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Site-Specific *recA*-Independent Recombination Between Bacterial Plasmids: Involvement of Palindromes at the Recombinational Loci

(DNA insertion/inverted genetic duplication/rotational symmetry/R-factor/translocation)

DENNIS J. KOPECKO AND STANLEY N. COHEN

Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT **A *recA*-independent recombinational event is described which results in insertion of an entire plasmid genome at a unique site of another plasmid, and coincident excision of a precisely defined DNA segment originally present at the point of the insertion. The resulting recombinant molecules subsequently can undergo site-specific translocation of their component segments or inversion of their original DNA sequence orientation. The events observed entail nonreciprocal exchange of genetic material, and involve a discrete nucleotide sequence that is duplicated in rotationally symmetrical reverse orientation on plasmid DNA (i.e., inverted repeat; palindrome).**

Autonomously replicating extrachromosomal genetic elements (plasmids) can undergo genetic recombination with both the host bacterial chromosome (1), and with other plasmids (2, 3). In *Escherichia coli*, general recombination between plasmids and bacterial chromosomes or between coexisting plasmids commonly occurs by a mechanism that involves the bacterial *recA* gene (3, 4), and which appears to be dependent upon physical breakage and reciprocal exchange of DNA in an area of extensive genetic homology.

During the past several years, indirect evidence has suggested that some bacterial plasmids can participate also in "illegitimate" recombinational events that involve the joining of DNA segments at precisely defined sites. In *Proteus mirabilis* (3) and *Salmonella typhimurium* (5), certain antibiotic resistance plasmids (R plasmids) can dissociate reversibly into RTF (transfer) and R-determinant component replicons. Dissociation and reassociation (i.e., recombination) of the components of such cointegrate (3) R-factors must involve specific DNA loci, since independently formed isolates of RTF components appear to be genetically and structurally identical (6, 7). Site-specificity of recombination between plasmid DNA segments is suggested also by the results of electron microscope heteroduplex studies, which show that a region of DNA sequence homology common to several related F-like plasmids terminates at a precisely defined "genetic boundary" (8). However, little is known about mechanisms that might lead to possible recombination between plasmid DNA segments at specific loci.

The present report describes certain properties of plasmids that have been formed in *E. coli* by *in vivo* recombination between a large F-like antibiotic resistance plasmid, and a small non-conjugative tetracycline resistance replicon. Our results indicate that genetic recombination between these molecular species occurs by *recA*-independent insertion of an entire plasmid genome at a specific DNA locus of another plasmid, and coincident excision of a precisely defined DNA segment originally present at the point of insertion. Moreover, we find that such site-specific nonreciprocal exchange (translocation) of genetic material involves a discrete nucleotide sequence that is duplicated in rotationally symmetrical reverse orientation on plasmid DNA [i.e., inverted repeat (8); palindrome].

Abbreviations: nal, nalidixic acid; Ap, ampicillin; Sm, streptomycin; Su, sulfathiazole; Tc, tetracycline; Cm, chloroamphenicol; CCC, covalently closed circular; OC, open circular; DS, double-stranded; SS, single-stranded; IR, inverted repeat; kb, kilobase.

MATERIALS AND METHODS

Escherichia coli strains JC1569 *recA*$^-$ (kindly provided by A. J. Clark) and C600 have been described (9, 10). Spontaneous mutants of these strains resistant to 100 μg/ml nalidixic acid (nal^r) were selected on nutrient agar plates. The *rec* character was assayed using *fec*$^+$ and *fec*$^-$ mutants of bacteriophage λ (11). The nonconjugative tetracycline-resistance plasmid pSC101 has been described (12, 13). pSC50 is a kanamycin-sensitive mutant, isolated in our laboratory, of the plasmid R1-19 (6). Neither plasmid was found to alter the *recA*$^-$ phenotype of JC1569.

