Intermediates in plasmid pT181 DNA replication

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ABSTRACT

Staphylococcus aureus plasmid pT181 is thought to replicate via an asymmetric rolling-circle mechanism. By studying pulse labeled replicative intermediates, here we report that pT181 replication involves: (1) a post-replicative hypersupercoiled monomer and (2) a partially replicated intermediate which lacks superhelicity but is unlike a typical rolling-circle intermediate in that only nascent strands of less than unit length are released by alkali denaturation. A model for pT181 replication is proposed to accommodate this apparent discrepancy.

INTRODUCTION

A plasmid replication cycle involves initiation, elongation, termination and maturation. Replication mechanisms have been worked out by analysis of replicative intermediates (1-15), of which two types have been described for circular replicons: the theta type observed with double-stranded plasmids such as ColE1 (1-9) and the rolling circle type found with single-stranded coliphages such as phiX174 (1, 10-13). The theta type of intermediate is generated when leading strand synthesis is initiated de novo by 3' extension of an RNA primer, followed immediately by (primase catalyzed) lagging strand initiation. Both template strands remain covalently closed but as replication proceeds, there is progressive loss of supercoiling. The final pre-termination form is a relaxed catenated dimer with nascent strands of monomeric length (2). Rolling circle replication, on the other hand, is initiated by the introduction of a nick in the plus strand origin followed by 3' extension of the nicked strand. This process results in nascent strands of longer than unit length (10,13). Here the intermediates are not supercoiled and the replication is usually asymmetric, the lagging strand initiating

only after the leading strand is complete or nearly complete (16).

In this paper, we have used a pulse-labeling method to isolate and characterize replicative intermediates of the S. aureus plasmid, pT181. First, we have observed a hypersupercoiled species which is probably a post-replicative form. Second, we have isolated partially replicated molecules whose properties are somewhat paradoxical in that they lack superhelicity but contain nascent strands of less than unit length. These results are discussed in the light of recent observations suggesting that pT181 may replicate by an asymmetric rolling circle mechanism.

MATERIALS AND METHODS

Isolation and analysis of in vivo replicative intermediates. Bacteria were grown in CY broth (17) to exponential phase with vigorous aeration in a 32°C shaking water bath and were then switched to 43°C for 20 minutes. At the end of this incubation, cells were transferred back to 32°C, incubated for 9 minutes, labeled with ³H-thymidine between 9 and 10 minutes and then frozen in a dry ice bath after addition of 4 volumes of 1:1 acetone-alcohol. The labeled cells were then rehydrated, treated with lysostaphin (100 µg/ml), lysed with 1% Brij-deoxycholate and centrifuged at 19,000 rpm for 70 minutes in Sorvall RC-5B centrifuge using an SS-34 rotor. Supernatants were alcohol precipitated, resuspended in 10 mM Tris, pH 8; 1 mM EDTA (TE) buffer and analyzed by sedimentation in a) 5-20% neutral sucrose gradients (0.05 M Tris, pH 8; 0.05 M EDTA, pH 8; 0.1 M NaCl), b) 5-20% alkaline sucrose gradients (0.3 M NaOH; 0.001 M EDTA; 1 M NaCl) and c) CsCl-propidiumdiiodide (CsCl-PDI) density gradients (500 µg/ml propidiumdiiodide). Neutral sucrose gradients were run in SW50.1 swinging bucket rotors at 45,000 rpm for 3.75 hours at 18°C, and alkaline at 44,000 rpm for 2.75 hours at 5°C.

Isolation and analysis of in vitro replicative intermediates. Cell-free extracts prepared as described by Khan et. al. (18) were used for replication in vitro. In brief, reaction mixtures (50 µl) contained 40 mM Tris.HCl, pH8; 100 mM KCl; 12 mM magnesium acetate; 50 µM each of dGTP, dCTP, dATP and

