**cmp**, a **cis-Acting Plasmid Locus That Increases Interaction between Replication Origin and Initiator Protein**

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**pT181**, a 4.4-kilobase multicopy plasmid of *Staphylococcus aureus*, encodes a trans-acting initiator protein, RepC, which was rate limiting for replication. Deletions in a 500-base-pair region of the plasmid external to the minimal replicon decreased the ability of the plasmid to compete with a coexisting incompatible plasmid. These deletions, which define a region called **cmp** (for competition), appeared to affect the interaction of RepC and the plasmid origin of replication. However, in the homoplasmid state the deletions affected neither copy number nor plasmid stability. The Cmp phenotype is orientation independent, and cmp defects could not be complemented in trans.

Many plasmids encode specific initiator proteins that are active in trans and may be produced in rate-limiting quantities; in such cases, the replication rate is regulated at the level of initiator protein synthesis (2, 26; N. Manch-Citron, M. L. Gennaro, S. Majumder, and R. P. Novick, Plasmid, in press). Because these proteins are necessarily present at very low concentrations, the plasmids must be able to utilize them very efficiently. A particularly sensitive measure of the efficiency of initiator utilization is the ability of a plasmid to compete with a coexistent plasmid that requires the same initiator. We were interested in genetic defects that reduce the competitiveness of the plasmid but do not occur in the elements essential for replication. Analysis of such genotypes should provide information on parameters that govern protein-DNA interactions and reveal essential features of plasmid sequence organization.

**pT181** is the prototype of an extended family of *Staphylococcus aureus* plasmids that control replication indirectly by countertranscriptional regulation of the production of a trans-acting, rate-limiting initiator protein (21, 23). Known elements of the basic replicon (Fig. 1) include an initiator gene, **repC** (14); a unique replication origin, ori, located within **repC**; a copy control determinant, **cop**, constituted by two small RNA species transcribed from the 5′-untranslated **repC** leader region in the anti-sense direction (2, 16); and a site for lagging strand initiation, **pala** (A. Gruss and R. P. Novick, submitted for publication). There is probably also a partitioning, **par**, system (4) that is as yet unmapped.

The **cop** determinant expresses an incompatibility known as Inc3A toward cognate replicons by inhibiting replication; the origin expresses an incompatibility known as Inc3B through its ability to utilize (and titrate) **RepC** (2).

Deletions affecting the **par** determinant of pSC101 affect competitiveness (Cmp) (27), as do certain mutations affecting **pT181** replication control (21). Here we describe a **pT181** segment that affects competitiveness but does not affect copy control, partitioning, or any other known element of the plasmid replicon. The **pT181** **cmp** region is contained within a 500-base-pair (bp) sequence and is active only in **cis**, but in either orientation. Replication of plasmids with **cmp** deletions is severely impaired in the presence of **cmp** derivatives of **pT181**, but no defect in copy number or stability is detected when **cmp** plasmids are maintained in the homoplasmid state. It is suggested that **cmp** affects the efficiency of interaction between the initiator protein **RepC** and the replication origin.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The strains used in this study were all derivatives of *S. aureus* NTCC 8325 (18). Plasmids used in this study are listed in Table 1. **pT181**, **pE194**, and **pC194** are naturally occurring small multicopy plasmids from *S. aureus* (9, 10) which belong to different incompatibility groups and exhibit no homology in their replication regions. **pSA5000** is a derivative of **pT181** in which the **Tc** determinant has been replaced by the Cmr determinant of **pC221** (12). It has the same copy number and incompatibility properties as **pT181**. **pT181** has four **DdeI** sites; two of the fragments, **B** and **D**, extending from nucleotide 769 through nucleotide 1930, can be removed without affecting plasmid copy number or stability. Derivatives of **pT181** and its copy mutants lacking these two fragments, referred to as **DdeI-B** + **D** derivatives, were prepared by S. Projan in this laboratory. **pEM9800** and **pEM9762** are **pT181** **cop-615** derivatives with **Bal31**-induced deletions within the **DdeI-B** + **D** region (kindly provided by E. Murphy). **pRN5101** is a thermosensitive replication (Trs) mutant of **pE194** and **pRN6321**, a derivative lacking a nonessential **TaqI** fragment (nucleotides 3383 through 587); both plasmids were isolated by A. Gruss in this laboratory. The **pT181** origin-containing **TaqI** C fragment (nucleotides 3942 through 158) was cloned to each of these **pE194** derivatives at their unique **ClaI** site to generate **pRN6366** and **pRN6326**. These two plasmids have equivalent properties and were used interchangeably to permit visualization on gels, depending on the size of other plasmids present. Both are referred to as **pE:orT** in the text. **pRN8061** (**pT181** **cop-623**) is a high-copy-number derivative of **pT181** which was used for most of the competition experiments. Other **pT181** derivatives were used for comparative purposes, including **pT181**, **pT181** **cop-615**, **pT181** **cop-615** **cop-619**, and their **DdeI-B** + **D** derivatives. Numbers given in parentheses after restriction sites throughout refer to the nucleotide coordinate of the site in the published sequence of the intact plasmid (7, 8, 15).