The conditions and procedures used for bacterial growth (14), conjugation (9), plasmid DNA transformation (12, 15), radioactive labeling and isolation of plasmid DNA (14), sucrose gradient and CsCl centrifugation (16), cleavage of plasmid DNA with the *Eco*RI restriction endonuclease, and agarose gel electrophoresis have been described (17, 18). The concentrations of antibiotics included in selection media were: ampicillin (Ap, 25 μg/ml); streptomycin (Sm, 20 μg/ml); sulfathiazole (Su, 250 μg/ml); tetracycline (Tc, 25 μg/ml); chloramphenicol (Cm, 25 μg/ml); and nalidixic acid (nal, 100 μg/ml). DNA was stored in TE buffer (17). The *Eco*RI restriction endonuclease was purified from *E. coli* strain RY13 according to Greene *et al.* (19) through the phosphocellulose chromatography step.

Heteroduplex analysis of plasmid DNA was carried out as described by Sharp *et al.* (8), using a Phillips EM201 electron microscope. Nicked PM-2 viral DNA and single-stranded circular molecules of pSC101 plasmid (13) or ϕX174 bacteriophage (8) DNA served as internal standards for contour length measurements.

RESULTS

Recombination Between pSC101 and pSC50 Plasmids in recA$^+$ or recA$^-$ E. coli. Earlier studies (12) have shown that mobilization (20) (transfer) of the nonconjugative pSC101 plasmid by a conjugally proficient plasmid can lead to re-

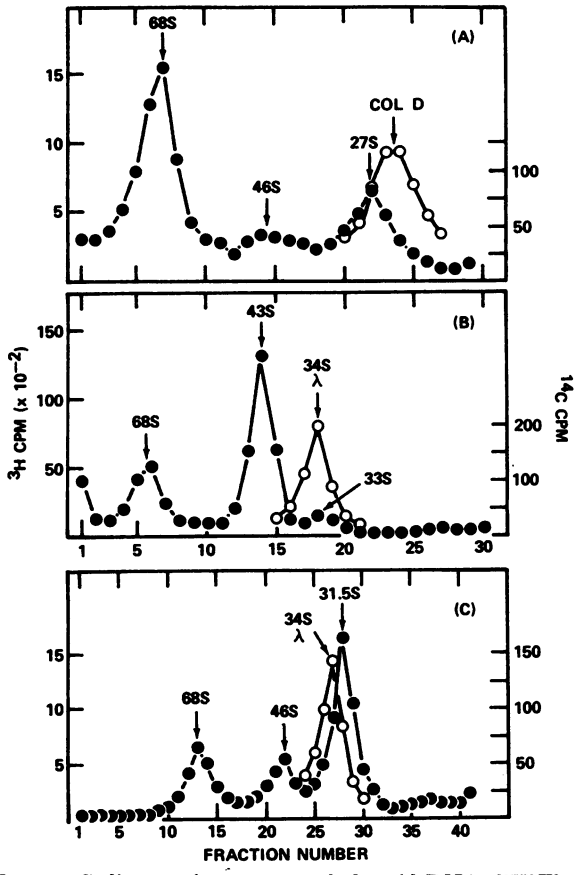

FIG. 1. Sedimentation pattern of plasmid DNA. [^{3}H]Thymidine-labeled CCC DNA (●) isolated from CsCl–ethidium bromide gradients was sedimented in 5–20% sucrose gradients at 39,500 rpm for 60–75 min in an SW50.1 rotor, as described (14), in the presence of ^{14}C-labeled reference DNA (○) (λ, 34S; Col D plasmid, 21.5S). Fractions were analyzed as described (6). (A) DNA from *E. coli* strain C600 carrying coexisting pSC101 and pSC50 plasmids. JC1569 showed identical patterns. Decrease in the 68S (CCC) peak and proportional increase of the 46S (OC peak) was observed after storage of DNA at 4° for 2 weeks. (B) DNA isolated from type I transconjugant showing CCC (43S) and OC (33S) forms of recombinant DNA in addition to pSC50 forms. (C) DNA from type II C600 transconjugant. DNA obtained from JC1569 transconjugants showed an identical sedimentation pattern.