^3H or alpha ^{32}P -dTTP; 2 mM ATP; 0.5 mM each of rCTP, rUTP, rGTP; 0.05 mM NAD; 0.05 mM cAMP; 0.5-1 μg template supercoiled plasmid DNA and 20 to 25 μl of the cell-extract (added last). Incubation was at 30°C and aliquots removed at various times were adjusted to 5% glycerol / 1% SDS / 1 mM EDTA / 0.02% bromophenol blue and subjected to electrophoresis on a 1% agarose gel using 89 mM Tris base/89 mM boric acid/0.25 mM EDTA (TBE) buffer, pH 8.3. Gels were run at 3.5 V/cm for 5 hours, stained with ethidium bromide, photographed and dried on Whatman number 1 filter paper at 37°C for 2 hours. Autoradiographs were prepared by exposure to Kodak RP-Royal X-omat X-ray films. For isolation of replicative intermediates, reactions were run for 10 minutes and were stopped by addition of SDS to a final concentration of 1%. These samples were subjected to two alcohol precipitations and were analyzed by sucrose and CsCl-PDI gradients as described above.

RESULTS

Isolation and characterization of in vivo replicative intermediates. The basic design for isolation of in vivo replicative intermediates was to use short pulses of labeled thymidine and identify by sedimentation analysis species that could be chased to supercoiled monomers. Initial experiments with wild type pT181 in exponential culture at 37°C , using 1 minute pulses, resulted in isolation of a chaseable species with the sedimentation properties of a nicked circular monomer. This was assumed to be the termination intermediate which would be predicted by either the theta or the rolling circle type of replication.

An attempt was next made to increase the proportion of replicative intermediates by pulse labeling during the burst of replication following temperature shiftdown of a plasmid mutant which is thermosensitive for replication (Tsr). Plasmid pSA0331, a Tsr copy mutant of pT181, has a copy number of about 250 per cell at 32°C and is unable to replicate at 43°C (19). A strain carrying pSA0331 (RN4416) was incubated at 43°C for 20 min and then shifted to 32°C . Following the shiftdown, there was a sharp burst of plasmid replication peaking between 5 and 10 minutes postshift (data not shown). Replicative intermediates were

labeled during this time by a 1 minute pulse of ^3H -thymidine and isolated by sedimentation of sheared crude lysates on neutral sucrose gradients. Two different species, (Fig.1A), which could be chased to supercoiled monomers (Fig.1B), were detected. One of these, species B, turned out to represent open circular monomers. The other, species A, appeared to represent hypersupercoiled monomer. This pulse labeled material, sedimenting ahead of supercoiled monomers (Fig. 1A), was collected and analyzed on CsCl-PDI and alkaline sucrose gradients and also on chloroquine-agarose gels. In CsCl-PDI gradients (Fig. 1C), it banded slightly lighter than the supercoiled marker. In a 5-20% alkaline sucrose gradient, species A cosedimented with the standard supercoiled marker (Fig. 1D). As treatment with pronase had no effect on its

Fig. 1. Sedimentation analysis of in vivo replicative forms of pSA0331 DNA. A. Cells carrying pSA0331 were grown in CY broth at 32°C to exponential phase and shifted to 43°C for 20 min. These were then transferred back to 32°C and pulse-labeled with ^3H -thymidine (100 $\mu\text{Ci}/\text{ml}$) from 9-10 minutes. Plasmid DNA was extracted and run on a 5-20% neutral sucrose gradient along with externally added ^{14}C -labeled supercoiled (21S) and relaxed (16S) forms of pSA0331 as sedimentation markers. The plot shows TCA-precipitable radioactivity of gradient fractions. B. Cells were labeled as described above, then chased for 15 min. with a 1000 fold excess of cold thymidine, and processed as above. C and D. Analysis of species A (see text), prepared without ^{14}C -labeled markers, on CsCl-PDI and alkaline sucrose gradients respectively. In all cases, ^{14}C -labeled supercoiled and relaxed/linear forms of pSA0331 were added as sedimentation markers. Fractions were collected and analyzed as before. Filled circles: ^3H ; unfilled circles: ^{14}C .

