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Structural and Functional Analysis of Cloned DNA Segments Containing the Replication and Incompatibility Regions of a Miniplasmid Derived from a Copy Number Mutant of NR1

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A 1.45-megadalton segment of DNA cloned from a miniplasmid derived in vivo from a copy number mutant of the R plasmid NR1 has been shown to contain all functions essential for incompatibility and autonomous plasmid replication in *Escherichia coli*. Specific endonuclease cleavage sites within this DNA segment that localize functions required for replication have been mapped. A 0.45-megadalton fragment that specifies the FII incompatibility of NR1 has been identified within the replication region, and DNA fragments containing this incompatibility region, but lacking other functions required for replication, have been cloned.

In order for a plasmid to be maintained stably in a bacterial host cell, it must be accurately replicated and partitioned during the cell division cycle. For plasmids occurring at one or a few copies per chromosome, this requirement appears to be especially stringent. The large cointegrate antibiotic resistance plasmid NR1 (25, 30), also referred to as R100 or R222, is one such low-copy-number plasmid (24, 47) that is inherited stably in *Escherichia coli* and several other *Enterobacteriaceae* (8, 25).

The control of replication of NR1 and related plasmids such as R1 can be affected by stable mutations that increase copy number (13, 24, 26, 40, 45, 47). Analysis of certain copy number mutants of NR1 and R1 has revealed that they can generate miniplasmids in vivo (12, 20, 40). Miniplasmids derived from various copy number mutants of NR1 contain a region necessary for autonomous replication but carry no antibiotic resistance determinants (20, 40).

Plasmid incompatibility has been defined as the inability of two genetically distinguishable plasmids to coexist in the same host cell in the absence of continued selection pressure (27). Thus, incompatibility interferes with the stable inheritance of the plasmids and leads to the distribution of the two plasmids during cell division, resulting in progeny cells that carry one or the other of the plasmids. Although its mechanism is not well understood, plasmid incompatibility is taken as a measure of relatedness among plasmids and has been used for the classification of naturally occurring plasmids for epidemiological purposes. On the basis of their mutual incompatibility, NR1, R1, and R6 have been placed in the FII incompatibility group (7). The FII incompatibility properties of NR1 also appear to be expressed by some miniplasmids generated by copy number mutants of this plasmid (20, 40).

Incompatibility and copy number control are believed to be closely associated functionally because some copy number mutants of R1 and NR1 have altered incompatibility properties (45; D. Taylor, unpublished data). Evidence has been obtained which indicates that certain copy number mutants may be defective in a *trans*-acting negative control factor that determines plasmid copy number (13, 45; D. Taylor and R. Rownd, manuscript in preparation) and regulates plasmid replication, as postulated by Pritchard et al. (32). These observations support the view (32) that components involved in copy number control may play a role in incompatibility.

Timmis et al. (43) have demonstrated that replication regions can be isolated from large cointegrate plasmids by a DNA cloning procedure that uses in vitro recombinant DNA techniques. The cloned replication region fragment of R6-5, a plasmid that has large segments of homology with NR1 (36), corresponds to the second largest *Eco*RI-generated fragment of R6-5 (43, 44) and NR1 (39). Miniplasmids generated in vivo from copy number mutants of NR1 all have in common a portion of that *Eco*RI fragment (21, 40). Thus, the size of the region from NR1 necessary for autonomous replication and incompatibility has been estimated from heteroduplex data to be no larger than 4.2 kilobases (kb) (21).

We report here DNA cloning experiments that localize more precisely the minimal amount of genetic information needed for autonomous rep-

lication of an NR1-derived miniplasmid. Endonuclease cleavage sites that must apparently remain intact for autonomous replication to occur, and which thus tentatively identify specific loci essential to this process, have been mapped within the replication region. DNA fragments that specify FII incompatibility but which lack other plasmid functions required for autonomous replication have been cloned from within the replication region. The tests used for identifying those fragments specifying incompatibility are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids utilized in this investigation are listed in Table 1. The culture media used included LB medium (22) and Penassay base agar (antibiotic medium no. 2, Difco), supplemented with thymine at a concentration of 20 μg/ml. When appropriate, antibiotics were added to the media at the following concentrations; tetracycline (Tc), 10 μg/ml; spectinomycin (Sp), 50 μg/ml; chloramphenicol (Cm), kanamycin (Km), and ampicillin (Ap), 25 μg/ml. All *recA* strains were tested regularly for UV sensitivity (23) to determine whether they were still Rec$^-$.

Isolation and manipulation of plasmid DNA. Plasmid DNA was prepared by using a cleared lysate procedure (44) modified by the use of Triton X-100 instead of Brij 58–deoxycholate for lysis. Some plasmid DNA preparations were purified further by chromatography on a Bio-Gel A-5m column as described previously (3). Endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and used as recommended. Digestions with two or more endonucleases requiring different buffers were performed sequentially, using the low-salt buffer first. The reaction was terminated by heating the initial digestion for 10 min at 65°C before digestion with the second endonuclease. Agarose gel electrophoresis of plasmid DNA was performed as described in reference 37, using a vertical slab gel apparatus, and gels were photographed as described in reference 3. The 13 largest *Eco*RI-generated fragments of pRR12 were used as molecular-weight standards, and the fragments' molecular-weight values used were those published by Tanaka et al. (39). The molecular masses of fragments smaller than 0.75 megadalton (Md), the smallest visible *Eco*RI fragment (M) of pRR12, were obtained by extrapolation.

Recombinant plasmids were constructed in vitro by ligation of endonuclease-generated fragments with T4 DNA ligase at a concentration of 1 to 1.5 U/ml at 12 to 14°C. DNA concentrations were varied between 10 and 50 μg/ml for ligation, depending on the desired outcome, in accordance with the considerations of Dugaiczyk et al. (11). Transformation of *E. coli* with plasmid DNA was performed as described (6).

Construction of miniplasmid derivatives. Km^r and Sm^r/Sp^r plasmid derivatives of pDPT101 were constructed in vitro by ligating *Eco*RI-cleaved pDPT101 DNA to *Eco*RI-cleaved pML21 (Km^r) or pFC012 (Sm^r/Sp^r) DNA. Km^r or Sm^r/Sp^r colonies were selected after transformation of C600 with the respective ligation mixtures. Colonies containing plasmids with Km^r or Sm^r/Sp^r on pDPT101, rather than the ColE1-derived parents pML21 or pFC012, were obtained by screening for colicin E1-sensitive clones, using crude preparations of colicin E1 (44). The presence of a pDPT101-derived replicon on the Km^r and Sm^r/Sp^r derivatives, designated pDPT103 and pDPT104, respectively, was confirmed by their ability to replicate independently of DNA polymerase I, that is, in a *polA*(Ts) host at the nonpermissive temperature (Table 2).

