Effects of the pSC101 partition (par) locus on \textit{in vivo} DNA supercoiling near the plasmid replication origin

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Received September 20, 1994, Revised and Accepted December 2, 1994

ABSTRACT

Previous work has shown that deletion of the partition (par) locus of plasmid pSC101 results in decreased overall superhelical density of plasmid DNA and concomitant inability of the plasmid to be stably inherited in populations of dividing cells. We report here that the biological effects of par correlate specifically with its ability to generate supercoils \textit{in vivo} near the origin of pSC101 DNA replication. Using OsO$_4$ reactivity of nucleotides adjoining 20 bp (G-C) tracts introduced into pSC101 DNA to measure local DNA supercoiling, we found that the wild type par locus generates supercoiling near the plasmid’s replication origin adequate to convert a (G-C) tract in the region to Z form DNA. A 4 bp deletion that decreases par function, but produces no change in the overall superhelicity of pSC101 DNA as determined by chloroquine/agarose gel analysis, nevertheless reduced (G-C) tract supercoiling sufficiently to eliminate OsO$_4$ reactivity. Mutation of the bacterial topA gene, which results in stabilized inheritance of par-deleted plasmids, restored supercoiling of (G-C) tracts in these plasmids and increased OsO$_4$ reactivity in par$^+$ replicons. Removal of par to a site more distant from the origin decreased supercoiling in a (G-C) tract adjacent to the origin and diminished par function. Collectively, these findings indicate that par activity is dependent on its ability to produce supercoiling at the replication origin rather than on the overall superhelical density of the plasmid DNA.

INTRODUCTION

Stabilization of plasmid inheritance by the partition (par) locus of plasmid pSC101 is mediated at least in part by the ability of par to generate negative supercoils in plasmid DNA (1). Deletion of par is associated with both plasmid instability and a decrease in overall DNA superhelicity, and host mutations that increase DNA superhelicity enable the partitioning of par-deleted pSC101 plasmids and a variety of other partition-defective extrachromosomal replicons (1-3). Conversely, mutations in \textit{E.coli} genes encoding DNA gyrase subunits reduce plasmid DNA superhelicity, and concurrently accentuate defects in par (1). While a strong DNA gyrase binding site in par (4) is congruent with the sequences that mediate plasmid stabilization (5), another DNA sequence that contains a comparably strong gyrase binding site (6) does not stabilize plasmid inheritance (7; Miller and Cohen, unpublished).

par locus partial deletions that decrease gyrase binding do not affect the overall superhelical density of the plasmid DNA as monitored by chloroquine/agarose gels (1,4). Moreover, despite the observed ability of superhelical density to affect plasmid stability, increased overall supercoiling is not invariably associated with stabilized inheritance; for example, active transcription, which leads to the transient build up of negative supercoils behind the transcription complex and a consequent increase in the overall superhelical density of the plasmid DNA, may or may not affect stability depending on the location and orientation of the transcriptional unit relative to the plasmid’s replication origin (8). This observation has suggested that local supercoiling may be more relevant to partitioning than overall superhelical density.

Domains of local supercoiling in plasmid DNA have been identified \textit{in vivo} by exploiting the observation that tracts of alternating (G-C) base pairs undergo a conformational change from right handed B form DNA to the left handed Z form when present in a region that contains a high concentration of negative supercoils (9-11). We used such (G-C) tracts to assess the effect of mutations in par, and of the location of par on the plasmid, on the supercoiling of a region of pSC101 DNA near the replication origin. Our results show that par in its native position induces supercoils locally in the origin region, and that mutations that reduce or eliminate par function dramatically affect the extent of such supercoiling. Additionally, displacement of par to a site distant from the origin reduces supercoiling of origin region (G-C) tracts and concurrently partially impairs par function. Collectively, these findings support the notion that stabilization of plasmid inheritance by the pSC101 par locus is dependent on the ability of par to increase the concentration of supercoils at the plasmid’s replication origin.

MATERIALS AND METHODS

Bacterial strains

All strains used are derivatives of \textit{E.coli} K-12: DPB635 (zch-2250::mini-kan) and DPB636 (zch-2250::mini-kan topA66)

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are also Cmp*. Cmp* refers to plasmids that were preferentially lost in the presence of pPM20. Par" plasmids were inoculated into liquid LB medium for 20 generations and scored for Ap' or Km'.