**Growth conditions.** Liquid and solid media for *S. aureus*, phage-mediated transduction, and protoplast transformation...
were as described previously (2). Plasmid stability and segregational incompatibility were scored by growing homo- or heteroplasmid strains, respectively, in liquid cultures nonselectively. Samples were plated at appropriate time intervals, and colonies were scored by replica plating.

**Isolation and analysis of plasmid DNA.** Plasmid DNA was analyzed in sheared whole-cell mini-lysates of exponentially growing cultures in nonselective medium by 1% agarose gel electrophoresis. Copy numbers were determined by fluorometric densitometry on ethidium bromide-stained gels (22). Plasmid DNA was prepared by ethidium bromide-cesium chloride centrifugation, as described previously (2).

**Restriction nuclease mapping, cloning, and DNA sequencing.** Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and Boehringer GmbH (Mannheim, Federal Republic of Germany) and were used as recommended by the manufacturers. For molecular cloning, standard procedures were carried out as described previously (2). DNA sequence analysis was done by the method of Sanger et al. (24).

**Plasmid constructions.** Plasmid constructions involved restriction fragment deletion, cloning of purified fragments to unique sites in vector plasmids, insertions of EcoRI linker 5′-GGAATTCC-3′ (from Collaborative Research, Inc., Waltham, Mass.) and digestion with Bal 31 nuclease. Procedures were carried out as described by Maniatis et al. (17).

**RESULTS**

**The Cmp effect.** Deletion of the region we call *cmp* caused a reduction in copy number of derivatives of pT181 when present in cells also containing the parental pT181 plasmid (Fig. 2). Normally an intact pSA5000 *cop-404* plasmid (pRN8044) has a competitive advantage over the incompatible plasmid pT181 (Fig. 2, lane 3), as has been reported previously (21). Deletion of *DdeI* fragments B and D (positions 769 through 1930; Fig. 1) from pSA5000 *cop-404*,

![Diagram of pT181](http://jb.asm.org/)
producing pRN8207, dramatically reversed the relative intensities of the two plasmid bands (Fig. 2, lane 5), changing the ratio of copy numbers of the two plasmids by a factor of at least 25 (Table 2). In the absence of the competitor plasmid, the copy number of the deletion-bearing plasmid (pSA5000 cop-404 ΔDdel-B + D) was unchanged (Fig. 2, lane 4).

As would be expected on the basis of reduced copy numbers and incompatibility, plasmids with deletions in the cmp region were destabilized by a co-resident cmp+ plasmid. Less than 1% of heteroplasmid cells retained the cmp- plasmid after overnight growth of colonies on nonselective medium. In the homoplasmid state, however, the cmp- plasmids were not detectably less stable than their cmp+ parents.