TABLE 1. *Bacterial strains and plasmids*

Designation	Genotype[a]	Reference
Bacterial strains		
C600	*thr-1 leu-6 thi-1 supE44 lacY1 tonA21* (λ$^-$) (F$^-$)	1
MM383	*polA12 thy rha lac rpsL* (λ$^-$) (F$^-$)	23
FL1699	*recA1* (λ$^-$) (F$^-$) (derived from Hfr KL16-99)	40
Bacterial plasmids		
pDPT101	Mini C (miniplasmid derived from pRR21 in vivo)	40
pRR12	*tet*$^+$ *cat*$^+$ *sul*$^+$ *aadA*$^+$ *mer*$^+$ *fus*$^+$ *inc*FII *tra*$^+$ *fin*$^+$ *cop*-12 (copy number mutant pRR12 derived from NR1)	24
pML21	*ice1*$^+$ *cea*$^-$ *aphA*$^+$	19
pFC012	*ice1*$^+$ *cea*$^-$ *aadA*$^+$	44
pBR313	*bla*$^+$ *tet*$^+$	3
pBR322	*bla*$^+$ *tet*$^+$	4
pSC305	*tet*$^+$ *aphA*$^+$ *rep*(Ts) (derived from pSC105 [5])	18
pSC304	*tet*$^+$ *bla*$^+$ *rep*(Ts) (pSC301::Tn*3*)	18

[a] Symbols are used in accordance with the proposal of Novick et al. (27).

RESULTS

Our goals were to reduce in size and isolate by cloning the replication region of NR1 and to dissect this region structurally and functionally by cloning endonuclease-generated fragments carrying separate replication-associated functions. The investigation was initiated with a miniplasmid, pDPT101, derived from pRR21 (40), a copy number mutant of NR1, because pDPT101 DNA could be obtained readily in high yields and, because of its small size (5.5 Md), was more easily manipulated than NR1.

Construction of a miniplasmid map. The pattern of cleavage of pDPT101 DNA by the endonucleases *Eco*RI and *Sal*I has been determined previously (39, 46). In the current studies, sites of cleavage (Fig. 1) by the site-specific endonucleases *Bgl*II, *Hinc*II, *Hin*dIII, *Pst*I, *Pvu*II, *Sma*I, and *Xho*I were located by double and triple digestions (see Fig. 2 for representative digests). The location of the *Bgl*II cleavage site on pDPT101, for example, was determined

TABLE 2. *Vector rescue by cloned fragments*

Plasmid designation	Vector	Cloned fragment[a]	Size (Md) of cloned fragment[b]	Frequency of plasmid-containing cells[c] at:	
				30°C	42°C
pDPT106	pBR313	*Hin*dIII	5.5	0.89	8.8×10^{-1}
pDPT111	pBR313	*Sal*I$_a$-*Sal*I$_b$	1.25	0.64	1.7×10^{-6}
pDPT113	pBR313	*Sal*I$_b$-*Sal*I$_a$	4.25	0.5	1.6×10^{-7}
pDPT122	pBR313	*Eco*RI-*Bgl*II	2.3	1.0	8.9×10^{-1}
pDPT132	pBR313	*Bgl*II-*Sma*I$_c$	1.45	1.0	5.9×10^{-6}
pDPT133	pBR313	*Sma*I$_b$-*Bgl*II	0.6	0.36	1.6×10^{-6}
pBR313				0.72	7.4×10^{-6}
pDPT161	pBR322	*Bgl*II-*Pvu*II$_d$	0.3	1.2	$<6.7 \times 10^{-6}$
pDPT165	pBR322	*Pvu*II$_c$-*Bgl*II	0.8	0.027	$<1.3 \times 10^{-6}$
pDPT251	pBR322	*Pst*I$_b$-*Pst*I$_c$ (D)	0.65	0.54	2.2×10^{-5}
pDPT253	pBR322	*Pst*I$_d$-*Pst*I$_a$ (B)	1.82	0.9	2.3×10^{-5}
pDPT254	pBR322	*Pst*I$_c$-*Pst*I$_d$ (A)	2.08	0.35	7.4×10^{-8}
pDPT255	pBR322	*Pst*I$_a$-*Pst*I$_b$ (C)	0.96	0.11	1.2×10^{-7}
pBR322				0.7	2×10^{-5}

[a] Fragments described by sites lettered sequentially clockwise from the *Eco*RI site and read in that order.

[b] Molecular weights of fragments determined by agarose gel electrophoresis, using *Eco*RI fragments of pRR12 (39) as standards.

[c] Frequency of Ap^r or Tc^r cells estimated at 30°C after growth at 42°C for about 20 generations in the absence of selection with the plasmids contained in MM383. The frequency represents the number of colonies appearing on Penassay base agar plates with Ap or Tc divided by the number of colonies growing on antibiotic-free Penassay base agar plates.

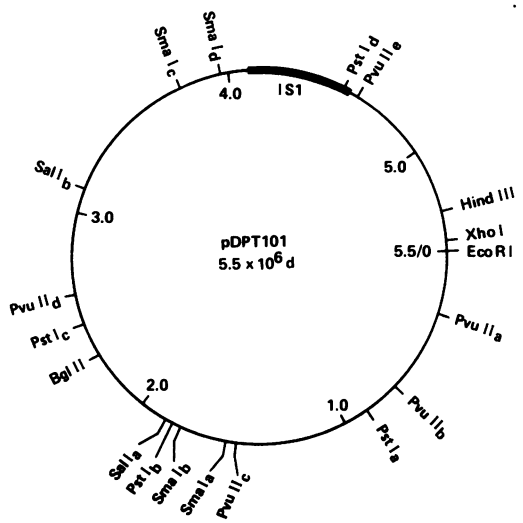

FIG. 1. *Map of restriction endonuclease sites contained on pDPT101. The sites of endonuclease cleavage were determined by double and triple digestions as described in the text, using the EcoRI fragments of pRR12 as molecular-weight standards. The coordinates are in megadaltons. The placement of IS1$_b$ on the map is described in the Discussion.*

from double digestions with *Sal*I (Fig. 2F) and with *Eco*RI (Fig. 2G). The single *Bgl*II site was localized within the smaller of the two *Sal*I fragments (Fig. 2D) of pDPT101, by cleaving this fragment into two fragments of 0.45 and 0.8 Md. The orientation of the fragments within the smaller *Sal*I fragment was determined by digestion of pDPT101 DNA with *Bgl*II and *Eco*RI. The size of the fragments thus produced (2.3 and 3.2 Md) allowed the *Bgl*II site to be located unambiguously on the pDPT101 map with respect to the *Sal*I and *Eco*RI sites. After the mapping of other sites on both pDPT101 and recombinant plasmids by similar means, the location of the *Bgl*II site was confirmed by additional digestions. Analogous procedures were used to map the location of the other endonuclease cleavage sites on the pDPT101 map shown in Fig. 1. No cleavage sites on pDPT101 were found for *Bam*HI, *Hpa*I, *Kpn*I, *Pvu*I, or *Sac*I.

