Inverted Repeats in the DNA of Plasmid pCU1

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Renaturable regions in the DNA strands of the N group plasmid pCU1 have been visualized as stem-loop structures by electron microscopy. Four such distinct structures are described, the smallest of which is within the loop of a larger one. The region of pCU1 in which these structures occur has several restriction sites. This and the availability of plasmid deletions and recombinants has permitted the mapping of these structures relative to one another and to the restriction and functional map of the plasmid. The replication and maintenance region of the plasmid is located within one of these stem-loop structures.

The bacterial plasmid pCU1 is a self-transmissible plasmid of the conjugative and incompatibility group N, a group of plasmids that has a relatively wide host range (1, 5, 15, 18). It is one of three members of this group that are being studied currently, the others being R46 (4) or its derivative, pKM101 (24, 25), and N3 (2). The origin of pCU1 has been described (20, 21), and a restriction map of the plasmid has been constructed, a map in which the various functions determined by this plasmid have been located (22). In addition to conferring phenotypes characteristic of plasmids of the N incompatibility and conjugative group (12, 19), the plasmid also confers resistance to the antibiotics ampicillin, streptomycin and spectinomycin (Ap′ Sm′ Sp′). This paper is concerned primarily with electron microscopic observations of pCU1 DNA after denaturation and renaturation (9, 28). The study was prompted by the observation that pCU1 gives rise to Sm′ Sp′ derivatives, but not Ap′ derivatives, spontaneously at a frequency of about 0.1% and that these derivatives are not revertible. Subsequently, some of them were shown to be deletions, and the gene coding for Ap′ was inferred to be close to the region concerned with the stable maintenance of the plasmid (22). Four distinct renaturable stem-loop structures have been found on pCU1. Their dimensions and positions with respect to one another and the restriction and functional map of the plasmid are described and discussed. Of particular interest is the assignment of the maintenance region of the plasmid to a location within one of these stem-loop structures and away from a neighboring region specifying Ap′. (Parts of this work have been presented by M.K.-K. as a Ph.D. thesis at Carleton University.)

MATERIALS AND METHODS

Bacterial plasmids. The bacterial plasmids studied are listed with their relevant properties in Table 1. These plasmids were maintained, tested, and isolated from Escherichia coli K-12 strain C600T (3).

Media and chemicals. Antibiotic medium 3 (Pensay broth; Difco Laboratories) was used as the standard organic medium to which antibiotics were added as required. For solid media, agar at 1.5% (wt/vol) was added to this medium before sterilization. Antibiotics were added after the rest of the media were sterilized and in amounts to give the following final concentrations (μg/ml): ampicillin, 30; streptomycin, 50; spectinomycin, 50. TE buffer was composed of 0.01 M Tris-hydrochloride and 0.001 M EDTA, pH 7.2.

Isolation of plasmid DNA. Plasmid DNA was isolated as described previously (22).

Restriction endonuclease digestion and electrophoresis of plasmid DNA. All restriction endonucleases and T4 DNA ligase used were purchased from New England Biolabs and used as recommended by the manufacturer, plasmid DNA preparations being digested to completion. Vertical electrophoresis of the digested DNA was performed by the procedure described by Tanaka and Weisblum (29).

Construction of recombinants. The vector DNA and the DNA to be cloned were separately digested to completion with the appropriate restriction enzymes. The reaction was stopped by exposure at 65°C for 10 min. The digested samples were mixed, and the DNA was precipitated by the addition of 2 volumes of ethanol and freezing at −80°C for 2 h. The DNA precipitate was pelleted by centrifugation, vacuum dried, and taken up in 50 μl of ligation buffer (50 mM Tris-hydrochloride [pH 7.8], 10 mM MgCl2, 20 mM dithiothreitol, 1.0 mM ATP, 50 μg of bovine serum albumin per ml, and 4 U of T4 DNA ligase per μg of DNA). The ligation reaction was for 16 to 18 h at 16°C, after which the mixture was used for transforming strain C600T.