The Cmp target. We suspected that the pT181 origin of replication was the target for the Cmp effect, so we tested the behavior of cmp- plasmids coresident with deletion derivatives of a pE194::pT181 cointegrate, pRN6019 (2). The Cmp effect was absent when the cointegrate lacked the pT181 origin and was present when the cointegrate maintained that region (data not shown). To extend this finding, we tested the cloned replication origin of pT181 against cmp- and cmp+ pT181 plasmids. The pT181 ori was cloned to pRN6231, a TsR derivative of the unrelated plasmid pE194. The resulting pE::oriT plasmid pRN6326 was then transferred by transformation to cells containing the copy mutant pT181 cop-623 or its cmp- derivative pRN8246, and relative copy numbers were measured. Very little, if any, increase of the copy number of the pE::oriT plasmid at 32°C (Fig. 3, lane 4) or complementation at 43°C (Fig. 3, lane 5) was observed in the presence of an intact cmp+ pT181 copy mutant. In contrast, a striking change in copy numbers occurred in cells containing the pE::oriT clone and the cmp- plasmid pRN8264 (Fig. 3, lanes 7 and 8), such that the relative copy number of the cmp- derivative was much lower than that of the cmp+ plasmid in the homoplasmid state. Similar results were obtained when Ddel-B + D deletion derivatives of the pT181 wild type and three other copy mutants were tested in the presence of the same pE::oriT plasmid (data not shown). Depending on the copy mutation of the cmp- plasmid, the pE::oriT plasmid was amplified to a greater or lesser extent. The copy number of the cmp- plasmid, however, was always greatly decreased.

Fine-structure analysis of the pT181 replication origin as the target of Cmp was carried out by introducing deletions into the pT181 ori region of the TsR pE::oriT plasmid pRN6366 (pRN5101::pT181 TaqI-C). Our goal was to determine whether origin function (assessed by the ability of the

### Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (kilobases)</th>
<th>Description</th>
<th>Copy no.*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT181</td>
<td>4.4</td>
<td>Tc' Inc3</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>pSA5000</td>
<td>4.0</td>
<td>Cm' Inc3</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>pRN8044</td>
<td>4.0</td>
<td>pSA5000 cop-404</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>pRN8207</td>
<td>2.8</td>
<td>pSA5000 cop-404 ΔDdel-B + D</td>
<td>120</td>
<td>S. Projan</td>
</tr>
<tr>
<td>pC194</td>
<td>2.9</td>
<td>Cm' Inc8</td>
<td>40</td>
<td>9</td>
</tr>
<tr>
<td>pE194</td>
<td>3.7</td>
<td>Em' Inc11</td>
<td>55</td>
<td>9</td>
</tr>
<tr>
<td>pRN5101</td>
<td>3.7</td>
<td>pE194 TsR Em' Inc11</td>
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<td></td>
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<td>pRN6321</td>
<td>2.8</td>
<td>pRN5101 ΔTaqI-C'</td>
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<td>19</td>
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<td>pT181::pE194 XbaI clone Em' Tc'</td>
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<td>pRN6326</td>
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<td>pRN6321::pT181 TaqI-C'</td>
<td>Tsr</td>
<td>A. Gruss</td>
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<tr>
<td>pRN6366</td>
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<td>Tsr</td>
<td>This study</td>
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<tr>
<td>pRN6379</td>
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<td>pRN6366 Δ63-78'</td>
<td>Tsr</td>
<td>This study</td>
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<tr>
<td>pRN6380</td>
<td>4.3</td>
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<td>This study</td>
</tr>
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<td>pRN6411</td>
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<td>pRN6366 Δ4409-43'</td>
<td>Tsr</td>
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<td>pT181 cop-623</td>
<td>300</td>
<td>2</td>
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<td>pRN8264</td>
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<td>pT181 cop-623 ΔDdel-B + D</td>
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<td>This study</td>
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<td>300</td>
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<td>pEM9701 Δ393'</td>
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<td>E. Murphy</td>
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<td>130</td>
<td>E. Murphy</td>
</tr>
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<td>pRN6378</td>
<td>5.0</td>
<td>pC194::pT181 Mbol-A</td>
<td>40</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Copies per cell.

† TsR. Temperature sensitive for replication: 55 copies per cell at 32°C; approximately 3 copies per cell at 43°C.