Cloning and characterization of endonuclease-generated fragments of pDPT101 containing plasmid replication functions. The plasmid-cloning vector pBR313 is a ColE1-like plasmid (3), and thus requires DNA polymerase I for its replication (15, 16) and is lost rapidly from a *polA*(Ts) bacterial host (e.g., MM383) grown at the nonpermissive temperature (42°C). The ability of the entire miniplasmid pDPT101 to "rescue" pBR313 at 42°C was demonstrated by the construction of the hybrid plasmid, pDPT106, containing the two replicons. The *Hin*dIII site on pBR313 is located near the gene for Tc^r (3) so that insertion of DNA, in this case *Hin*dIII-cleaved pDPT101 DNA (Fig. 2C), into this site can lead to inactivation of Tc^r. Thus, insertional inactivation (42) can be used to detect the hybrid recombinant plasmid. Rescue of the pBR313 component of the composite plasmid (pDPT106) by the pDPT101 compo-

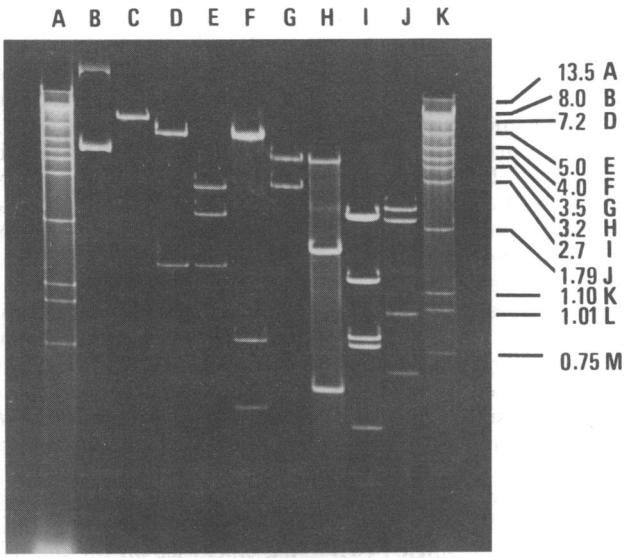

FIG. 2. *Agarose gel electrophoresis of miniplasmid DNA cleaved with various endonucleases. The EcoRI-generated fragments of pRR12 are included as molecular-weight standards (A and K) and the sizes of the fragments (39) in megadaltons are listed at the left; although EcoRI fragment C is present, its size (7.5 Md) is not listed for convenience. Undigested pDPT101 DNA is included (B) for comparison. pDPT101 DNA digested with HindIII (C), SalI (D), EcoRI and SalI (E), SalI and BglII (F), EcoRI and BglII (G), SmaI and BglII (H), PvuII and BglII (I), and PstI (J) were subjected to electrophoresis on a 1% agarose slab gel as described in the text.*

nent occurred (Table 2).

Unlike smaller miniplasmids, pDPT101 contains two *SalI* sites and includes a *SalI* fragment (E) of NR1 (46). Earlier experiments have shown that an NR1-derived plasmid, pRR12 (24), when isolated from *Proteus mirabilis* (46) contained an origin of replication of the *SalI* fragment E. To determine whether this small *SalI* fragment is capable of rescuing the pBR313 vector, the two *SalI* fragments of pDPT101 (40; Fig. 2D) were cloned separately onto the vector, using insertional activation of the Tc^r gene, which contains the only *SalI* cleavage site on the vector plasmid (3).

The presence of replication functions on the cloned *SalI*-generated DNA fragments was tested by determining whether each of these fragments could rescue the vector plasmid pBR313 when grown under nonpermissive conditions. The two *SalI*-generated miniplasmid fragments (Fig. 2D), when inserted into pBR313 (as in pDPT111 and pDPT113), were incapable of rescuing the vector (Table 2), indicating that functions located on both of the miniplasmid *SalI* fragments are required for replication or that the $SalI_a$ site is located within an essential structural gene, or both. Insertion of either of these two *SalI* fragments into pBR313 in the opposite orientation (e.g., pDPT112 and pDPT114) also failed to rescue the vector (data not shown). In separate experiments, the two smallest *SalI* fragments of NR1 were cloned onto pBR313, in either orientation, tested directly for their ability to rescue pBR313 under nonpermissive growth conditions, and found incapable of doing so (data not shown).

The 4.2-kb (2.8-Md) segment that the smallest miniplasmids and the *Eco*RI-generated replication region fragment from R6-5 have in common does not include the $SalI_b$ cleavage site. To determine the extent of the information between $SalI_a$ and $SalI_b$ sites required for replication, the *Eco*RI-*Bgl*II-generated miniplasmid fragment which contains $SalI_a$ (the smaller fragment in Fig. 2G) was cloned onto pBR313. This was done by ligating the *Eco*RI- and *Bgl*II-cut pDPT101 DNA with pBR313 DNA cleaved with *Bam*HI and *Eco*RI, utilizing the homology between the single-stranded tetranucleotide sequence produced by *Bgl*II (31) and *Bam*HI (34). The substitution of an *Eco*RI-*Bgl*II miniplasmid fragment for the pBR313 *Eco*RI-*Bam*HI fragment, which carries the promotor-proximal portion of the Tc^r structural gene (3), results in an Ap^r Tc^s recombinant plasmid. Vectors that had incorporated the *Eco*RI-*Bgl*II miniplasmid fragment containing the $SalI_a$ site (pDPT122) were rescued under the nonpermissive conditions (see Table 2).

To determine the limits of the DNA segment

required for replication between the *SalI*$_a$ and *Eco*RI sites, *Bgl*II-*Sma*I-generated miniplasmid fragments (Fig. 2H) were cloned onto pBR313, which had been cleaved with *Bam*HI and *Sma*I. In this instance, the miniplasmid fragments replaced the promotor-distal portion of the Tc^r structural gene, resulting in Ap^r Tc^s recombinant plasmids. Neither of the *Bgl*II-*Sma*I miniplasmid fragments (the second and third largest fragments in Fig. 2H, contained on pDPT132 and -133, respectively) was able to rescue pBR313 under the nonpermissive conditions (Table 2).

A larger segment that contained the *SalI*$_a$ site of the miniplasmid was generated by digestion with *Bgl*II and *Pvu*II. Miniplasmid DNA fragments thus obtained (the third and sixth largest fragments in Fig. 2I) were cloned onto the pBR313-derived cloning vector, pBR322 (4), which had been cut with *Bam*HI and *Pvu*II to produce pDPT161 and pDPT165. A single *Pvu*II site on pBR322 is located conveniently between the end of the Tc^r gene and the origin of replication, whereas the two *Pvu*II sites on pBR313 are situated in a less useful arrangement (D. Taylor, unpublished data). The *Pvu*II$_c$-*Bgl*II miniplasmid fragment on pDPT165 which contains the *SalI*$_a$ site was unable to rescue pBR322 (Table 2). Moreover, recombinant plasmids that carry this fragment (Table 2), as well as other Tc^s plasmids, which appear to be the pBR322 plasmid with the *Bam*HI-*Pvu*II fragment deleted (data not shown), are unstable in a *polA*(Ts) host grown under permissive conditions.

The four fragments generated by digestion of pDPT101 with *Pst*I (Fig. 2J) were also cloned onto pBR322. The resulting recombinant plasmids (pDPT251 to -255) were detected by the inactivation of the Ap^r gene by insertion of *Pst*I fragments into the single *Pst*I site within this gene on pBR322 (4). None of the *Pst*I fragments from the miniplasmid was capable of rescuing the vector in a *polA*(Ts) host growing at 42°C (Table 2). We thus concluded that functions essential for replication are located on more than one of the *Pst*I fragments of pDPT101.