Isolation of in vitro deletion and fusion derivatives of the plasmid. Deletion of plasmid DNA was obtained by two methods. In one, purified DNA of pCU1 was
treated with XmaI and ligated with T4 DNA ligase, and the preparation was then used to transform strain C600T by the transformation procedure of Cohen et al. (8). In a second procedure, used with pCU1 or one of its derivatives, the ligation step was omitted. In both cases, an antibiotic resistance marker (Ap' or Sp') on the plasmid was used for the selection of transformants. The transformant was purified, and plasmid DNA was extracted from it and examined by agarose gel electrophoresis after digestion with appropriate enzymes to determine which fragment(s) was deleted. The plasmid deletions obtained by these procedures are listed in Table 1 along with a spontaneously arising deletion that had lost Sm' and Sp' (pCU8). Plasmid pCU26 (Table 1) is a recombinant plasmid which was obtained after the fusion of pCU16 and pACYC184 at their single BamHI cleavage sites. BamHI is known to inactivate tetracycline resistance (Tc') in plasmid pACYC184 (6). Screening for chloramphenicol-resistant (Cm') Tc' derivatives allowed the selection of fusion derivatives. Restriction analysis of plasmid DNA from such a derivative confirmed that it was the expected fusion derivative.

**Electron microscopy of plasmid DNA.** Samples of plasmid DNA isolated from various strains were prepared for electron microscopy as described earlier (11, 13, 21). Plasmid pML2 DNA (8.8 × 10^6 daltons; 17-M. Fiandt, personal communication) was included in the preparations and served as an internal length standard.

To detect and visualize inverted-repeat loop structures, a sample containing plasmid DNA in a solution of 50% formamide in 0.1 M NaCl-0.008 M Na₂EDTA-0.1 M NaHCO₃ (pH 8.0) was heated to 80°C for 2 min. It was then allowed to reanneal for 2.5 h at room temperature. The plasmid DNA was then spread for electron microscopy. To map the location of the inverted-repeat loop structures, deletion derivatives of plasmid pCU1 containing single sites for different restriction endonucleases (BamHI, HindIII, SalI, Smal) were used. Circular molecules were linearized with a particular endonuclease and then were prepared for electron microscopy as described above. The location of the inverted-repeat loop structures was determined with reference to the ends of the linearized molecules and the restriction map that has been described (22) and which is extended in this study.

**RESULTS**

**Four stem-loop structures in pCU1 DNA.** Plasmid pCU1 DNA was heat denatured, allowed to renature as described above, and examined by electron microscope. Four distinguishable types of stem-loop structures were observed. Rarely could all four structures be visualized on the same molecule, but an example of this is shown in Fig. 1. Figure 2 is an electron micrograph showing two such stem-loop structures, the smaller of the two (structure B) being within the loop of the larger one (structure A). In Fig. 2 (inset), the B stem-loop structure is not formed, and this then gives rise to a structure composed of the stem of A and a loop the length of which is the sum of the loop of A and the total length of the B stem and loop. Two other stem-loop structures, C and D, were also observed. The dimensions of these four structures are given in Table 2. Since the morphology of each stem-loop was fixed, the inverted-repeat sequences that renature must have a constant position on the plasmid DNA. As will be shown, the measurement of distances between the four structures also supported this conclusion.

**Stem-loop structures in pCU8.** Plasmid pCU8 is a spontaneous mutant that had irreversibly lost its Sm' and Sp' markers. Contour length measurements on its double-stranded DNA indicated that it had suffered a 3.0-kilobase (kb) deletion (22). When examined for stem-loop structures, it was observed to have A, B, and D but not C (2.6 kb). These two markers must therefore be either contained within C or in its vicinity. This conclusion will be discussed further.