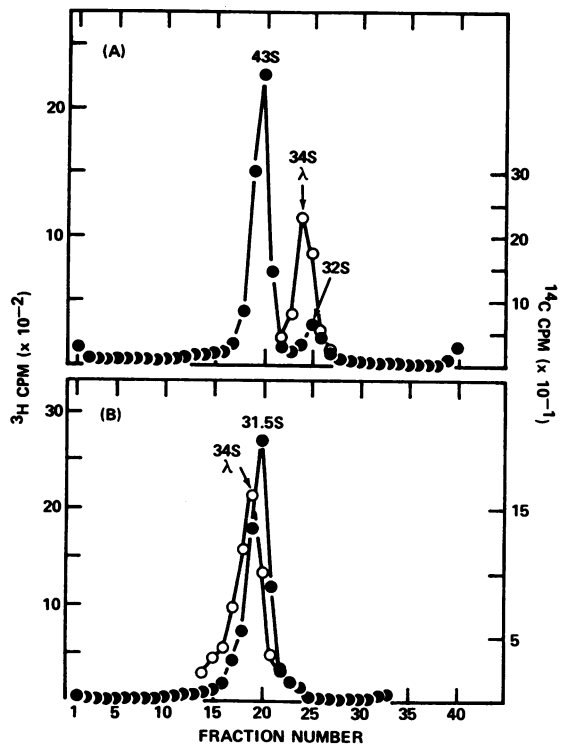

FIG. 2. Sucrose gradient analysis of recombinant plasmid DNA. Radioactively labeled plasmid DNA isolated from transformed clones by detergent lysis and CsCl–ethidium bromide centrifugation was sedimented and analyzed as described in Fig. 1. (A) DNA of type I recombinant plasmid, pSC120. (B) DNA of type II recombinant plasmid, pSC184.

combination between these genetic elements. In the current experiments, independent isolates of *E. coli* strains C600 or JC1569 carrying coexisting autonomous pSC101 and pSC50 plasmid replicons (Fig. 1A) were separately mated with nal^r mutants of these respective strains, and Tc-resistant recipient bacteria (transconjugants) were selected and cloned. Covalently closed circular (CCC) DNA obtained from 11 randomly chosen C600 transconjugants showed two different types of sucrose gradient sedimentation profiles. Type I isolates (2 of 11) (Fig. 1B) contained new DNA peaks sedimenting at 43 S and 33 S, in addition to a 68S CCC DNA peak characteristic of the pSC50 plasmid (unpublished data); type II isolates (9 of 11) (Fig. 1C) contained a 31.5S DNA species, in addition to pSC50 CCC and open circular (OC) DNA peaks. CCC DNA obtained from single colony isolates of JC1569 that had been randomly selected from eight separate matings carried out entirely in the *recA*$^-$ strain yielded only type II isolates. Although both types of transconjugants expressed Tc-resistance, they lacked the 27S pSC101 plasmid CCC DNA species present in the donor (Fig. 1A), suggesting that the Tc gene of pSC101 was incorporated into a recombinant plasmid species.

The usefulness of transformation for the separation and cloning of coexisting plasmids has been demonstrated (14, 17). In order to isolate the putative recombinant plasmids from transconjugants, *recA*$^+$ and *recA*$^-$ *E. coli* strains were transformed with unfractionated CCC DNA obtained from type I or type II clones, and transformed bacteria were selected on media containing Tc, Ap, Sm, Cm, or various combinations of these antibiotics.

Three groups of transformants resulted from the above selection: (*1*) bacteria selected on media containing Cm also expressed resistance to Ap and Sm, and carried CCC and OC DNA species characteristic of the pSC50 plasmid. (*2*) Clones selected on media containing Tc plus Cm carried both the pSC50 plasmid and a recombinant molecular species, and showed sedimentation profiles similar to that seen in Fig. 1B and C. (*3*) Selection of transformants on media containing Ap plus Tc enabled isolation of clones that carry *only* a recombinant plasmid, and which lack the DNA species characteristic of pSC50. The cloned type II plasmids (Fig. 2B) expressed resistance to only Ap and Tc, and their CCC DNA had a sedimentation value (31.5S) consistent with a molecular weight of 8.5×10^6. The type I recombinants (43S CCC DNA, 18.5 megadaltons) (Fig. 2A) carried resistance to Sm and Su in addition to Ap and Tc.