‡ There are two different TaqI C fragments referred to throughout this paper: pE194 TaqI-C is a dispensable fragment which is deleted in pRN6321; pT181 TaqI-C contains the plasmid replication origin.

§ Figures indicate deletion endpoints in the pT181 sequence. pEM9800 and pEM9762 conserve 49 and 94 bp of the original 171-bp insert, respectively.

EcoRI linker octanucleotide.

### Table 2. Plasmid copy numbers

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Copy no. in heteroplasmid state</th>
<th>Total copy pool*</th>
<th>Homoplasmid copy no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT181</td>
<td>pSA5000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRN8044</td>
<td>2</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>pT181 and pRN8044</td>
<td>3</td>
<td>17</td>
<td>115</td>
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<tr>
<td>pRN8207</td>
<td>4</td>
<td>20</td>
<td>&lt;5</td>
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<tr>
<td>pRN8207</td>
<td>5</td>
<td>20</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* From Fig. 1.

† Total plasmid copies in the heteroplasmid state.
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pT181 ori to drive replication of the recombinant plasmid at temperatures nonpermissive for the vector plasmid) and the Cmp effect were parallel in the various deletion derivatives tested. Removal of the 16-bp HpaII fragment (positions 62 through 78) internal to the origin (plasmid pRN6379) abolished origin activity; Bal 31-generated deletions starting at the PvuI (nucleotide 1) site and extending to nucleotide 39 (plasmid pRN6380) or to nucleotide 43 (plasmid pRN6411) impaired, but did not abolish, origin activity in vivo. These deletions affected the Cmp phenomenon in parallel with their effect on origin function (Fig. 4). Thus we conclude that reduction in activity of the cloned origin is paralleled by a reduction in the ability to compete with a coresident cmp- plasmid.

The cmp region. Various sequence rearrangements affecting the cmp region of pRN8061 (pT181 cop-623) were tested for Cmp activity (Fig. 5). Deletion of the HindIII C fragment (nucleotides 885 to 1444) resulted in the Cmp- phenotype (Fig. 5, lanes 7 to 8), whereas inversion of this fragment was without effect (Fig. 5, lanes 10 to 11). Insertion of a synthetic octamer (EcoRI linker) into either the HindIII (nucleotide 885) site or the FnuDII (nucleotide 1687) site had no effect (Fig. 5, lanes 16 to 17 and 19 to 20, respectively), whereas insertion into the Rsal (nucleotide 1236) site produced a partial Cmp- phenotype (Fig. 5, lanes 13 to 14); the copy number of the pE::oriT plasmid increased fourfold and that of the pT181 replicon decreased by a factor of two. Deletion by Bal 31 of sequences between nucleotides 1687 and 1181 gave the Cmp+ phenotype, whereas deletion of nucleotides 1687 to 1393 was without effect (data not shown). These results (Fig. 1) suggest that cmp is located entirely within the HindIII C fragment (nucleotides 885 to 1444) and includes the Rsal site at position 1236. This was confirmed by the observation that cloning back the HindIII C fragment to the cmp- plasmid pRN8264, deleted for the larger Ddel-B + D region, restored the Cmp+ phenotype (data not shown). As pointed out above, none of these manipulations had any detectable effect on the copy number of pT181 cop-623 in the homoplasmid state.

There is a large open reading frame in the cmp region that encodes a site-specific recombinase, the pre gene product (M. L. Gennaro and R. P. Novick, submitted for publication). Pre mediates formation of cointegrates between nonhomologous plasmids carrying the recombination site RS_A (Fig. 1). Deletions affecting the pre gene alter neither plasmid copy number nor stability. The pre gene product is almost certainly not involved in the Cmp phenomenon; neither linker insertion at FnuDII (nucleotide 1687) or HindIII (nucleotide 885) nor inversion of HindIII-C (nucleotides 885 through 1444) had any effect on the Cmp phenotype, though all of these changes destroyed the pre reading frame. Although we did not generate cmp- derivatives retaining an intact pre gene, we showed that the cloned pre product has no effect on the Cmp phenomenon in trans.