Plasmid constructions

The chimeric plasmids pDLC11, pDLC87 and pDLC92 used in this study contain the following relevant DNA fragments: the Ndel–PstI fragment of a pSC101 derivative containing an intact or mutated par locus, the pSC101 origin of replication, the repA gene, and the upstream portion of the bla gene. pDLC11 contains an wild type par locus from pZC20 (Par") (16); pDLC87 contains a par locus having a 4 bp deletion at HaeII of the third PR segment (5) from pZC119 (Cmp-') (7); and pDLC92 contains a par locus from pZC127 (Par") (7) having a deletion from EcoRI to HaeII. Each pSC101 derivative fragment was ligated to the Ndel–PstI fragment of pBR322 containing the ColE1 replication origin and the downstream portion of the bla gene. pDLC11 and pDLC87 contain a single copy of the 20 bp (G-C) sequence inserted into the AwaI site. pDLC92 contains a single copy of the 20 bp (G-C) sequence inserted into the EcoRI site. The (G-C) sequence was obtained by digesting an annealed double-stranded oligomer having the sequence 5'-CTATGCAGAATTCTGCGCCGCCGCGCAATTCCCTAGGG-3' (purchased from the PAN facility, Stanford University, Stanford, CA). A diagram of the chimeric plasmids is shown in Figure 2.

Plasmids pDLC18, pDLC93 and pDLC94 were constructed from the chimeric plasmids by deleting the ColE1 origin-containing PstI–AflII fragment of each plasmid and replacing it with the PstI–AflII fragment of the pSC101 derivative, pZC20. The resultant constructs contain only the pSC101 replicon. Plasmid pDLC103 contains the intact par locus on an EcoRI fragment [derived from pDLC11 and containing no (G-C) tract] introduced into the HaeII site downstream of repA by blunt end ligation. The entire par locus near the origin has been deleted from the EcoRI site to the AwaI site and the EcoRI site restored (Biek, D., unpublished). The (G-C) tract was introduced at the restored EcoRI site.

Assay of OsO4 reactivity

The OsO4 reaction (11), the sample preparation and primer extension assays (19) were performed as described for the higher copy number chimeric plasmids. For experiments involving the lower copy number pSC101-derived plasmids (10 copies per cell at division), the OsO4 reaction and sample preparation were increased in scale to obtain sufficient DNA for primer extension reactions. In this case, 30 ml of cells grown to OD600 = 0.8 were concentrated to 2 ml and treated as described. Alternatively, 200 ml of cells were concentrated to 13 ml and reacted with OsO4 as described, followed by an alkaline lysis procedure and CsCl gradient purification (20).

The primer (designated Ava3) used for the extension reaction has the sequence 5'-AAAAAGGATGTCGCACAACCGC-G-3' (purchased from Operon, Inc., Alameda, CA) and hybridizes to a region between the AwaI site and the pSC101 origin at bp 4956–4975 (21). The primer was 32P end-labelled using γ-ATP and T4 kinase 7-Deaza-dGTP (Boehringer Mannheim) was substituted for dGTP in the primer extension reactions. The bottom strand of the plasmid sequence was extended using this primer.
Gel electrophoresis was performed as described (11) except that 8% acrylamide/bis-acrylamide gels (Long Ranger, J. T. Baker, Phillipsburg, NJ) and 40% formamide (Ultrapure, J. T. Baker) were used. Fixed and dried gels were exposed to film (Amersham, Arlington Heights, IL) according to manufacturer's instructions.

RESULTS

Effects of par on supercoiling in the pSC101 origin region in vivo

To assess the ability of par in its native location to induce superhelicity in the origin region, a 20 bp tract containing alternating dG and dC nucleotides was inserted at the AvaI site between par and the origin of replication (Figs 1 and 2), creating EcoRI sites at the junctions of the insert and the plasmid. In regions of sufficiently high superhelicity, this (G-C) tract converts from B form to Z form DNA, distorting and dissociating A-T pairs at the interface between the Z form (G-Q tract and the adjacent B form plasmid sequences (9-11). This distortion can be detected by treatment of cells containing the construct with OSM4, which oxidizes thymidine residues at the dissociated base pairs; the resulting single strand breaks are seen as sites of chain termination during primer extension analysis (11). Thymidine residues located in EcoRI sites adjacent to (G-C) tracts that have a superhelical density too low to produce Z form DNA show no detectable OSM4 reactivity.