Molecular Properties of Recombinant Plasmids. Agarose gel electrophoresis of *Eco*RI-restriction-endonuclease-treated

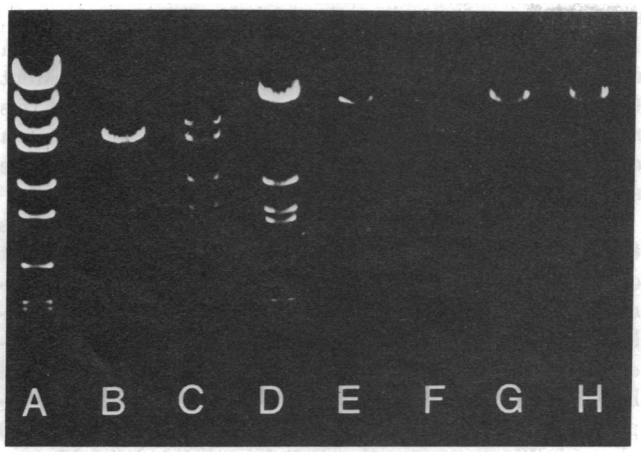

FIG. 3. Agarose gel electrophoresis of *Eco*RI restriction endonuclease digests of parental and recombinant plasmids. Conditions for DNA cleavage and analysis of fragments in agarose slab gels have been described (17, 18). Electrophoresis was from top to bottom. Molecular weights of DNA fragments were calculated from their mobility in gels relative to the mobility of fragments of *Eco*RI-cleaved R6-5-plasmid DNA (13). (A) pSC50. The molecular weights, in millions, for the nine fragments seen in this figure are (from top to bottom) 17.0, 10.0, 6.5, 6.1, 3.6, 2.8, 1.82, 1.40, and 1.30. Two additional fragments (molecular weights 1.12 and 0.90 $\times$ 10^6) were observed when large amounts of DNA were analyzed. (B) pSC101 (5.8 million, see ref. 12). (C) pSC120 (molecular weights of fragments = 6.5, 5.1, 3.4, 2.7, and 1.12 million). An additional fragment (about 0.46 $\times$ 10^6) was seen when large amounts of DNA were analyzed. (D) pSC179 (molecular weights of fragments = 9.0, 3.4, 2.7, 2.45, and 1.12 million). A 0.46 million fragment was observed when large amounts of DNA were analyzed. (E), (F), (G), and (H) pSC174, 175, 183, and 190, respectively. Molecular weight about 9 million.

DNA representing both types of recombinant plasmids is shown in Fig. 3. All eight type II recombinant plasmids that had been formed and cloned entirely in *recA*$^-$ bacteria in separate experiments and all type II (31.5S) recombinant plasmids isolated from separate *recA*$^+$ clones contained a single *Eco*RI cleavage site. The molecular weight of the resulting linear *Eco*RI-generated plasmid DNA fragments estimated from their mobility in the gel is 9.0 $\times$ 10^6, which is in close agreement with the values calculated by sucrose gradient sedimentation (Fig. 2). In contrast, *Eco*RI-treated type I plasmid DNA showed two different gel electrophoresis patterns (Fig. 3C and D). The antibiotic resistance patterns, molecular weights, and buoyant densities of the parent plasmids and representative recombinant plasmids are summarized in Table 1.

DNA Nucleotide Sequence Relationships Between Parent and Recombinant Plasmids. A representative heteroduplex between the pSC183 recombinant plasmid (type II) and its pSC101 parent is shown in Fig. 4a. The heteroduplex contains a double-stranded region (DS) about 3 μm in length, corresponding to the region of the recombinant molecule showing homology with pSC101, and a smaller single-strand loop (SS) which represents the contribution of pSC50 to the recombinant plasmid. The absence of a detectable deletion loop within the homologous region, which is equal in length to the entire pSC101 molecule, indicates that all of the DNA sequences of pSC101 are contained contiguously in each recombinant plasmid. At the junction of the circular duplex