Autonomous replicators generated by *PstI*. The region between *PstI*$_a$ and *PstI*$_c$, which includes the two smaller *Pst*I-generated fragments C and D from the miniplasmid, was tested for its ability to replicate autonomously by attempting to clone a replication region from the miniplasmid after digestion of pDPT101 DNA with *Pst*I (Fig. 2J). The selection was provided by using a *Pst*I fragment derived from pRR12; this fragment (second largest fragment in Fig. 3B), which encodes Cm^r and Sm^r/Sp^r, was inserted into a *Pst*I site on pSC304 to form pDPT37 (Fig. 3B and 4). This plasmid can be contraselected by its inability to replicate at 42°C and eliminated by screening against Tc^r as an unselected marker. Miniplasmid DNA, partially digested with *Pst*I, was ligated with pDPT37 DNA that had been completely digested with *Pst*I. Transformants were selected as Cm^r Sp^r at 42°C and later screened for Tc sensitivity. Plasmid DNA from the Cm^r Sp^r Tc^s clones, including pDPT211, was analyzed by agarose gel electrophoresis after digestion with *Pst*I. Among eleven clones examined, all were found to contain three (or more) of the four miniplasmid *Pst*I fragments, in addition to the Cm^r Sp^r *Pst*I fragment from pDPT37. However, only the two smallest *Pst*I fragments of the miniplasmid are common to all the recombinant plasmids, which suggests that these two fragments are sufficient for autonomous replication.

To establish that the *PstI*$_a$-*Bgl*II region (reading clockwise on the miniplasmid map) is sufficient for autonomous replication, additional autonomous replicators were constructed. One of the *Pst*I-generated plasmids, pDPT211, which contains *Pst*I miniplasmid fragments B, C, and D, and the Cm^r Sp^r *Pst*I fragment (Fig. 3D and 4) was "scrambled" by treating the plasmid concurrently with the *Pst*I, *Bgl*II, and *Bam*HI endonucleases. After ligation of the resulting frag-

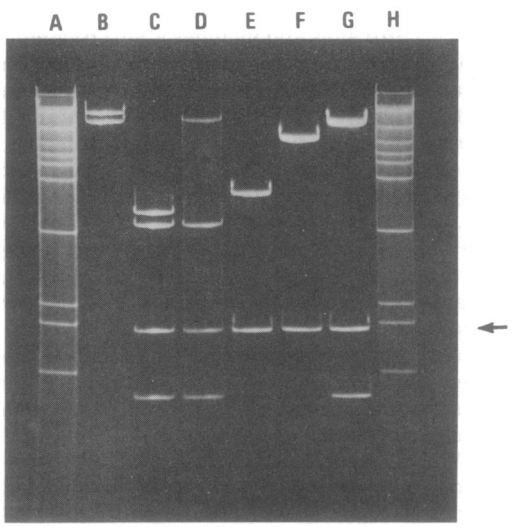

FIG. 3. *PstI-generated fragments contained on autonomous replicators cloned from the miniplasmid. The following DNAs were digested with PstI and subjected to electrophoresis on a 1% agarose gel: (B) pDPT37, (C) pDPT101, (D) pDPT211, (E) pDPT234, (F) pDPT240, (G) pDPT270. The EcoRI-generated fragments of pRR12 (A and H) are included as standards. The arrow indicates the PstI fragment C common to pDPT101 and its recombinant plasmid derivatives.*

ments and transformation into an *E. coli* K-12 host, clones resistant to either Cm or Sp were selected. The recombinant plasmids isolated from these clones were of two types, one carrying Sm^r/Sp^r (Fig. 3E) and the other carrying Cm^r (Fig. 3F). The maps of two representative plasmids, pDPT234 (Sm^r/Sp^r) and pDPT240 (Cm^r), are shown in Fig. 4. We conclude that all the functions required for autonomous replication of the miniplasmid must be included within the region defined by the $PstI_a$ to *BglII* sites, contains the *PstI* fragment C (indicated by arrow in Fig. 3), and has a molecular weight of 1.45×10^6 (2.2 kb). Analogous autonomous replication re-

