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Cloning of Replication, Incompatibility, and Stability Functions of R Plasmid NR1

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The region of R plasmid NR1 that is capable of mediating autonomous replication was cloned by using *Eco*RI, *Sal*I, and *Pst*I restriction endonucleases. The only *Eco*RI fragment capable of mediating autonomous replication in either a *pol*$^+$ or a *polA* host was fragment B. *Sal*I fragment E joined in native orientation with the part of *Sal*I fragment C that overlapped with *Eco*RI fragment B, and also two contiguous *Pst*I fragments of sizes 1.6 and 1.1 kilobases from *Eco*RI fragment B-mediated autonomous replication. When these individual *Sal*I fragments were cloned onto plasmid pBR313 or the individual *Pst*I fragments were cloned onto plasmid pBR322, none of these single fragments could rescue the replication of the ColE1-like vectors in a *polA* host, even in the presence of a compatible "helper" plasmid derived from a copy mutant of NR1. In contrast to the results reported for closely related R plasmid R6, *Eco*RI fragment A of NR1 could not rescue the replication of ColE1 derivative RSF2124 in a *polA*(Am) mutant or in a *polA*(Ts) mutant at the restrictive temperature. Although capable of autonomous replication, *Eco*RI fragment B of NR1 (or smaller replicator fragments cloned from it by using other restriction enzymes) was not stably inherited in the absence of selection for the recombinant plasmid. When *Eco*RI fragment B was ligated to *Eco*RI fragment A of NR1, the recombinant plasmid was stable. Thus, *Eco*RI fragment A contained a stability (*stb*) function. The *stb* function did not act in *trans* since *Eco*RI fragment B was not stably inherited when a ColE1 derivative (RSF2124) ligated to *Eco*RI fragment A was present in the same cell. A cointegrate plasmid consisting of *Eco*RI fragment B of NR1 ligated to RSF2124 was also not stably inherited, whereas only *Eco*RI fragment B was unstable when both RSF2124 and *Eco*RI fragment B coexisted as autonomous plasmids in the same cell. The incompatibility gene of NR1 was shown to be located within the region of overlap between *Sal*I fragment E and the *Pst*I 1.1-kilobase fragment. A copy mutant of NR1 (called pRR12) was found to have greatly reduced incompatibility with NR1; this Inc$^-$ phenotype is *cis* dominant.

Most self-transmissible plasmids are present in a low copy number in host cells (4, 10, 25, 26). Since these plasmids are stably inherited, their replication must be controlled by a mechanism which ensures that the plasmids are replicated during each cell division cycle and that at least one replica then segregates to each daughter cell at division. The nature of the mechanism which controls DNA replication and segregation is presently not understood in any detail. Although a number of models have been proposed, most available data seem more consistent with a negative control mechanism rather than a positive control mechanism. Pritchard and his colleagues have proposed a repressor dilution model which postulates that an inhibitor or repressor specified by a gene on a replicon interacts with a specific receptor on the DNA to control the frequency of initiation of replication (23, 24). In addition to accounting for plasmid copy number control, this model can account for the inability of two plasmids which share the same replication control mechanism to coexist stably in descendants of the same host cell (incompatibility). Mutations in either the repressor or its binding site would lead to less stringent control; consequently plasmid copy number would be increased. Plasmid copy number mutants have been isolated for a number of plasmids, and many, but not all, have been found to have altered incompatibility properties (23, 34; this study; C. Barton, A. Easton, J. McKell, and R. Rownd, unpublished data).

R plasmid NR1 (also called R100 and R222) is a self-transmissible plasmid, 90 kilobases (kb)

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in size, which belongs to the FII incompatibility group. NR1 confers resistance to chloramphenicol (Cm), fusidic acid (Fa), streptomycin/spectinomycin (Sm/Sp), sulfonamides (Su), tetracycline (Tc), and mercuric ions (Hg^{2+}). We have previously determined the locations of the cleavage sites of several restriction endonucleases and mapped the locations of the drug resistance genes of NR1 and related FII R plasmids R6 and NR84 (1, 18, 28). Timmis et al. (31) have shown that *Eco*RI fragment B (12 kb) of R6-5 is capable of autonomous replication and confers FII incompatibility. Miniplasmids which contained only 5 to 6 kb of *Eco*RI fragment B have been isolated in vivo from several copy number mutants of NR1 (17, 30). These miniplasmids are capable of autonomous replication and are incompatible with the copy number mutant. In this communication we present cloning experiments which localized the replication and incompatibility functions of NR1 to a 2.7-kb region within *Eco*RI fragment B which consisted of two contiguous *Pst*I fragments 1.6 and 1.1 kb in size. Neither *Pst*I fragment was capable of autonomous replication alone, although the 1.1-kb fragment conferred incompatibility. The 2.7-kb replicator region, as well as the larger *Eco*RI fragment B on NR1 was not stably inherited in the absence of selection and required a function, located on *Eco*RI fragment A, for stability (*stb* function). A copy number mutant of NR1 was shown to be compatible with NR1 (or its derivatives), and this Inc^- phenotype was *cis* dominant. The *cop* mutant also had much less dependence on the *stb* function for stable inheritance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and previously constructed plasmids are listed in Table 1. The media and antibiotic concentrations were as described previously (18). Penassay agar plates were made by adding 1.4% agar (Difco Laboratories) to Penassay broth (Difco).