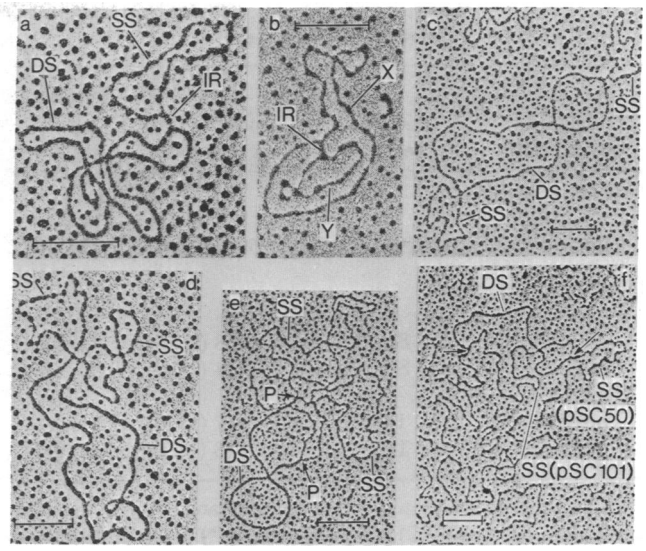

FIG. 4. Heteroduplex analysis of recombinant plasmids. Double-stranded (DS), single-stranded (SS), and inverted repeat (IR) are indicated where appropriate. Procedures for heteroduplex formation and analysis are indicated in *Materials and Methods*. The scale on each photo indicates 0.25 μm. All measurements were calculated from five or more molecules [SEM (DS) = 4%; SEM (SS) = 10%]. (a) pSC183/pSC101 heteroduplex. (b) Single strand of DNA from pSC184 recombinant plasmid. The molecule is divided into pSC50 (Y) and pSC101 (X) component segments by the inverted repeat (IR). (c) Heteroduplexes between two recombinant type II plasmids (pSC184 and pSC186) formed and cloned in *recA*$^-$ bacteria. Single-stranded (SS) regions indicate pSC50 DNA segments that have recombined at different locations of the pSC101 plasmid. (d) Heteroduplex between plasmids pSC184/pSC185 showing single-stranded noncomplementary pSC50 loops emerging from the same site of a duplex region representing the pSC101 segment of the recombinant molecules. (e) Heteroduplex between pSC184/pSC185 showing substitution loops (SS) emerging at sites (P) from a duplex region that corresponds to the contribution of pSC50 DNA to both molecules. (f) Cropped photo of pSC175/pSC50 heteroduplex showing a 4.4 kb double-stranded region common to both plasmids. The arrows indicate the ends of the duplex region. The contribution of the pSC101 plasmid to the recombinant molecule and the nonhomologous pSC50 (single-stranded) DNA are identified. The length of the pSC50 molecule seen in this heteroduplex (complete molecule not shown) is 25.6 μm, which is consistent with a molecular weight about 51 $\times$ 10^6.

(DS) and single-stranded (SS) regions of the molecule is seen a short linear duplex segment of DNA (IR), 0.04 $\pm$ 0.007 μm in length, which has the appearance associated with intramolecular renaturation between complementary nucleotide sequences contained on the same DNA strand (i.e., inverted repeats; see ref. 8). The existence of such an inverted duplication of DNA nucleotide sequences at the junction of the pSC101 and pSC50 component regions of the recombinant molecule is further demonstrated on examination of self-annealed *single* strands of the recombinant plasmid (Fig. 4b); 0.04 μm segments of the same strand are seen annealing with each other at a site that divides the molecule into two precisely defined segments corresponding in length to its pSC101 and pSC50 components. Inverted duplications in self-annealed, single-stranded molecules were observed at the junction of the pSC50 and pSC101 DNA segments of all other independently isolated 31.5S (type II) and 43S (type I) recombinant plasmids examined.