Fig. 2. Chloroquine-agarose gel analysis. A. DNA samples (species A) were subjected to electrophoresis through a vertical agarose gel (0.8%) in 50mM Tris-phosphate buffer, pH 7.2, containing 20 ug/ml chloroquine. The gel was run at 1-2 V/cm for approximately 20 h at room temperature. It was then stained with ethidium bromide (1 ug/ml), destained in distilled water and photographed with UV light using Polaroid type 665 positive/negative film. B. The negative was scanned by a Shimadzu model CS910 densitometer. Direction of migration is from top to bottom in panel A and from left to right in panel B. Both the arrow and the dashed line represent positions of relaxed circular plasmid DNAs. Track 1 represents native pSA0331 DNA and track 2, species A.

sedimentation pattern (data not shown), it appears that bound proteins are not responsible for its anomalous buoyant density in CsCl-PDI. Instead, as shown by chloroquine-agarose gel electrophoresis (Fig. 2), it appears that species A has a greater negative superhelical density than standard plasmid monomers. In

Fig. 2A is shown the photograph of such a gel and Fig. 2B a densitometric scan of the photographic negative. Lane 1 and the corresponding scan represent non-replicating supercoiled pSA0331 DNA whereas lane 2 and the corresponding scan represent species A. At the chloroquine concentration used (20 $\mu\text{g/ml}$), native supercoiled pSA0331 DNA showed a topoisomer distribution with a peak at the position of relaxed material (that is, with an average linking number of zero), whereas with species A, the peak was at a linking number of about -5. Higher concentration of chloroquine was found to produce higher degree of positive supercoiling in pSA0331 DNA (data not shown). As species A is labeled during a 1 minute pulse and can be chased to the standard monomeric form, it is clear that the hypersupercoiled form is involved in replication.

Replication in vivo inhibited by hydroxyphenylhydrazinouracil (HPU). As it seemed likely that the attempt to isolate partially replicated molecules of pSA0331 was unsuccessful because the polymerization time is likely to be very short for a 4 kb plasmid (~10 seconds), we utilized HPU to reduce the polymerization rate. HPU has been shown to inhibit DNA replication in Bacillus subtilis by forming a ternary complex with DNA polymerase III, the primer and the DNA template. This reaction is reversible but thermodynamically favored (20). HPU has also been shown to be effective as an antimicrobial agent for several gram positive bacteria but ineffective for E. coli and eukaryotic organisms (20). To determine if HPU inhibited DNA replication in S. aureus and, if it did, to determine an appropriate concentration of HPU that can facilitate obtaining partially replicated molecules, we analyzed the kinetics of incorporation of label into cellular DNA of S. aureus at different HPU concentrations. RN4416 was grown to mid-exponential phase at 32°C, shifted to 43°C for 20 minutes and then returned to 32°C. After 8 minutes at this temperature, samples were labeled in the presence of various concentrations of HPU. Cells were withdrawn at various intervals and their TCA precipitable counts were determined (data not shown). It was observed that HPU did inhibit cellular DNA replication in S. aureus. Furthermore, an HPU concentration of 15 μM and a pulse time of 50 seconds

appeared to yield partially replicated molecules and these conditions were used in the following experiments.

Pulse-chase experiments were done essentially as outlined above except that 8 minutes after the cells were downshifted to 32°C, HPU was added to a concentration of 15 μ M and 3 H-thymidine was added 1 minute later. Labelling was stopped after 50 seconds (pulse) and cells were processed as described above. In a parallel experiment, an excess of cold thymidine was added at the end of the labeling period and incubation continued for 15

Fig. 3. Sedimentation analysis of pSA0331 DNA pulse labeled in the presence of HPU. A and B. Cells carrying pSA0331 were grown in CY broth at 32°C to exponential phase and shifted to 43°C for 20 min. These were then transferred back to 32°C. HPU was added to a final concentration of 15 μM after 8 min at 32°C and ^3H -thymidine (100 $\mu\text{Ci}/\text{ml}$) 1 min later. Labelling was carried out for 50 sec and was then either stopped (Pulse; A) or continued for another 15 min in presence of 1000 fold excess cold thymidine and then stopped (Chase; B). Cells were processed and analyzed on 5-20% neutral sucrose gradients as described in the legend to Fig. 1. C and D. Fractions 13-17 from a neutral sucrose gradient of the ^3H -labeled pulse material as shown in panel A (run without ^{14}C -labeled markers) were pooled, alcohol precipitated and analyzed on CsCl-PDI (C) and alkaline sucrose gradients (D) as described in the legend to Fig. 1. Filled circles: ^3H ; unfilled circles: ^{14}C .