**Relative position of the inverted-repeat loop structures on the restriction and functional map.** A restriction map of plasmid pCU1 and deletions spanning different regions of the plasmid has been available (22; Thatte, unpublished observations; see Fig. 5). To determine the position of the inverted-repeat loop structures, three artificially constructed deletion mutants of the plas-
mid, each with single cleavage sites for the enzymes BamHI and HindIII, SalI, or Smal, were used. These three mutants are called plasmids pCU16, pCU13, and pCU10, respectively. Their properties and the regions deleted in them are shown in Table 1; see also Fig. 4. Circular molecules of pCU16 and pCU13 were cleaved with BamHI, HindIII, or SalI and were denatured and renatured as described above. Upon visualization of the resulting linear molecules, each stem-loop structure could be mapped with reference to the enzyme-generated ends. Electron micrographs of the A + B structures in such molecules are shown in Fig. 3. The plasmid pCU13 DNA showed the A + B structure 5.3 kb away from the single SalI site (Fig. 3a) as determined by measurements on 42 different molecules. Figure 3b and c show the results of similar procedures for the enzymes HindIII and BamHI (see also figure legend). The precise position of stem-loop B within the loop of A was determined by analyzing single-stranded molecules of plasmid pCU10 digested with Smal, which cleaves within the loop A. The location of structure B in relation to the Smal-generated end and also to the stem of structure A is shown in Fig. 3d. Analogous procedures were used for positioning the structures C and D, the intervening distances between pairs of these structures being measured. These results (not shown as electron micrographs) are summarized diagrammatically in Fig. 4.

**Location of Ap' gene in relation to the A + B structure.** It has been shown previously that the gene expressing the Ap' phenotype is located in the vicinity of the region controlling plasmid pCU1 stable maintenance (22). This region has BamHI site 1 (Fig. 5). To determine the precise location of the Ap' gene, pCU1 DNA was digested with BamHI to give a large fragment (clockwise from kilobase coordinate 26.9 to coordinate 21.0) and a small fragment (from 21.0 to 26.9; see Fig. 5). The preparation of linear molecules was then used to transform strain C600T selecting for Ap'. In repeated experiments, no Ap' transformants were detected. This suggests that either BamHI site 1 was within the Ap' region or else it was so close to such a site that even small deletions generated from this site in vivo affected the region. To distinguish between these two possibilities, we used the plasmid pCU16, a derivative of pCU1 in which the region between HindIII sites 1 and 2 is deleted (Fig. 4). This deleted region includes BamHI site 2, so that

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**FIG. 1.** Electron micrograph of a single-stranded molecule of plasmid pCU1 in which all four stem-loop structures (A, B, C, and D) were formed. Bar, 0.5 μm.
FIG. 2. Electron micrograph showing single-stranded molecule of plasmid pCU1 with two stem-loop structures A and B, the stem-loop B being within the loop of A. Inset, Molecule in which loop B is not formed. Bar, 0.5 μm.

pCU16 has only the BamHI site 1 near the Ap' locus. The plasmid pACYC184 also has only one BamHI site, which is located within its Tc' gene. Fusions of plasmids pACYC184 and pCU16 at their respective BamHI sites gave rise to pCU26 (Table 1). Since pCU26 is also Ap', it could be concluded that the BamHI site is not within the Ap' region. It has been shown previously (22) that deletion of the region from EcoRI site 1 to EcoRI site 2 results in the loss of the Sm' and Sp' phenotypes but not the Ap' phenotype. This placed Ap' in the region between EcoRI site 1 and BamHI site 1. The region from BgIII site 5 to BgIII site 6 could be cloned into the BamHI site of plasmid pACYC184 since these two enzymes generate homologous 5' extensions. Although the resulting recombinant (plasmid pCU54) could not be used for the recovery of the two components, its size and the phenotype it conferred (Ap' Sm' Sp' Cm' Tc') were consistent with its being a recombinant of plasmid pACYC184 and the BgIII site 5 to BgIII site 6 fragment of pCU1. Thus, all of the antibiotic resistance markers of pCU1, including that of Ap', are contained within this fragment, the Ap' locus being in the interval between the EcoRI site and BgIII site 5. This interval contains two sites for the enzyme PstI (Fig. 5). Deletion of the region between these two sites gave rise to plasmid pCU19, a derivative which was Ap'. These experiments have thus narrowed the Ap' locus to a region between PstI site 1 and BglII site 5, 1.0 kb to the left (or clockwise) of the replication and maintenance region as shown in Fig. 4. One of the nine cleavage sites for HpaI is located between BglII site 5 and PstI site 1.