FIG. 2. The Cmp effect. Whole-cell sheared mini-lysates of S. aureus strains containing the indicated plasmids were separated on 1% agarose in Tris borate buffer for 18 h at 2.5 V/cm, stained with ethidium bromide, and photographed. Samples corresponding to equivalent numbers of starting cells (approximately 10⁷ cells per sample) were used to permit comparison of copy numbers as calculated from these gels. Lane 1, pT181; lane 2, pRN8044 (pSA5000 cop-404); lane 3, pT181 and pRN8044; lane 4, pRN8207 (pSA5000 cop-404 ΔDdel-B + D); lane 5, pT181 and pRN8207. The heavy upper band corresponds to sheared chromosomal DNA. The supercoiled plasmid bands are indicated by arrows. Intermediate plasmid bands correspond to topoisomerases and multimers.

FIG. 3. The Cmp effect with the cloned pT181 ori gene. pE::oriT plasmid pRN6326 at 32°C (lane 1) and 43°C (lane 2); pRN8061 (pT181 cop-623) (lane 3); plasmids pRN6326 and pRN8061 at 32°C (lane 4) and 43°C (lane 5); pRN8264 (pT181 cop-623 ΔDdel-B + D) (lane 6); and plasmids pRN6326 and pRN8264 at 32°C (lane 7) and 43°C (lane 8) are shown.
Here, pT181 MboI-A (nucleotides 376 through 2496) was cloned to pC194, and the derivative plasmid pRN6378 was shown to express the Pre function in trans (Gennaro and Novick, submitted for publication), but to have no effect on the competition between a coresident cmp⁻ cmp⁻ pair of plasmids (A. Gruss and R. P. Novick, unpublished data). Additionally, the cloned cmp region did not express incompatibility toward cmp⁺ or cmp⁻ plasmids (data not shown).

**DISCUSSION**

We identified a plasmid function, cmp, that affects the ability of pT181 to coexist with cognate replications but has no effect on the copy number of the plasmid by itself. cmp is located within a 500-bp region, about 500 bp away from initiator protein, origin, and replication control determinants. Because the pT181 origin of replication is both necessary and sufficient for a cmp⁻ plasmid to outcompete a cmp⁻ derivative which also carries the pT181 origin, we hypothesize that the pT181 ori on a cmp⁻ plasmid competes more effectively for the rate-limiting initiator protein RepC than the same origin on a cmp⁻ plasmid. During the course of these studies a similar observation was made by Iordanescu (11).

The cloned pT181 ori gene expresses incompatibility (referred to as Inc3B) toward the pT181 wild type and certain mutants (2), which has been attributed to RepC titration. High-copy-number mutants, such as cop-623 (300 copies per cell), are completely indifferent to Inc3B incompatibility at the copy level of the ori-carrying plasmid pE194 (55 copies per cell) (S. K. Highlander, Ph.D. thesis, New York University, New York, 1985). Deletion of the cmp locus caused all the copy mutants tested to become sensitive to Inc3B (i.e., to be lost at a high rate from cells containing a coresident cloned pT181 ori), regardless of their copy number. Furthermore, mutations in the origin of replication diminished origin

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**FIG. 4.** Analysis of the cmp target. Interaction between deletion derivatives of pE::oriT plasmid pRN6366 and Cmp⁺ plasmid pRN8156 (pT181 cop-615 cop-619 ΔDdel-B + D). Plasmid pRN6366 at 32°C (lane 1) and 43°C (lane 2); pRN8156 (lane 3); plasmids pRN6366 and pRN8156 at 32°C (lane 4) and 43°C (lane 5); plasmids pRN6379 (Δ nucleotides 63 to 78) and pRN8156 at 32°C (lane 6) and 43°C (lane 7); plasmids pRN6380 (Δ nucleotides 4394 to 39) and pRN8156 at 32°C (lane 8) and 43°C (lane 9); and plasmids pRN6411 (Δ nucleotides 4409 to 43) and pRN8156 at 32°C (lane 10) and 43°C (lane 11) are shown.