Isolation and manipulation of plasmid DNA. DNA was purified by the Triton X-100 cleared-lysate procedure (14). Stationary-phase cells cultured in Penassay broth were used as a source of plasmid DNA.

Restriction endonucleases *Eco*RI and *Sma*I were gifts from F. W. Farrelly and J. T. Barnitz. *Sal*I endonuclease and T4 ligase were purchased from Miles Laboratories; *Bgl*II, *Hae*III, *Hpa*II, and *Pst*I endonucleases were purchased from New England Biolabs; and *Hin*dIII endonuclease was purchased from Bethesda Research Laboratories. Digestion of DNA by these enzymes was performed as recommended by the suppliers. Ligation of restriction fragments, agarose gel electrophoresis, and transformation of *Escherichia coli* cells have been described previously (18).

TABLE 1. *Bacterial strains and plasmids*

Strain/plasmid	Marker	Genotype/description	Reference/source
E. coli			
KP245		*met trp his thy lac gal*	18
KP435		*trp thy ilv rpsL recA*	T. Miki
JG112		*met thy rpsL polA*	19
DK100ts214		*his arg met leu thy xyl lac rpsL polA*(Ts)	12
Plasmid			
NR1	Cm^r, Fa^r, Hg^r, Sm^r/Sp^r, Su^r, Tc^r	Natural isolate	21
pRR37	Ap^r, Cm^r, Fa^r, Hg^r, Sm^r/Sp^r, Su^r, Tc^r	NR1::Tn*3* (insertion into *Sal*I fragment D in *Eco*RI B)	This paper
pRR250	Ap^r, Tc^r	RTF[a] of NR1::Tn*3* (insertion into *Eco*RI fragment B)	This paper
pRR12	Cm^r, Fa^r, Hg^r, Sm^r/Sp^r, Su^r, Tc^r	*cop-12* mutation of NR1	20
pBR313	Ap^r Tc^r		2
pDW1	Ap^r	pBR313 + *Sal*I fragment E of NR1	7
pDW2	Ap^r	pBR313 + *Sal*I fragment D of NR1	7
pBR322	Ap^r Tc^r		3
RSF2124	Ap^r	ColE1::Tn*3*	27
pRR101	Ap^r, Km^r/Nm^r	RSF2124 + *kan* fragment of R6-5	18
pRR138	Ap^r	RSF2124 + *Eco*RI fragment A of NR1	18
pRR149	Ap^r	RSF2124 + *Eco*RI fragment B of NR1	18

[a] The RTF is an NR1 derivative which has deleted the region between IS*1a* and IS*1b* which contains genes for resistance to chloramphenicol (Cm), fusidic acid (Fa), streptomycin/spectinomycin (Sm/Sp), sulfonamides (Su), and mercuric ions (Hg).

Plasmid copy number determinations. Copy numbers of NR1, pRR12, and the *Eco*RI B replicator fragment cloned from these plasmids were determined by using ethidium bromide-cesium chloride gradients as described previously (35).

Incompatibility assay. To determine whether two plasmids were incompatible, a *recA E. coli* strain harboring one of the two plasmids was transformed with the DNA of the second plasmid. After transformation, the cells were cultured for 90 min in drug-free L broth, and appropriate dilutions of these transformed cultures were spread on nutrient agar plates containing a single antibiotic to which resistance was conferred only by the donor transforming DNA. After 36 h of incubation at 37°C, 10 individual transformant colonies were suspended in dilution buffer and then streaked onto drug-free Penassay agar plates. From each of these streaks 10 single colonies were picked, using sterile toothpicks, and patched onto drug-free nutrient agar plates. The patches were tested for the drug resistances conferred by either the donor or the resident plasmid by replica plating to nutrient agar plates containing appropriate antibiotics. Using this procedure, we examined cells for their drug resistance pattern approximately 30 generations after the donor plasmid DNA was introduced by transformation into the recipient cells which initially harbored another plasmid.