Position of the inverted-repeat loop structures in relation to functional regions on the plasmid. We had previously assigned the vegetative repli-

### Table 2: Different types of inverted repeat-loop structures observed in single-stranded DNA molecules of plasmid pCU1

<table>
<thead>
<tr>
<th>Inverted-repeat loop structure</th>
<th>No. of molecules measured</th>
<th>Size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A + B</td>
<td>52</td>
<td>300 ± 45</td>
</tr>
<tr>
<td>B</td>
<td>35</td>
<td>135 ± 15</td>
</tr>
<tr>
<td>C</td>
<td>17</td>
<td>150 ± 15</td>
</tr>
<tr>
<td>D</td>
<td>36</td>
<td>225 ± 30</td>
</tr>
</tbody>
</table>

*Calculated from the relationship 10^6 daltons of DNA = 1.5 kb (10).
FIG. 3. Electron micrographs showing the location of stem-loop structures A and B in relation to various single restriction endonuclease cutting sites in the DNA of plasmid pCU13 or pCU16. (a) Single-stranded molecule of pCU13 linearized with SalI. (b and c) Single-stranded molecules of pCU16 linearized with BamHI (b) or HindIII (c). (d) Single-stranded molecule of pCU10 linearized with SmaI, the site for which is within loop A.
Arrows in the electron micrographs point to structure B. Interpretative line diagrams are included in each electron micrograph. Bar, 0.5 μm.
FIG. 4. Position of the four stem-loop structures on the restriction map of plasmid pCU1 (see Fig. 3). The enlarged area which extends clockwise from KpnI site 2 to HindIII site 2 shows the position of the stem-loop structures in relation to the restriction endonuclease and functional map of pCU1 (see also Fig. 5). The ends of each structure are indicated by arrows. The nomenclature for the enzymes used is presented in an abbreviated form. The cutting sites are shown for the following enzymes: BamHI (B), BglII (BglI), BsrEI (BsiI), EcoRI (E), HindIII (HIII), KpnI (K), PstI (P), and Smal (Sml). The cleavage sites recognized by restriction enzymes are indicated by superscript numerals. Some of the pCU1-associated phenotypes were localized through analysis of various deletions and insertion derivatives of the plasmid. The lower part of this figure shows the extent of deletions (shaded areas) in derivatives of plasmid pCU1 (pCU8, pCU102, pCU13, pCU16, pCU19, and pCU26). The phenotypes of these derivatives are included in Table 1. The insertion of plasmid pACYC184 (cross-hatched areas) into the BamHI site of pCU26 is marked by an arrow.
cution and maintenance functions of the plasmid to a 4-kb region extending clockwise from Smal site 2 (22; see also plasmid pCU102 in Fig. 4). In the previous section, we have shown that a part of this region that includes the Ap’ locus and which lies outside the A + B structure is not essential for maintenance. Neither is the small structure B, which is inserted within A because this structure spans the region between Smal site 1 and BglII site 4, which could be deleted without affecting replication and maintenance (22; see also Fig. 5). These essential functions can thus be localized as shown in Fig. 4 to a region within the loop of structure A. The structure C which is clockwise to A is also of potential interest as the mapping data indicate that it includes part, but not all, of the region specifying Sm’ and Sp’. No functions can be as yet
assigned to B and D, nor is it known at the present time whether any of these structures is transposable.