We analyzed supercoiling of (G-C) tracts in pSC101-derived replicons that have three different phenotypes related to par locus function (Table 1). pDLC 18 (par™) carries an intact par locus and consequently is retained by 100% of viable cells after 100 generations of growth in non-selective medium (13). pDLC93 lacks 4 bp of par sequence at the HaeII cleavage site (par11). This Cmp- plasmid (5) ordinarily is stably inherited but is lost preferentially in the presence of a pSC101 replicon that carries an intact par locus. pDLC92 contains an EcoRI-AvaI deletion (par(EA)) that removes the entire par locus and results in rapid plasmid loss in the absence of selection (5). Introduction of (G-C) tracts into the corresponding parental plasmids to generate these constructs had no detectable affect on the Cmp or Par phenotype (data not shown).
Table 1. Relevant features of plasmid constructs used in the analysis of supercoiling in vivo

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Replicon</th>
<th>Insertion site of (G-C) tract*</th>
<th>Status of par locusb</th>
<th>Phenotypec</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDLC18</td>
<td>pSC101</td>
<td>Aval</td>
<td>intact</td>
<td>Cmp*</td>
</tr>
<tr>
<td>pDLC93</td>
<td>pSC101</td>
<td>Aval</td>
<td>Δ4 bp HaeII</td>
<td>Cmp*</td>
</tr>
<tr>
<td>pDLC94</td>
<td>pSC101</td>
<td>Aval</td>
<td>ΔEcoRI-HaeII</td>
<td>Par</td>
</tr>
<tr>
<td>pDLC103</td>
<td>pSC101</td>
<td>HaeII</td>
<td>intact, distant from origin</td>
<td>Cmp*</td>
</tr>
<tr>
<td>pDLC11</td>
<td>ColEI/pSC101</td>
<td>Aval</td>
<td>Δ4 bp HaeII</td>
<td>Cmp*</td>
</tr>
<tr>
<td>pDLC97</td>
<td>ColEI/pSC101</td>
<td>Aval</td>
<td>ΔEcoRI-HaeII</td>
<td>Par</td>
</tr>
<tr>
<td>pDLC92</td>
<td>ColEI/pSC101</td>
<td>Aval</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a All (G-C) insertions are 20 bp tracts of alternating dG-dC, plus the flanking EcoRI sites and Aval sites where applicable.
b Deletions in par locus unless par is intact. For pDLC103, an intact par locus was inserted at the HaeII site downstream of repA. The orientation of the par locus, which functions in either orientation (13) is unknown.
c The Cmp phenotypes of pDLC18, pDLC93, pDLC94, pDLC95 and pDLC103 were determined experimentally in this study. The Cmp phenotype listed for chimeric plasmids refers to the phenotype of the parent pSC101 component (1,8).

While earlier work has shown that deletion of the entire par locus can decrease supercoiling of plasmid DNA as assayed by electrophoresis in chloroquine/agarose gels, small deletions that alter the DNA gyrase binding site within par and produce the Cmp- phenotype have no detectable effect on overall superhelical density (1,4). However, as seen in Figure 3, pDLC 93, which contains a 4 bp par locus deletion that yields the Cmp- phenotype (5) and affects other par-dependent functions such as the formation of the origin region protein/DNA complex (22) and pSC101-related incompatibility (23) showed reduced local supercoiling in a (G-C) tract adjacent to the pSC101 replication origin when assayed by the OsO₄/primer extension assay. While sites of potential reactivity of pSC101 DNA with OsO₄ are present at both junctions of the (G-C) tract, only one junction showed significant cleavage; analogous differential reactivity at junctions of (G-C) tracts has been observed previously on other plasmids (11).

Whereas Rhamouni and Wells observed that supercoiling of plasmid DNA induced by transcription from strong promoters can lead to OsO₄ reactivity at interfaces between (G-C) tracts and plasmid DNA (11), we were unable to detect effects of transcription on OsO₄ reactivity at the junctions of the 20 bp (G-C) tracts we inserted into pSC101 derivatives that were stabilized by transcription initiated near the origin region of the plasmid (data not shown). Our finding that transcription sufficient to stabilize plasmid inheritance (8) fails to produce detectable supercoiling in 20 bp (G-C) tracts is consistent with results showing that such transcriptionally stabilized plasmids cannot compete with Cmp+(pDLC18) plasmids, for which supercoiling was observed (Fig. 3).

Effects of par on supercoiling near the pSC101 replication origin are independent of origin function and are accentuated by topA mutation

We wished to know whether the observed par-induced effects on pSC101 DNA supercoiling in the vicinity of the pSC101 replication origin require functioning of this origin. Previous work has shown that the pSC101 replication origin is not used on chimeric plasmids comprised of pSC101 and ColEI when the ColEI origin, which maintains the plasmid at an elevated copy number, can function (24). As seen in Figure 4A, OsO₄/primer
extension analysis of (G-C) tracts introduced into chimeric replicons comprised of pSC101 and the ColEI-related plasmid pBR322 indicated that the topological effects of par on a (G-C) tract inserted near the pSC101 replication origin is independent of whether that origin is being used to initiate DNA synthesis. Additional bands representing DNA breaks at various other locations in the chimeric plasmids were observed in these experiments. The cause of these bands is not known; however, they cannot be due to OsO₄ reactivity as they were observed also in untreated controls (Fig. 4A).