Plasmid stability assays. To examine the stability of plasmid inheritance, cells harboring various plasmids were cultured in Penassay broth containing a low concentration of an antibiotic to which resistance was conferred by the plasmid. These cells were then repeatedly subcultured in drug-free Penassay broth by being diluted 10^6-fold and incubated overnight at 37°C until the cultures reached stationary phase. After each subculture, appropriate dilutions of the cells were spread on drug-free Penassay agar plates, and the drug resistance patterns of individual colonies were tested by replica-plating as described above for the incompatibility assay.

RESULTS

***Eco*RI fragments of NR1 capable of autonomous replication.** To isolate the *Eco*RI fragments of NR1 that were capable of autonomous replication, NR1 DNA and pRR101 DNA (RSF2124 plus the fragment of R plasmid R6-5 specifying resistance to kanamycin and neomycin [Km/Nm] ["*kan* fragment"] [11]) were digested separately with restriction endonuclease *Eco*RI (Fig. 1a and b), mixed, and ligated. The ligated DNA was then used to transform *E. coli* JG112 *polA* to Nm^r. Since RSF2124 cannot replicate in a *polA* host (12), all transformants should have carried a plasmid which had a replicator fragment derived from NR1. A similar experiment was also carried out with pRR12, a copy mutant of NR1 (18, 20). Thirteen Nm^r transformants from the experiment with NR1 and 11 Nm^r transformants from the experiment with pRR12 were characterized, and all were found to harbor recombinant plasmids consisting of *Eco*RI fragment B of the R plasmid DNA and the *kan* fragment (Fig. 1c). Further analysis with *Sal*I restriction endonuclease revealed that all of these plasmids contained the two *Sal*I fragments within *Eco*RI fragment B (*Sal*I fragments D and E). Also present were *Sal*I fragments of molecular weights expected from the joining, in either orientation, of *Eco*RI fragment B and the *kan* fragment which contained a single cleavage site for *Sal*I (data not shown). In similar experiments, NR1 DNA was digested with *Eco*RI, ligated, and used to transform recipient cells to either Su^r or Sm^r. All transformants were found to harbor plasmids which contained *Eco*RI fragments B and G of NR1 (Fig. 1d). *Eco*RI fragment G confers resistance to sulfonamides and streptomycin/spectinomycin (18). The *Eco*RI and *Sal*I maps of NR1 are shown in Fig. 2; the *Eco*RI fragment pattern of pRR12 was indistinguishable from that of NR1. The locations of the *Sal*I, *Bgl*II, *Pst*I, and *Sma*I cleavage sites in *Eco*RI fragment B are shown at the bottom of Fig. 2.

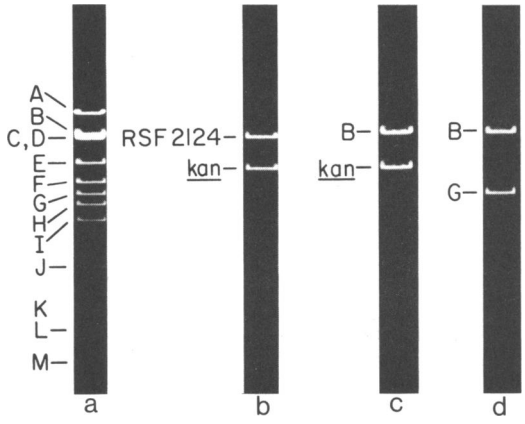

FIG. 1. *Agarose gel electrophoresis of EcoRI fragments of recombinant plasmids prepared from NR1 and pRR101. (a) NR1; (b) pRR101 (RSF2124 plus kan fragment); (c) pRR104 (EcoRI fragment B plus kan fragment; (d) pRR177 (EcoRI fragments B and G).*

Since a *polA* recipient was used in the previous cloning experiments, it was possible that fragments other than *Eco*RI B of NR1 were capable of autonomous replication but that they required DNA polymerase I. The DNA of an NR1 derivative (pRR37) that carried ampicillin transposon Tn*3* in *Sal*I fragment D (*Sal*I-D::Tn*3*), which is within *Eco*RI fragment B (*Eco*RI-B::Tn*3*), was digested with *Eco*RI, ligated, and used to transform *E. coli* KP245 *pol*$^+$ to Su^r, which is conferred by *Eco*RI fragment G. Of 210 Su^r transformants, 199 were also found to be Ap^r. Thus, these transformants presumably carried *Eco*RI fragment B as the replicator frag-