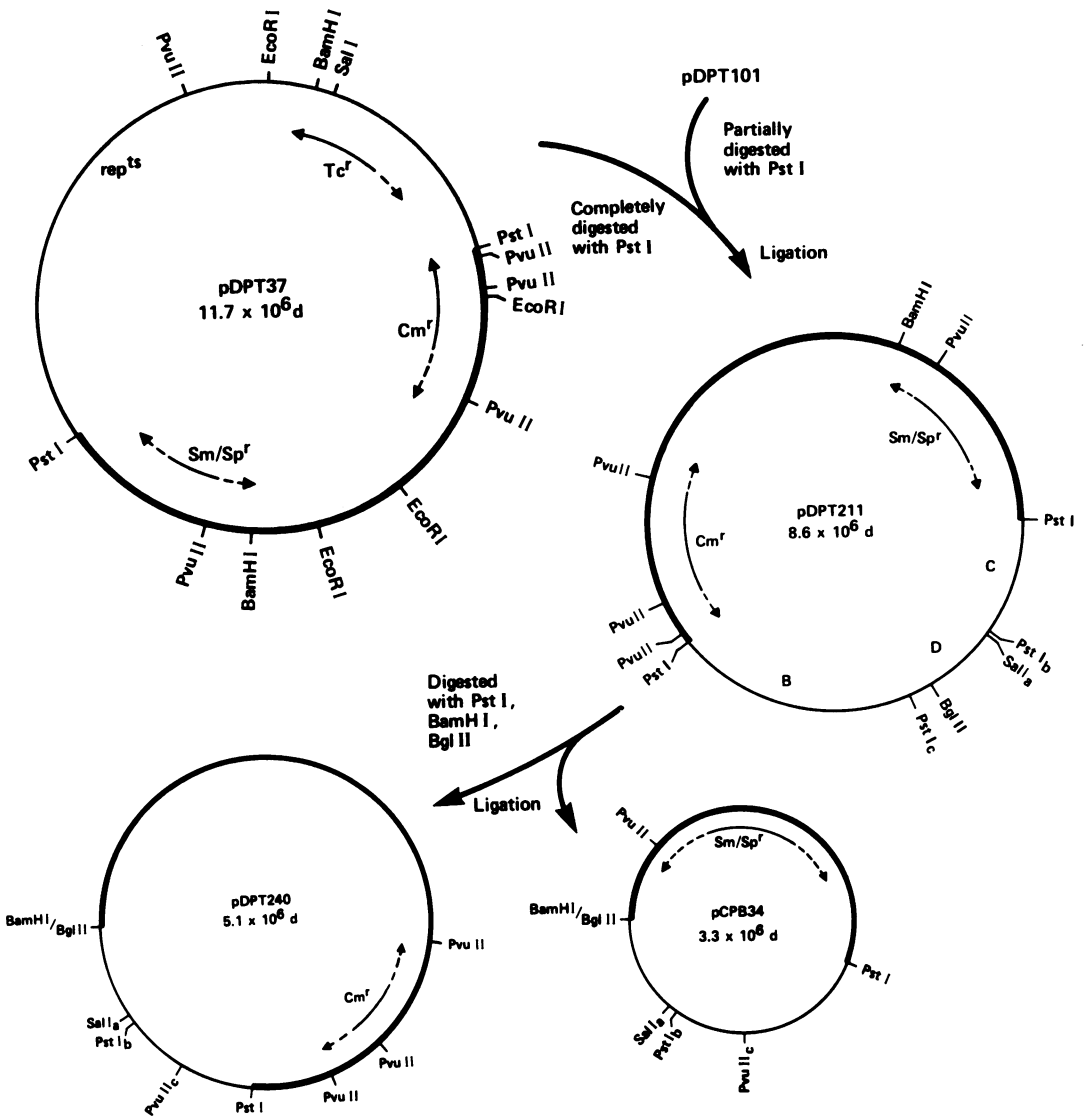

FIG. 4. *Construction of autonomous replicators from pDPT101. pDPT101 DNA, partially digested with PstI, was ligated with pDPT37 DNA which had been completely digested with PstI. Sp^r Cm^r transformants of C600 were selected at 42°C, and Tc^s clones were obtained by screening. pDPT211 DNA, from a representative transformant, was digested with PstI, BamHI, and BglII and ligated, resulting in the formation of pDPT234 (Sp') and pDPT240 (Cm'). The thin segments on pDPT211, -234, -240 are those regions derived from pDPT101. The thick segment on these plasmids is that region derived from the PstI fragment obtained from pRR12 which carries Cm^r and Sm^r/Sp^r. The site labeled BamHI/BglII on pDPT234 and -240 represents the junction at which the BamHI and BglII sites were fused in vitro but which can no longer be cleaved by either endonuclease.*

gions have also been derived from NR1 (data not shown).

Identification of specific sites essential for replication. To determine whether autonomous replication of the segments cloned in pDPT234 and pDPT240 requires structural continuity of the *SalI*, *PstI*, or *PvuII* sites located within the region of replication activity, the following experiments were performed.

The requirement for continuity of the $SalI_a$ site was tested by introducing a segment of DNA into the *SalI* sites on the miniplasmid. The *SalI* fragment inserted contained the structural gene for Km^r and was obtained from pDPT31 (Fig. 5). This plasmid was constructed from pSC305, a derivative of pSC105 (5) that is defective in replication at 42°C and carries an *Eco*RI fragment from R6-5, which contains the Km resistance determinant (18). pSC305 DNA was cut with *Eco*RI, ligated, and transformed into C600 at 30°C; two of six Km^r transformants contained a plasmid in which the *Eco*RI Km^r fragment had been reoriented, so that now a Km^r fragment (the second largest fragment in Fig. 6B) could be obtained from pDPT31 by digestion with *SalI*.

pDTP31 DNA, digested with *SalI*, and pDPT101 DNA, partially digested with *SalI*, were mixed and ligated; Km^r transformants were selected at 42°C and screened for Tc^s clones. In this manner, miniplasmids carrying the Km^r *SalI* fragment were obtained. Plasmid DNA from Km^r Tc^s clones was isolated and analyzed by digestion with *SalI* to confirm that these

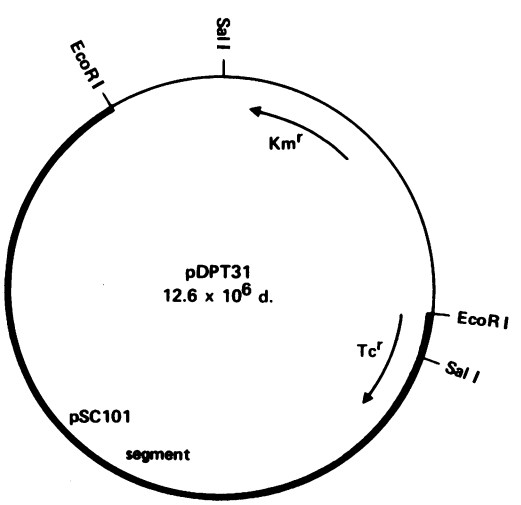

FIG. 5. *Map of pDPT31. pSC305 DNA was digested with EcoRI; after ligation, transformants were selected and screened to obtain a plasmid with the Km^r EcoRI fragment (thin segment) in the alternate orientation relative to the pSC301 (thick segment).*

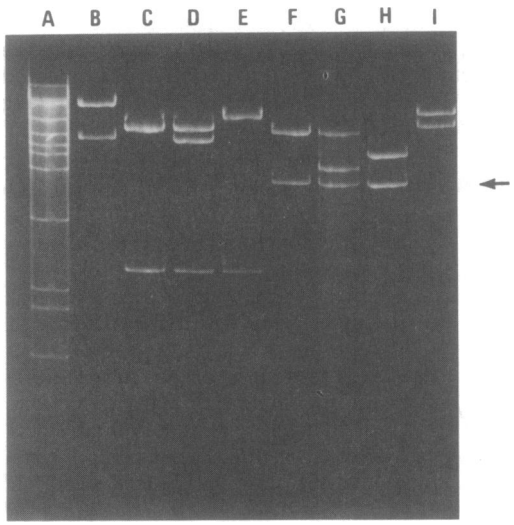

FIG. 6. *Insertion of DNA into SalI sites on pDPT101. DNA containing Km^r from pDPT31 was inserted into pDPT101 as described in the text. The EcoRI-generated fragments of pRR12 (A) are included as molecular weight-standards, and SalI-digested pDPT31 (B) and pDPT101 (C) DNAs are shown for comparison. Representative recombinant plasmid DNAs of pDPT143 (D) and pDPT152 (E) were digested with SalI and subjected to electrophoresis on a 1% agarose gel. Digestion of pDPT152 (F), pDPT143 (G), pDPT101 (H), and pDPT31 (I) DNA with EcoRI and BglII demonstrates the conservation of a fragment, indicated by the arrow, on pDPT101, -143, and -152 which contains the $SalI_a$ site.*

miniplasmids (e.g., pDPT143) contained the Km^r *SalI* fragment (cf. Fig. 6B and D). Digestion of these DNAs with *Eco*RI and *Bgl*II indicated that the $SalI_a$ site was intact in these plasmids because they contained an *Eco*RI-*Bgl*II fragment identical to one from pDPT101 (indicated by arrow in Fig. 6G and H).

The experiment was repeated but, in addition, the pDPT31 and pDPT101 DNAs were completely digested with *Eco*RI before ligation. The results obtained with the plasmids (e.g., pDPT152, Fig. 6E) examined in this experiment were similar to those just described using only *SalI*. In all, a minimum of six independently derived plasmids have been examined, and all produce a fragment (indicated by arrow in Fig. 6F and H) identical in size upon digestion with *Eco*RI and *Bgl*II to the one from the miniplasmid which contains the $SalI_a$ site. These results imply that preservation of the $SalI_a$ site is essential for autonomous replication, whereas $SalI_b$ is not required. The second experiment with *SalI* and *Eco*RI also demonstrates that the DNA segment extending from the *Eco*RI site to the $SalI_b$ site on pDPT101 is sufficient for replica-

tion, as was anticipated because this region is contained within the smaller miniplasmids and is common to the *Eco*RI-generated replication region of R6-5.

The possibility that the *Pst*I$_b$ site may be at a location that is essential was examined by analyzing the autonomous replicators obtained after scrambling pDPT211 with *Pst*I, *Bam*HI, and *Bgl*II. If continuity of the *Pst*I$_b$ site is not essential for replication, then the orientation of the *Pst*I fragments after scrambling should be random. However, double digestions of 12 independently produced recombinant plasmids, including pDPT211, -234, and -240, with *Sal*I and *Pvu*II produced the same unique fragment (0.35 Md; indicated by arrow in Fig. 7C–E) as is produced with pDPT101 DNA when digested similarly, indicating that the *Pst*I site is conserved or regenerated in the formation of these recombinant plasmids. The recombinant plasmids generated independently with *Pst*I alone, including pDTP211, also produced a unique fragment (0.6 Md; data not shown), identical in size to a fragment from the miniplasmid after digestion with *Bgl*II and *Sma*I, which is consistent with the above interpretation.