Whereas supercoiling near the pSC101 origin of replication was not seen for par-mutated plasmids in wild type hosts, it was observed (Fig. 4B, lanes 5 and 6) in bacteria defective in topoisomerase I, which antagonizes the negative DNA supercoiling produced by DNA gyrase (25) and leads to stabilization of a variety of partition defective plasmid replicons (1). While OsO₄ reactivity at the (G-C) tract was observed in the topA66 mutant for plasmids that contain full or partial deletions of par, it was much greater in the construct containing only the 4 bp deletion. Additionally, the topA66 mutation dramatically increased OsO₄ reactivity at (G-C) tract junctions on plasmids containing an intact par locus, enabling the detection of cleavages at the junction furthest from par as well as at the par-proximate junction (pDLCl1, Fig. 4B, lanes 2 and 3). Based on the relationship between insert length and supercoil density elucidated by Zacharias et al. (10), we estimate the negative supercoil density at the midpoint of the B to Z transition for a 20 bp (G-C) tract to be 0.029. Thus the wild type pSC101 plasmid has a local superhelical density of at least $-\sigma = 0.029$ near the origin in a wild type E.coli host strain. This is an increase above the effective superhelix density of $-\sigma = 0.025$ for plasmids in E.coli (10). Increased OsO₄ reactivity of par-mutant plasmids in a topA66 strain indicates that this host mutation increases the percentage of mutant plasmids having a superhelical density of at least $-\sigma = 0.029$ near the origin.

The par locus generates negative supercoils in the origin region when present at an alternative location

Is the proximity of par to the pSC101 replication origin important to its ability to generate supercoils in the origin region? Earlier work has shown that displacement of par to a site distant from the origin does not eliminate its ability to stabilize inheritance of the plasmid (13; C. A. Miller, unpublished). We confirmed that pSC101 stability did not decrease when par was displaced to site ~2.2 kb from the origin in the counterclockwise direction and 1 kb from the origin in the clockwise direction (plasmid pDLCl03, Fig. 2). The retention of OsO₄-reactivity at the (G-C) tract located near the origin (Fig. 5) is consistent with the observed ability of par to act at a distance; however, the primer extension band representing OsO₄ cleavage at this site was only 15-20% of the intensity, by densitometry, of the band seen at the same position when par was present at its native location (Fig. 5).
propose that the stabilizing effects of par on the inheritance of pSC101 result from its ability to allow the multiple copies of the plasmid in the intracellular pool to be recognized as individual molecules (5). The findings reported here suggest that supercoiling induced by par at the pSC101 replication origin is at least in part responsible for the effects of par on plasmid stability. While the mechanism by which topological changes in plasmid DNA might affect the physical or functional separation of plasmids in the intracellular pool not known, our results support the view that the target of par-induced supercoiling is the origin region DNA/protein complex that has been implicated in both partitioning and replication of the plasmid (22,23,30,31).

Using distortion at the junctions of (G-C) tract insertions to assess local plasmid DNA supercoiling near the replication origin, we found that a 4 bp par locus deletion that does not reduce the overall supercoiling of pSC101 DNA, as determined by chloroquine/agarose gel analysis, nevertheless reduced supercoiling in the origin region to a level that did not convert the (G-C) tract to Z form DNA. Displacement of par to a site distant from the origin also reduced supercoiling in a (G-C) tract inserted adjacent to the origin region, while the supercoiling that did occur was sufficient to allow stable inheritance of the plasmid, par function was impaired, as indicated by the inability of construct pDLC103 to compete equally with a pSC101 replica containing par at its native location. As pDLC103 was preferentially retained in the presence of a Cmp- pSC101 plasmid, its phenotypic expression of the par-determined Cmp function is intermediate between Cmp+ and Cmp- plasmids, and therefore the plasmid was designated a Cmp+ replicon. The extent of origin region OsO4 reactivity observed for the pDLC103 plasmid was intermediate to Cmp+ and Cmp- replicons.

Origin region supercoiling induced by the intact par locus was increased greatly in a host mutated in the topA gene (Fig. 4B). In topA mutant bacteria the OsO4/primer extension assay we used also detected (G-C) tract supercoiling in par-defective plasmids defective in par, consistent with the known ability of topA mutations to stabilize the inheritance of even plasmids that lack the entire par locus (1). The extent of OsO4 reactivity was substantially greater for plasmids that carry a small par deletion than for a plasmid deleted for the entire locus.

ACKNOWLEDGEMENTS

These studies were supported by NIH grant GM26355 to SNC. DLC was supported by NIH Predoctoral Training Grant 2732 GM 07790. We thank Chris Miller and Hanne Ingmer for helpful discussions and for providing certain plasmids.

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