TABLE 1. *Summary of properties of parental and recombinant plasmids*

Plasmid designation	Antibiotic resistance	Size in megadaltons or (kilobases)	Buoyant density g/cm³
Parental plasmids			
pSC101	Tc	5.8 (8.7)	1.7100
pSC50	Ap, Sm, Su, Cm	50.9 (77.0)	1.7110
Recombinant plasmids			
Type I			
pSC120	Ap, Sm, Su, Tc	18.8 (28.5)	1.7145
pSC179	Ap, Sm, Su, Tc	18.4 (28.0)	1.7145
Type II			
pSC174–178	Ap, Tc	8.7 (13.0)	1.7095
pSC183–190*	Ap, Tc	8.8 (13.0)	1.7095

All recombinant plasmids were formed and cloned independently in *E. coli* strain JC1569 (indicated by *) or C600. Antibiotic resistance was assayed on nutrient agar containing drugs at concentrations indicated in *Materials and Methods*. Molecular size of each plasmid was calculated from contour lengths of 20 or more molecules based on a molecular weight of 5.97 million for bacteriophage PM-2 reference DNA included on grid preparations (SEM $< \pm 4\%$). Kilobase (kb) pairs rounded to the nearest 0.1 are given in parentheses (see ref. 8). Buoyant densities of recombinant plasmids were determined by analytical centrifugation in CsCl as described previously (16).

Fig. 4c and d show heteroduplexes between different type II plasmids that have been formed and cloned independently in *recA*⁻ bacteria. The double-stranded region of homology (DS) in both heteroduplexes represents the entire pSC101 plasmid, which is common to all of these recombinant molecules. The spacing of two single-strand deletion loops (SS) in Fig. 4c, each the size of the DNA segment derived from the pSC50 plasmid, indicates that linkage with the pSC50 segment has occurred at widely separated sites of the pSC101 plasmid in the pSC184 and pSC186 recombinants.

A heteroduplex between the pSC184 and pSC185 plasmids (Fig. 4d) shows two deletion loops emerging from the same site of the duplex pSC101 segment common to both plasmids. The occurrence of an alternative pSC184/pSC185 structure (Fig. 4e) in 23 of 66 heteroduplexes counted indicates that both plasmids contain the same region of the pSC50 molecule, but that this segment has been inserted in different orientations with respect to the pSC101 genome in pSC184 and pSC185. In this alternative structure, the pSC50 segments of both strands form a duplex region, while the noncomplementary, inverted pSC101 segments are seen as single-stranded substitution loops, indicating that insertion of the pSC101 genome occurred at the same site of the pSC50 plasmid in both of the recombinants. The absence of expected substitution loops in Fig. 4d may be due to self-annealing of the complementary DNA nucleotides sequences located at the bases of the two pSC50 DNA segments. In certain heteroduplexes, substantial tangling of adjacent single-stranded loop structures emerging from the pSC101 duplex region was observed.

Comparison of heteroduplexes formed by eight different combinations of the type II recombinant plasmids listed in Table 1 supports the interpretation that all of the recombinant molecules contain identical segments of the pSC50 and pSC101 plasmids, but that these segments have recombined in different DNA sequence orientations in the different plasmids. Moreover, analysis of a typical heteroduplex formed between a recombinant plasmid DNA species and the pSC50 parent (Fig. 4f) indicates that the segment of pSC50 involved in this recombinational event is a continuous region, rather than a composite of nucleotide sequences derived from two or more separated regions.

The simplest interpretation of these aggregate findings is that the entire pSC101 plasmid can be inserted in two different orientations at a unique site of pSC50 DNA, and that a segment of DNA originally present in the pSC50 plasmid is deleted at the point of the insertion. The site of insertion and excision bisects an axis of rotational symmetry, which is defined by inverted DNA sequence duplications located on the component of the recombinant molecules derived from the pSC50 plasmid. When a preparation containing DNA derived from a single cloned type II recombinant plasmid is denatured and *self*-annealed, a small number of heteroduplexes identical to those shown in Fig. 4c, d, and e are seen along with the expected homoduplexes. Thus, the pSC50 and pSC101 segments of these recombinant plasmids can undergo site-specific translocation of plasmid component segments, or inversion of their original DNA sequence orientation, after the initial recombinational event has occurred.