Autonomously replicating plasmids have been constructed by combining the separately cloned *Pst*I fragments C and D of pDPT101 and the *Pst*I fragment from pDPT37 carrying Cm^r Sm^r/Sp^r. The construction of the recombinant plasmids, pBR322 vectors carrying *Pst*I D (pDPT251) and *Pst*I C (pDPT255), was described earlier. pDPT251 and pDPT255 DNAs, as well as pDPT37 DNA, were digested with *Pst*I, mixed, and ligated. Cm^r Sp^r clones were selected at 42°C after transformation of C600 and then screened to insure that they were Tc^s. Plasmid DNA from one clone, pDPT270, contained *Pst*I C and D as expected (cf. Fig. 3C with 3G) and produced a fragment (indicated by arrow in Fig. 7F) similar to the one produced by digestion of pDPT101 DNA with *Pvu*II and *Sal*I (Fig. 7B), as described above. Thus, *Pst*I C and D necessarily derived from different plasmids must be rejoined in the same orientation as in pDPT101, and the *Pst*I$_b$ regenerated to produce a DNA segment capable of autonomous replication. A total of 13 plasmids independently produced with *Pst*I have been shown to contain the *Pst*I$_b$ site intact. Thus, the DNA sequence relationships that constitute the *Pst*I$_b$ site must remain intact for autonomous replication to occur.

To determine whether continuity of the *Pvu*II$_c$ site on *Pst*I fragment C of the miniplasmid is also essential for replication, pDPT240 was scrambled with *Pvu*II, which produces three fragments from this plasmid. The derivatives all still retained a *Pst*I fragment indistinguishable from *Pst*I C of pDPT101 (data not shown), indicating that the *Pvu*II$_c$ site is conserved or regenerated in these derivatives. Similarly, pDPT211 was scrambled with *Pvu*II or *Pvu*II, *Bgl*II, and *Bam*HI; the Sp^r clones from each scrambling were examined and found to have an intact *Pst*I C fragment (data not shown). If continuity of the DNA sequences comprising the *Pvu*II$_c$ site were not essential, the *Pvu*II fragments would be expected to occur in either of two possible orientations, or in different orders, on the various *Pvu*II-scrambled plasmids. Since only one order and orientation was observed in 10 independently produced clones, an intact *Pvu*II$_c$ site also appears to be required for autonomous replication. We do not presently know whether the *Sma*I$_a$ or *Sma*I$_b$ sites are essential.

Incompatibility. In testing for the expression of incompatibility functions by cloned fragments, two tests were used. We refer to the first test as the establishment test because it measures the ability of an incoming plasmid to establish itself in the presence of a resident plasmid. The test is performed by transforming a *recA* host carrying a resident plasmid (e.g., pDPT103) with DNA from the plasmid to be tested, the incoming plasmid. Transformants are obtained by selecting for the antibiotic resist-

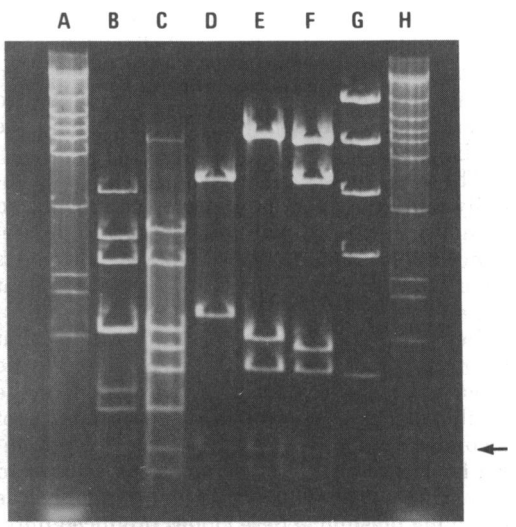

FIG. 7. *Conservation of a PvuII-SalI fragment on autonomous replicators of pDPT101. Digestions of pDPT101 (B), pDPT211 (C), pDPT234 (D), pDPT240 (E), pDPT270 (F), and pDPT37 (G) DNA with PvuII and SalI were subjected to electrophoresis on a 1% agarose gel to demonstrate that a 0.35-Md fragment, indicated by the arrow, is common to pDPT101 and autonomous replicators derived from pDPT101. The EcoRI-generated fragments of pRR12 (A and H) are included as standards.*

ance determinant on the incoming plasmid. In addition, transformants that contain both the incoming and resident plasmids can be selected. The ratio of the frequencies of transformants carrying both plasmids to transformants selected for carrying only the incoming plasmid is analogous to the incompatibility index of Timmis et al. (43). When the value of this index is low, the degree of incompatiblity reaction is considered high.

If the incoming and resident plasmids are incompatible, then selection for the establishment of the incoming plasmid should result in the loss of the resident plasmid. This can be detected by replica-plating the transformants (which were selected for carrying the incoming plasmid) onto plates containing an antibiotic that will test for the presence of the resident plasmid. By using replica-plating, plate-to-plate variability in the efficiency of plating can be avoided, especially when comparisons must be made between incoming plasmids that carry different antibiotic resistances.

The second test, which we term the maintenance test, examines the ability of two plasmids to be maintained in the same cell after establishment. Transformants that have been selected to contain both the resident and incoming plasmids are transferred to broth containing antibiotics that select for both plasmids. After reaching stationary phase, the culture is diluted 10^6-fold into antibiotic-free medium and allowed to grow to stationary phase again. The culture is assayed on nonselective plates and is replica-plated to determine the frequency of cells containing one or the other of the two plasmids.

These two tests have been applied sequentially so that if incompatibility was not observed with the establishment test, then the maintenance test was performed. Only if both tests were negative was it concluded that a fragment did not express incompatibility. If the results of either test were positive, it was concluded that incompatibility functions were present on the fragment being tested. In all cases where the establishment test gave clear-cut evidence of incompatibility, the frequency of clones containing both plasmids was less than 10^{-2} compared with those cells selected to contain only the incoming plasmid. These clones produced unreliable results when examined by the maintenance test; the unreliability of the maintenance test in such instances is probably the result of mutation or deletion of incompatibility functions on one of the two plasmids being forced to coexist by the selection procedure (see Discussion).

The miniplasmid pDPT101 was inferred to express plasmid incompatibility functions because it segregated from the copy number mutant plasmid, pRR21, from which it was derived (40). To test directly the expression of incompatibility by the miniplasmid, a *recA* strain of *E. coli* was transformed simultaneously with pDPT103 and pDPT104 DNA (pDPT103 and pDPT104 are Km^r and Sp^r derivatives, respectively, of pDPT101, constructed as described in Materials and Methods). Doubly transformed cells were selected as Km^r Sp^r and were maintained initially under continuous selection by growth in the presence of both antibiotics. After growth in the absence of either antibiotic for approximately 20 generations, 53% of the colonies tested had lost either pDPT103 or pDPT104, indicating that the miniplasmid can specify all the components necessary for incompatibility as determined by the maintenance test (Table 3). pDPT103 has also been shown to be incompatible with NR1 (unpublished data).

