC is no more than 16 nucleotides. The cleavage site of RepC was determined to lie immediately 5' to the AA dinucleotide (between nucleotides 9 and 10 in Table 1). The size of the cleavage product was determined by comparing the results of the cleavage reaction with the products of limited digestion of the 16-mer with snake venom phosphodiesterase (Figure 5B). When the 16-mer was cleaved by RepC the major product migrated with a 9-mer product of phosphodiesterase digestion. Similar results were obtained when the phosphodiesterase products were incubated with RepC (Figure 5B, lane 4). These results show that the synthetic oligonucleotide was cleaved by RepC at the same site as in the supercoiled pT181 DNA (3,4).

DISCUSSION

We have previously shown that RepC protein nicks pT181 DNA at a specific site within the origin of replication (3,4). This nick at the origin contains a free 3' end which is likely to be used as a primer for DNA replication by the rolling circle mechanism (3,4). Replication of pT181 by such a mechanism is supported by the evidence that RepC protein has both single-stranded endonuclease and ligation activities. Further, only the leading strand of DNA near the origin is replicated in vitro in the presence of high concentrations of dideoxynucleotides (4). Single-stranded pT181 plasmid DNA has been shown to be present during replication both in vivo (6,7,21) and in vitro (unpublished data). Finally, pT181 leading strand synthesis in vitro does not require ribonucleotides (4). In these respects RepC protein has activities reminiscent of the gene A protein of bacteriophage ϕX174 and the gene 2 protein of f1 and fd phages (14,15).

In this report we have investigated the sequence requirements for RepC protein cleavage in detail. Our results show that RepC protein cleaves DNA at additional sites when the DNA is present in single-stranded form. However, RepC protein cleaves supercoiled or linear double-stranded DNA only at a unique site within the origin (3). Thus, at least part of the requirements for specific RepC cleavage at the origin is the presence of double-stranded regions within the DNA. Since the primary nick site at the origin is also efficiently cleaved by RepC protein when the DNA is in a single-stranded form, RepC is capable of recognizing and cleaving at the primary site in single-stranded DNA. However, when single-stranded DNA is treated with RepC protein, the presence of a large number of secondary cleavage sites is revealed (Figures 1 and 2). Similar results were obtained when single-stranded ϕ X174 DNA was cleaved with gene A and A* proteins (12,13). However, gene A protein cleaves single-stranded DNA at only one secondary site in addition to the primary nick site (12). The RepC protein cleaves the synthetic hexadecamer 5' GGCTACTCTAATAGCC 3' at the 3' side of the T residue at position 9. This cleavage point corresponds to the site where pT181 supercoiled and linear (single and double-stranded) DNA is cleaved by RepC protein. These results also show that the actual recognition sequence for cleavage by RepC protein is no longer than 16 nucleotides.

Analysis of 16 major secondary cleavage sites of RepC protein on single-stranded DNA leads to the consensus sequence 5' TPyNT^CPyAAN 3'. This sequence has a strong resemblance to the primary nick site of RepC protein. Especially noteworthy is the presence of an AA dinucleotide which was an absolute requirement for cleavage by RepC protein both at the major and minor cleavage sites.

The mechanism of pT181 DNA replication has many of the features of ϕ X174 RF DNA replication (16,17). The gene A protein becomes covalently attached to the 5' terminus after cleavage at the ϕ X174 origin (17). The 5' end at the RepC cleavage site is blocked, suggesting a covalent attachment (3). The secondary cleavage site of gene A protein on single-stranded ϕ X174 DNA is masked in the presence of SSB protein, while the primary site is still available for cleavage (13). Our results demonstrate that SSB and the T4 gene 32 protein (data not shown) inhibit cleavage by RepC protein at secondary sites but not at the primary nick site on single-stranded DNA (Figure 4). A *S. aureus* protein analogous to SSB protein may provide specificity for cleavage at the primary nick site in vivo. Gruss and Novick (21) have shown that a sequence about 500 basepairs upstream of the pT181 origin acts as the

origin of lagging strand synthesis. This would imply that the strand displaced as a single strand during replication would not be copied into double-stranded DNA until near the end of a round of replication. A protein analogous to SSB would be required in vivo to protect this exposed parental single strand until lagging strand synthesis could be completed. RepC protein recognizes and cleaves the DNA at the origin in the presence of SSB. Thus, if RepC protein is involved in the termination of replication (as implied by its ligation activity) it would have to recognize the origin region in the presence of SSB. We have shown that this is, in fact, the case.

Additionally, since a small percentage of pT181 DNA synthesized in vivo (21) and in vitro is single-stranded, RepC protein must cleave the displaced leading strand (presumably bound by SSB) at the origin to release a single-stranded circular DNA. These results imply that the origin sequence is either not bound by SSB because of a secondary structure or bend (22) or that the strength of the interaction between RepC and DNA is able to displace SSB (and possibly other proteins) bound to the origin.

The ability of RepC protein to cut a series of related sequences in single-stranded DNA could be useful in applications requiring a single strand endonuclease activity. Such an activity could be useful in mapping open or "active" sites in chromatin, or in the mapping of naturally occurring single stranded DNAs.

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