DISCUSSION

The experiments reported here describe recombinational events that result in insertion of an entire plasmid genome at a unique site of another plasmid, and excision of a DNA segment originally present at the point of this insertion. The events described are independent of the general recombination functions specified by the *E. coli recA* gene, and involve a tandemly duplicated inverted DNA sequence approximately 130 nucleotides long which occurs on both sides of the inserted DNA segment. Although the insertion site of the pSC50 plasmid segment is uniquely defined by the limbs of this genetic duplication, recombination with pSC50 can occur at four separate sites of the pSC101 plasmid (in preparation). Subsequent to their initial formation, the recombinant plasmids can undergo inversion of the initial DNA nucleotide sequence orientation of their components, or translocation of the pSC50 component to another of the recombinational loci of the pSC101 plasmid.

A schematic representation of the events reported here is shown in Fig. 5. Although heteroduplex analysis demonstrates that both limbs of the inverted repeat in recombinant plasmids are contained in a DNA segment derived from the pSC50 plasmid (Fig. 4a) and that this segment is contained continuously in the parent molecule (Fig. 4f), inverted genetic duplications have not been identified in the pSC50 plasmid. While no explanation for this apparent paradox is yet available, one can speculate that a tandemly duplicated DNA sequence originally present in *direct* order on the pSC50 plasmid may have been excised and subsequently inverted during the recombinational event. Such *en bloc* excision of a precisely defined DNA segment and reinsertion of this segment in inverted orientation has been found to occur with insertion sequence (IS) segments (21) which have been identified recently on plasmid DNA (22, 23).

The site of insertion of the pSC101 replicon into the pSC50 plasmid segment bisects an axis of 2-fold rotational symmetry of the type proposed by Bernardi to be involved in the inter-

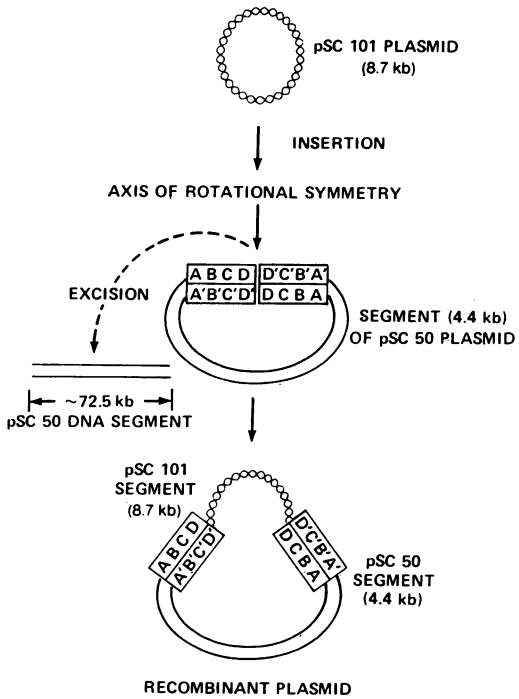

FIG. 5. Schematic representation of events reported here. Excision of a segment of the pSC50 plasmid DNA and insertion of the entire pSC101 plasmid genome has occurred at a site which bisects an axis of rotational symmetry defined by the inverted repeats (palindromes): ABCD ---- D'C'B'A'.

action of spleen DNase with its substrate (24). Because of the antiparallel arrangement of DNA polynucleotide chains, such sequences read the same backward and forwards. Similar rotationally symmetrical sequences (palindromes) have been identified at the DNA interaction site for the *ter* enzyme of phage λ (25), at promotor and operator sites associated with λCI and *E. coli lac* operon repression (26, 27), and at the site of action of several restriction and modification enzymes (28, 29), suggesting that inverted genetic duplications may provide highly specific recognition sites for protein–DNA interactions. Moreover, palindromes appear to be a general feature of DNA from higher eukaryotes (30).

Recent observations indicating that certain palindrome-site-specific endonucleases are coded by plasmid genes (28) raise the possibility that similar enzymes may have a role in the recombinational events described here. In any case, these *recA*-independent events involve a process that appears to be relevant to an understanding of the formation and evolution of bacterial plasmids. The possible role of inverted and direct genetic duplications in the structural evolution of other types of complex genomes remains to be determined.

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