Incompatibility expressed by cloned DNA fragments. Using the Km^r miniplasmid derivative, pDPT103, as the resident test plasmid, we tested various recombinant plasmids for their ability to exhibit incompatibility. The hybrid plasmid, pDPT106, which contains pBR313 and the entire miniplasmid linked at their *Hin*dIII sites, and pDPT104 were incompatible with pDPT103 (Table 3). The constructed plasmids, pDPT111 and pDPT113, each containing one of the two *Sal*I fragments from pDPT101 linked to pBR313, were tested against pDPT103. The smaller *Sal*I fragment on pDPT111 was found to express incompatibility functions, whereas the larger *Sal*I fragment on pDPT113 did not. The orientation of insertion of the *Sal*I fragments on pBR313 did not alter the result. When the smaller *Sal*I fragment was in the opposite orientation on pBR313 (e.g., pDPT112), it still showed incompatibility with pDPT103 with the establishment test (Table 3).

Similarly, pDPT122, which contains the *Eco*RI-*Bgl*II (reading clockwise on the pDPT101 map) fragment carried on pBR313 and which can rescue pBR313 under conditions that are nonpermissive for pBR313 replication, was also incompatible with pDPT103. pDPT133, which consists of pBR313 carrying the *Sma*I$_b$-*Bgl*II fragment, also expressed incompatibility; however, pDPT132, which contains the *Bgl*II-*Sma*I$_c$ fragment, failed to do so by either test. When the *Pvu*II$_c$-*Bcl*II fragment cloned onto pBR322 was tested (e.g., pDPT165), it also expressed incompatibility (Table 3).

The four *Pst*I fragments of pDPT101 cloned onto pBR322 were tested, and only those plasmids carrying *Pst*I fragment D expressed incompatibility. Plasmids carrying this *Pst*I fragment in either orientation (e.g., pDPT251 and

TABLE 3. *Incompatibility tests*[a]

Plasmid	Antibiotic resistance expressed by incoming plasmid	Establishment test		Maintenance test			
		Ab^r[b] Km^r/Ab^r	% Ab^r Km^s	% Ab^r Km^r	% Ab^r Km^s	% Ab^s Km^r	% Ab^s Km^s
pDPT104	Sp	1×10^{0}	0	45	18	35	2
pDPT106	Ap	1.4×10^{0}	68	62	33	4	1
pDPT111	Ap	$<4.5 \times 10^{-4}$	100				
pDPT112	Ap	2.5×10^{-4}	100	100	0	0	0
pDPT113	Ap	3.8×10^{-1}	0	100	0	0	0
pDPT122	Ap	9.6×10^{-1}	0.3	10	82	7	1
pDPT132	Ap	3.5×10^{-1}	1.3	99	0	1	0
pDPT133	Ap	2.8×10^{-3}	99.5	10	3	83	5
pBR313	Ap Tc	6.3×10^{-1}	0.8	99	0	1	0
pDPT165	Ap	2.4×10^{-2}	99.5	0	0	97	3
pDPT171	Ap	1×10^{0}	0	100	0	0	0
pDPT251	Tc	6.3×10^{-4}	100	0	100	0	0
pDPT261	Tc	1×10^{-4}	100	35	64	0	0
pDPT255	Tc	1.6×10^{-1}	3.2	100	0	0	0
pDPT180	Ap	1×10^{-1}	1	100	0	0	0
pDPT184	Ap	2×10^{-4}	100	0	0	99	1
pBR322	Ap Tc	5.8×10^{-1}	0	100	0	0	0
pDPT234	Sp	3.4×10^{-1}	4.7	16	1	84	0
pDPT270	Cm Sp	1.3×10^{0}	1.3	10	1	90	0

[a] Transformation was performed with FL1699 (pDPT103) as the *recA* recipient with a Km^r resident plasmid. The percentages given are representative values; most incompatibility measurements have been done two or more times. An analysis of the variance with those plasmids for which four or more comparable tests have been performed indicates that the standard error usually does not exceed ±5%. The incompatibility tests were performed by transforming FL1699 (pDPT103) with 5 μg of plasmid DNA.

[b] Ab^r, Antibiotic resistance.

pDPT261) were examined, and again orientation of insertion did not alter the results (Table 3). Incompatibility was also expressed by an analogous NR1-derived *Pst*I D fragment cloned onto pBR322 in either orientation (data not shown).

The *Sal*I-*Bgl*II fragments of pDPT101 were cloned onto pBR322 and tested for incompatibility. The smaller *Sal*I$_a$-*Bgl*II fragment contained on pDPT184 expressed incompatibility, whereas the larger *Bgl*II-*Sal*I$_b$ fragment on pDPT180 was compatible with pDPT103. The autonomous replicators cloned from pDPT101 (e.g., pDPT234 and pDPT270) have also been tested and found to express incompatibility (Table 3).

DISCUSSION

A 1.45-Md (2.2-kb) region isolated from a miniplasmid derived from a copy number mutant of NR1 has been found capable of autonomous replication. This region corresponds to DNA between the *Pst*I$_a$ site and the *Bgl*II site of the miniplasmid and can be translated into the R100-1 (or NR1) heteroduplex map coordinates. Mickel et al. (21) have shown that a series of miniplasmids they obtained from pRR12, a copy number mutant of NR1, contain the insertion element which corresponds to IS*1*$_b$ (14, 33). A structural analysis of various independently derived miniplasmids has implicated the terminus of IS*1*$_b$ in miniplasmid formation (21). Although the particular miniplasmid described, pDPT101, has not been examined directly for the presence of IS*1*, extrapolation of the data of Mickel et al. (21) has led us to include IS*1*$_b$ on the miniplasmid map (Fig. 8).

Earlier, the *r*-determinant proximal terminus of IS*1*$_b$ of R100-1 or NR1 was assigned the coordinate of 12.9 kb by Sharp, Cohen, and Davidson (SCD) (36). To facilitate comparison of DNA regions on different plasmids in the R100/NR1/R1/R6 series, it was agreed recently that this location be reassigned the coordinate of 0/89.3 kb (21, 44). The *Eco*RI site on the miniplasmid is located at 87.2 kb (10.8 kb using SCD coordinates). The 2.2-kb replication region is situated between the *Pst*I$_a$ site at 85.9 kb (9.5 kb SCD) and the *Bgl*II site at 83.7 kb (7.3 kb SCD) (Fig. 8) and presumably contains the *repA* locus described by Yoshikawa (48).