minutes (chase). Both the pulse and the chase DNAs were analyzed on 5-20% neutral sucrose gradients. In the pulse experiment (Fig. 3A), two rather broad peaks were obtained. One of them banded at approximately 21S with a faster moving shoulder that varied from experiment to experiment. Thus this material behaved similar to species A of Fig. 1A. The other broad peak, not observed previously, banded primarily at 16-18S and could be effectively chased (>70%; Fig. 3B). This material (corresponding to fractions 13-17 in Fig. 3A) was pooled, dialyzed against TE buffer, and then analyzed on CsCl-PDI and alkaline sucrose gradients. In CsCl-PDI gradients (Fig. 3C), there was one major peak with a density slightly greater than that of the nicked circular marker. This suggested that these molecules were either rolling circle forms with single-stranded tails or nearly complete theta intermediates with a minimal degree of residual superhelicity. There was also a minor peak between the supercoiled and the relaxed forms, which probably corresponds to mixed catenated dimers (21). In alkaline sucrose gradients (Fig. 3D), the majority of the ^3H -labeled material banded at a position corresponding to linear single stranded plasmid DNA of less than unit length with an average size of about 25% of the full-length strand. This material appears to represent nascent strands whose size would suggest *de novo* initiation. This is problematic in view of other evidence favoring the rolling circle mode and is discussed below.

Isolation and characterization of in vitro replicative intermediates. A cell free extract capable of supporting replication of pT181 (18) was used to prepare *in vitro* replicative intermediates. The reaction was carried out in presence of ^3H -dNTPs with pRN8008 (copy mutant of pT181; 18) supercoiled DNA. Samples were withdrawn at different time intervals and were subjected to 1% agarose gel electrophoresis and fluorographed (Fig. 4). The amount of labeled material in the supercoiled form was found to increase with time. An increase in the relaxed form was also observed. A prominent feature of the fluorograph was a broad band of material with mobility intermediate between those of the supercoiled and the nicked forms of the plasmid DNA. This material, which showed an increase

Fig. 4. Replication of pRN8008 DNA in vitro using cell free extracts. Incubation was carried out under standard assay conditions (Materials and Methods) in presence of ^3H -dTTP. Aliquots were withdrawn at different intervals of time, mixed with glycerol/SDS/bromophenol blue, run on 1% agarose gel and autoradiographed. Supercoiled and relaxed forms of the plasmid DNAs are shown by arrows. Lanes A-I represent time intervals : 0, 2, 5, 10, 15, 20, 30, 45 and 60 minutes respectively.

in labeling and a decrease in mobility with time up to about 45 minutes, is presumed to represent partially replicated molecules. Material from the 10 minute time point in such an experiment, stopped by the addition of SDS and EDTA to 1% and 1 mM respectively, was alcohol precipitated and run on 5-20% neutral sucrose gradients. As shown in Fig. 5A, a broad peak of ^3H -labeled material was obtained spanning the 21S and the 16S markers, and having a leading shoulder. In some experiments, two peaks were observed, similar to those shown in Fig. 3A. The material sedimenting between 16S and 21S was pooled, dialyzed against TE buffer, alcohol precipitated and analyzed on CsCl-PDI and alkaline sucrose gradients. The results of this analysis, shown in Fig. 5, were very similar to those obtained with the corresponding material prepared from HPU-inhibited whole cells (Fig. 3). In CsCl-PDI (Fig. 5B), the material was slightly more dense than the nicked forms and in alkaline sucrose (Fig. 5C), the label sedimented more slowly than unit length single

strands. Again, the average length of the nascent chains was about 25% of unit length.

DISCUSSION

Data presented here suggest that replication of pT181 DNA proceeds via a relaxed intermediate that contains shorter-than-unit-length nascent strands; this intermediate appears to give rise to hypersupercoiled and nicked circular forms in uncertain order, followed by processing to monomeric supercoiled molecules of standard superhelix density. Since the partially replicated molecules are devoid of superhelicity, it is likely that the nicked circular forms precede the hypersupercoiled intermediates. The maturation pathway would then involve ligase followed by gyrase activity. The hypersupercoiled species would then be due to gyrase "overshoot", presumably followed by a corrective relaxation. It has been shown that during the initiation of replication of oriC plasmids in vitro the supercoiled DNA template is converted at the prepriming stage to a form having higher negative supercoiling, which serves as the functional template for replication (22). In that case, it was presumed that the increased torsional strain facilitates initiation of replication. Whether the elevated torsional strain in the hypersupercoiled molecules of pT181 is utilized biologically is unknown.