**FIG. 5.** Analysis of the cmp region. pE::oriT plasmid pRN6326 at 32°C (lane 1) and 43°C (lane 2); pRN8061 (pT181 cop-623) (lane 3); plasmids pRN6326 and pRN8061 at 32°C (lane 4) and 43°C (lane 5); pRN8251 (pT181 cop-623 ΔHindIII-C) (lane 6); plasmids pRN6326 and pRN8251 at 32°C (lane 7) and 43°C (lane 8); pRN8252 (pT181 cop-623 inverted HindIII-C) (lane 9); plasmids pRN6326 and pRN8252 32°C (lane 10) and 43°C (lane 11); pRN8249 (pT181 cop-623 EcoRI-linker at Rsal-1236) (lane 12); plasmids pRN6326 and pRN8249 at 32°C (lane 13) and 43°C (lane 14); pRN8250 (pT181 cop-623 EcoRI-linker at HindIII-885) (lane 15); plasmids pRN6326 and pRN8250 at 32°C (lane 16) and 43°C (lane 17); pRN8232 (pT181 cop-623 EcoRI-linker at FnuDII-1687) (lane 18); and plasmids pRN6326 and pRN8232 at 32°C (lane 19) and 43°C (lane 20) are shown.
function and Cmp effect in parallel, supporting the hypothesis that cmp affects RepC-origin interaction.

The Cmp effect has been seen with at least three other S. aureus plasmids. A pT181-related plasmid, pC221 (23), also shows the Cmp phenomenon with its own cloned ori, as does an unrelated plasmid pair, pUB110 and pBC16 (J. Polak and R. Novick, unpublished data). Moreover, the observation made throughout this study that the pT181 ori, when cloned to temperature-sensitive derivatives of the unrelated plasmid PE194, is complemented at high temperature by a cmp " but not by a cmp" plasmid suggests that PE194 has a sequence element analogous to cmp. There may be a hierarchy of Cmp strengths with pT181 cmp" > PE194 > pT181 cmp", such that the stronger replicon outcompetes the weaker replicon for available repC gene product. Analysis of these cmp elements will allow us to determine whether there is any homology between them and whether the cmp region has replicon specificity.

The competition effect observed with certain deletions affecting the partitioning (par) determinant in pSC101 (27) has been attributed to partial inactivation of the par function. Stable plasmid maintenance, however, may be determined by factors other than the par function, namely, multimer resolution and copy number variance in a cell population (D. K. Summers and D. J. Sherratt, Proceedings of the Conference on Plasmids in Bacteria, Urbana, Ill., 1985, p. 909). Failure to correct fluctuations in copy number efficiency is predicted to broaden the frequency distribution of plasmid copies and thus increase the probability of plasmid loss (R. Novick, submitted for publication). We propose that the pSC101 cmp element, like that of pT181, is required for efficient utilization of the rate-limiting plasmid Rep protein; by this means, the cmp element could facilitate correction of copy number fluctuations and supplement the par system to ensure stable maintenance.

We demonstrated that the pT181 cmp element is equally effective in either orientation and is cis acting; these properties are reminiscent of enhancers, cis-specific DNA elements that modify DNA-protein interactions at sites removed from the modifying element itself. Such elements have been identified for each of the basic activities of DNA replication, including homologous (25) and site-specific recombination (3), transcription (1, 6), and replication. The replication enhancer of M13 (13) is contiguous with the primary initiator binding site of the plus strand origin and may be merely a part of that site. In the case of polyomavirus it has been shown that the transcriptional enhancer is required for virus replication (5). Because Cmp plasmids do not show any detectable replication defect, it is still unclear whether the cmp element can properly be considered an enhancer. Examination of the sequence of the cmp region did not reveal structural or sequence homology with any known genetic function other replicons. However, we have recently observed that cmp derivatives of pT181 have a lower superhelical density than their cmp" progenitor. The role of cmp as a determinant of DNA structure is currently under investigation.

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LITERATURE CITED