In cloning the DNA segments that were capable of autonomous replication, two different antibiotic resistance determinants were used for selection. Thus, it is highly likely that information contributed by the DNA segment carrying the resistance determinant is not required to obtain autonomous replication and that the *Pst*I$_a$-*Bgl*II region is sufficient for autonomous replication. Within the replication region, three separate endonuclease sites, *Sal*I$_a$ (84.4 kb),

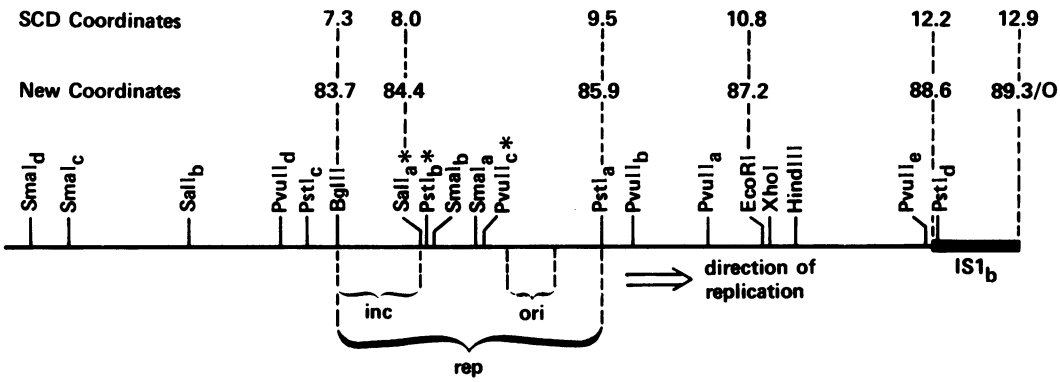

FIG. 8. *Summary of results of mapping incompatibility and replication functions on pDPT101. The map of pDPT101 is shown with the appropriate heteroduplex map coordinates assigned as described in the Discussion. The regions labeled inc and rep are those regions identified in this study as the minimal amount of DNA required for expression of incompatibility and replication functions, respectively. The asterisk indicates those sites identified tentatively as essential for autonomous replication. The origin of replication determined by Silver et al. (38) and by Ohtsubo et al. (28) lies within the bracketed region labeled ori, and the direction of replication is indicated by the arrow.*

*Pst*I$_b$ (84.7 kb), and *Pvu*II$_c$ (84.9 kb), have been tentatively identified as being essential for replication (Fig. 8). These sites may identify the location of a site or structural gene that is required for replication.

Recently, origins of replication utilized by R100-1 (38) and by miniplasmids (28) replicating in *E. coli* have been mapped. These origins are located within the *Pst*I fragment C and are indicated as *ori* on the miniplasmid map (Fig. 8). In both studies, replication was unidirectional and proceeded toward IS*1*$_b$. Although the map positions determined previously for the origin of replication utilized in *E. coli* (28, 38) suggest that they may be 0.4 kb apart, these origins may in fact be coincident loci that have been assigned different locations because of the inexactness inherent in localizing such sites on large plasmids or in translating coordinates from one plasmid map to another. The origins of replication on NR1 (or on its copy number mutant, pRR12) that are reported to be utilized in *P. mirabilis* (30, 46) either do not lie within the 2.2-kb replication region identified in our studies or, alternatively, must be extremely close to the *Bgl*II site, if within this region.

The fragment that is identified as expressing the FII plasmid incompatibility function(s) and which is common to all plasmids incompatible with pDPT103, is located between the *Sal*I$_a$ site at 84.4 kb and the *Bgl*II site at 83.7 kb (Fig. 8). Reports which map incompatibility on R100-1 (29, 49) are in agreement with our results; however, the present investigation provides a more precise localization of the gene(s) that specifies incompatibility.

The *Sal*I$_a$-*Bgl*II fragment has a molecular weight of approximately 0.46×10^6 (0.7 kb). If this fragment codes for a single protein, the maximum size of this protein could be no more than 23,000 daltons. Examination of proteins synthesized by minicells containing pDPT234, -240, and -251 has revealed a protein of approximately 14,500 daltons (P. Lemaux, personal communication).

Recent studies by Timmis et al. (41) involving the cloning of replication and incompatibility regions derived from R6-5 have yielded results similar to those reported here: *Pst*I fragments 4 and 6 (analogous to *Pst*I fragments C and D of pDPT101) of the pSC102 plasmid (5, 44), which contains an *Eco*RI-generated replication region from R6-5, are required for autonomous replication. The incompatibility functions of pSC102 are contained on a *Sal*I fragment and analogous *Pst*I fragment. However, expression of incompatibility functions was detected for only one of two possible orientations of these fragments, as opposed to our finding that the orientation of the *Pst*I D and the smaller *Sal*I fragments of pDPT101, or the analogous *Pst*I and *Sal*I fragments of NR1, has no effect on expression of incompatibility functions.

Similar experiments involving the cloning of autonomous replication regions from copy number mutants of R1 by Kollek et al. (17) have resulted in assignment of R1 replication functions to a 1.8-kb region contained on two *Pst*I fragments (E plus F). Neither of these two *Pst*I fragments, when cloned separately on pBR322, was found to express incompatibility, indicating that incompatibility functions are not in this region or are distributed between the two *Pst*I fragments. Direct comparison of these results to

ours is difficult because the *Pst*I maps of the replication regions from NR1 and R1 do not appear to be identical. Kollek et al. have also reported that their "basic replicon" cloned from pKN102, a copy number mutant of R1, does not express incompatibility. However, in the cloning experiments reported here for NR1 and by Timmis et al. (41) for R6-5, no autonomous replication regions have been isolated which do not also contain incompatibility.

In assigning incompatibility functions to cloned fragments, we have used two tests that we believe measure incompatibility reactions of different degrees. The establishment test can detect the high degree of incompatibility that occurs when fragments cloned onto high-copy-number vectors are tested. The weaker reactions, such as those observed with autonomous replicators, require the use of the maintenance test to detect incompatibility. However, the maintenance test can be unreliable; regions that express incompatibility by the establishment test may appear to be compatible in the maintenance test (see results for pDPT112, Table 3). Since the transformants that contain both the resident and the incoming plasmids are rare when the plasmids are severely incompatible, selection for such transformants can result in cells which contain compatible plasmids due to a mutation/deletion in one of the incompatible plasmids (initially observed with pSC101:: miniColE1 hybrids by P. Meacock, this laboratory). This was the case when clones containing pDPT103 and pDPT165 were examined by the maintenance test. The plasmids segregated from some clones but not from others. The plasmids from a nonsegregating clone were examined; pDPT165 was found to have suffered a deletion sufficient to have removed most if not all of the cloned *Pvu*II$_c$-*Bgl*II fragment (unpublished data). The pDPT165 plasmid containing the deletion, pDPT171, was retested and was found to be compatible with pDPT103 (Table 3). Such problems inherent in using a single test for incompatibility may be the reason for the differences between results we have reported here and those of Timmis et al. (41) and Kollek et al. (17).

Although evidence is presented in Table 3 which shows that the recombinant plasmid can be lost asymmetrically in the maintenance test for incompatibility, no plasmid DNA segment uniquely produces this phenomenon. Therefore, our results do not support the postulation by Timmis et al. (41) of another mechanism, such as plasmid partitioning, to explain the asymmetric loss of recombinant plasmids in incompatibility tests.

The size of the miniplasmid replication region described here is considerably larger than the 363-base-pair replication region obtained from a high-copy-number ColE1-related plasmid, pMB1 (2). The difference in size is not surprising because the pMB1 replication region can replicate in the presence of C_m (2) and thus does not appear to require continuing protein synthesis; like ColE1 (10), it may not require any plasmid-coded proteins. However, an *Hae*II site identified on the pMB1 replication region must remain intact for replication to occur (2). Studies of bacteriophage lambda replication confirm that λ-coded proteins are required for replication with the origin located in the structural gene *O* (9). The replication of NR1 appears to be more similar to lambda replication than to ColE1.

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