The partially replicated pT181 molecules seem atypical of those observed either for intermediates of the theta type or of the rolling circle type. Theta replication typically involves

Fig. 5. Analysis of pRN8008 DNA labeled in vitro. A. pRN8008 DNA was labeled in vitro in the presence of ^3H -dTTP for 10 min under standard assay conditions (Materials and Methods). The reaction was stopped by addition of SDS to a final concentration of 1% and EDTA to 1 mM. The material was alcohol precipitated and run on a 5-20% neutral sucrose gradient. ^{14}C -labeled supercoiled (21S) and relaxed (16S) or linear forms of pRN8008 DNA were used as sedimentation markers and analysis was carried out as described in the legend to Fig. 1. B and C. Fractions 13-16 of a neutral sucrose gradient as shown in panel A (run without ^{14}C -markers) were pooled, alcohol precipitated and subjected to CsCl-PDI gradient (panel B) and 5-20% alkaline sucrose gradient (panel C) centrifugation along with ^{14}C -labeled markers. Fractions were collected and analyzed as described in the legend to Fig. 1. Filled circles: ^3H ; unfilled circles: ^{14}C .

Fig. 6. Model of pT181 replication cycle. Origin specific binding of the RepC protein to a supercoiled plasmid DNA (A) and subsequent nicking of the (+) strand initiate replication of the molecule (B). The protein remains bound to the 5'-end while the host replication machinery synthesizes nascent (+) strand from the 3'-end, causing the parent (+) strand to peel off (C). However, the displaced strand with bound RepC remains associated with the origin in this replicative intermediate (C) and upon completion of the nascent strand catalyzes a strand exchange that generates a relaxed covalently closed molecule (D) plus a single stranded circle (E) that may or may not have initiated lagging strand synthesis. Gyrase would act on the relaxed daughter molecules to produce a hypersupercoiled form (F) which, upon corrective relaxation, gives rise to a supercoiled molecule of standard superhelix density (A). In the presence of alkali, the bound RepC protein is activated to cleave the bond between the parent and the nascent DNA strands releasing a covalently closed (-) strand (G), a linear parent (+) strand (H) and a linear nascent strand (I).

intact parent strands so that there is a gradual reduction in overall superhelix density as polymerization proceeds. The pT181 intermediates showed no superhelicity, from which it would appear that at least one parent stand is nicked. A relaxed covalently closed circular parent molecule (template) cannot be ruled out but seems unlikely as negative superhelicity is most likely

required to initiate replication. On the other hand, rolling circle replication typically involves a relaxed intermediate with a nascent leading strand that grows from monomeric to dimeric length as polymerization proceeds (10,13). Here we found that the pT181 intermediates released nascent strands of less than unit length in the presence of alkali. Previously we and others (18) had found that the predominant product of the cell free in vitro replication system used here is the rifampicin-resistant leading plus strand indicating that the majority of the nascent chains obtained from in vitro pT181 intermediates in the alkaline gradients represents plus strands. Since both in vitro and in vivo replicative intermediates have similar sedimentation properties, we believe that most of the in vivo nascent chains also delineate plus strands.

Independently of these studies, it has recently been observed that RepC, the pT181 initiator protein (19,23), has sequence-specific nicking-closing activity at the pT181 origin, that the protein appears to remain bound to the displaced 5' end and that the initiation of replication in vitro appears to involve 3' extension of the nicked plus strand (24). Additionally, we have observed that pT181 replication is asymmetric, leading to the accumulation of single strand circular monomers specific for the displaced plus strand (25), and that RepC is directly involved in termination (S. Iordanescu, S. Projan and R. Novick, unpublished data). The only finding, therefore, that is not consistent with a rolling circle type of replication is that the nascent strands appear shorter than unit length. A possible explanation for this is as follows: we suggest that like phiX174 cisA protein (1,10,13), RepC is involved in a strand exchange reaction by which replication is terminated and continuity of the newly replicated and displaced template strands are restored. We suggest that unlike the situation with phiX174, the displaced 5' strand of pT181 may remain associated with the origin and that alkali treatment activates the bound RepC to sever the bond between the parent and the nascent DNA at that point (see Fig. 6), as is the case with protein-DNA relaxation complexes that have been observed for several plasmids (26-29).

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