DISCUSSION
This study was prompted by the observation that plasmid pCU1 gave rise spontaneously to Sm\(^{\text{R}}\) and Sp\(^{\text{R}}\) deletion derivatives at a relatively high frequency (0.1%). The literature on bacterial plasmids records many instances in which deletions are promoted by the presence in the plasmid DNA of sequences that are most often repeated in inverted orientation to one another. If these sequences and the DNA region between them are large enough, they can be visualized as stem-loop structures by electron microscopy. This approach has led to the observation that plasmid pCU1 DNA contains four inverted-repeat loop structures that are morphologically distinguishable from one another and that range in size from about 1.2 to 4.9 kb. It should be noted, however, that the stem sizes, particularly for structures B and C, can be overestimated. It has been reported for an ampicillin transposon (Tn3) that the stem size of 140 base pairs as determined by electron microscopy (16) was in fact only 38 base pairs as determined by nucleotide sequence (26). Stems of structures B and C are small and similar in length (135 ± 15 and 150 ± 15 base pairs, respectively). This raises the possibility that there is sequence homology between them. If this is the case, one would expect to see larger structures produced by reannealing stem B with stem C. We did not, however, observe such structures. The structures observed on denatured strands of pCU1 have the appearance of some bacterial transposons, although it is not known whether these structures are transposable. Since their morphology is known, it should be possible to determine whether they are transposable.

All four structures are grouped together in one region of the plasmid. One of the structures, B, has been found to be within a larger structure, A. Another naturally occurring double loop structure has been reported previously (23). In that case, both of the structures were transposable (independent of one another), the smaller structure containing the Ap\(^{\text{R}}\) gene and the larger one containing the Sm\(^{\text{R}}\) and sulfonamide resistance (Su\(^{\text{R}}\)) genes.

It has been shown that for most of the common restriction endonucleases with hexanucleotide specificity, restriction sites are not symmetrically distributed on plasmid pCU1 (22). The region in which the four stem-loop structures are located does, however, have several restriction sites (Fig. 4 and 5). This circumstance has allowed us to locate the structures with a greater degree of certainty than might otherwise have been possible. Using a combination of plasmid deletions and plasmid recombinants in conjunction with electron microscopy, we have mapped the position of the four inverted-repeat loop structures in relation to the restriction and functional map of the plasmid. This has allowed the conclusion that the region on the plasmid DNA concerned with its vegetative replication and maintenance is contained within structure A but outside structure B. Deletions of DNA which include structure B do not affect replication and maintenance of plasmid pCU1 (22; also see Fig. 4). At the present time we are attempting to determine whether structure A is a transposable element. If so, it could be a naturally occurring transposable replicon. A similar observation has been reported by Langer et al. (25) for another plasmid. This plasmid also belongs to the N group and is generally similar to pCU1 in its structural and functional organization, although there is no evidence that it carries the structure B. Using in vitro methods, Haykinson et al. (14) and Cohen et al. (7) have been able to construct an artificial transposon that contains genetic regions for plasmid replication and maintenance.

The ability to visualize structure C is correlated with a deletion of 3.0 kb of DNA in the region where this structure occurs and with the simultaneous loss of the Sm\(^{\text{R}}\) and Sp\(^{\text{R}}\) phenotypes. It has not been observed that these two phenotypes are lost independently of one another. The small EcoRI fragment within which these two phenotypes map lies partly within the loop of C and partly outside. However, this constitutes a maximum estimate for the region, and it is possible that one or both of these two phenotypes are determined by regions within the loop or outside but close to it. No phenotype has been as yet conclusively associated with structure D. Preliminary mapping data (Thatte, unpublished observations) suggest that the kill phenotype specified by this plasmid (27) is in the region of this structure.

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LITERATURE CITED