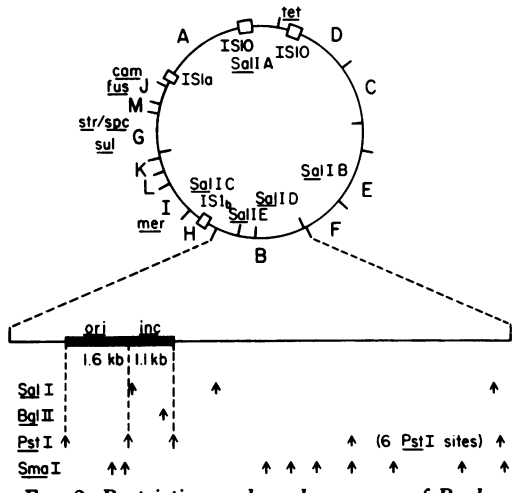

FIG. 2. *Restriction endonuclease map of R plasmid NR1. The EcoRI and SalI restriction sites were determined previously (1, 28). The EcoRI fragments are shown on the outside of the circular map and are designated A through M in order of decreasing size. The SalI fragments are shown in the inside and are designated A through E in order of decreasing size. The locations of the restriction sites within EcoRI fragment B were determined by cleavage of the cloned fragments and miniplasmid derivatives (30) with the indicated enzymes in the appropriate combinations. EcoRI fragments of pRR12 DNA and HaeIII fragments of φX174 DNA were used as molecular weight standards. The locations of the drug resistance genes (18) and the origin of replication in E. coli (22; R. Warren and R. Rownd, unpublished data) have been reported previously. The location of the incompatibility locus was determined in this study and also by Timmis et al. (33) and Taylor and Cohen (29).*

ment. The plasmid DNAs of the 11 Ap^s transformants were analyzed and found to carry the *Eco*RI fragments B::Tn*3* and G. Analysis of these plasmid DNAs with *Sal*I revealed the two *Sal*I fragments contained within *Eco*RI fragment B::Tn*3* (*Sal*I-D::Tn*3* and E) and two *Sal*I fragments expected from the joining of *Eco*RI fragments B and G in either orientation (data not shown). The reason that these 11 transformants did not express ampicillin resistance has not been examined. Taken together, these experiments show that the only *Eco*RI fragment of NR1 capable of mediating autonomous replication in either a *pol*$^+$ or *polA* host was *Eco*RI fragment B.

The copy numbers of NR1, pRR12, and recombinant plasmids consisting of *Eco*RI fragment B of these plasmids ligated to the *kan* fragment (pRR104 derived from NR1 and pRR114 derived from pRR12) were estimated from the proportion of covalently closed circular plasmid DNA in *E. coli* KP245 harboring these plasmids. The copy numbers of NR1 and pRR104 were approximately 1 per chromosome, whereas those of pRR12 and pRR114 were 4 and 6 to 8, respectively.

Timmis et al. (32) have reported that *Eco*RI fragments A and D of the closely related IncFII R plasmid R6-5 can rescue the replication of ColE1 at the restrictive temperature in a *polA*(Ts) host. In our experiments, however, both RSF2124 and pRR138 (RSF2124 plus *Eco*RI fragment A) were cured to the same extent in a *polA*(Ts) host at the restrictive temperature, whereas pRR149 (RSF2124 plus *Eco*RI fragment B) replicated under these conditions (Table 2). Not all of the *polA*(Ts) cells harbored pRR149 in this experiment. As will be discussed subsequently, the *Eco*RI B fragment of NR1 was not stably inherited by host cells, and this fragment also conferred instability on RSF2124 when the two were ligated together to form a hybrid plasmid. We also examined the ability of pRR138 and pRR149 to replicate in *polA*(Am) mutant JG112. The transformation frequency of JG112 to Ap^r, using pRR138 DNA, was less than 5×10^{-6} of the frequency of transformation with the DNA of pRR149 (Table 3). Taken together, these experiments indicate that *Eco*RI fragment A could not rescue the replication of RSF2124 in the *polA* host.

SalI cloning of replicator region of NR1. To locate more precisely the replication functions of NR1, *Eco*RI fragment B was subcloned by using *Sal*I and *Pst*I. *Eco*RI fragment B contains entire *Sal*I fragments D and E but only parts of *Sal*I fragments B and C (which will be

TABLE 2. *Inability of EcoRI fragment A of NR1 to rescue replication of RSF2124 at the restrictive temperature in a polA(Ts) host*[a]

Incubation temp (°C)	Ap^r cells at indicated temperature (%)		
	TS214 (RSF2124)	TS214 (pRR138)	TS214 (pRR149)
Starting culture	100	100	68
30	100	99	60
34	100	100	81
37	91	76	71
40	4	4	82
42	2	10	79

[a] *E. coli* TS214 harboring either RSF2124, pRR138, or pRR149 was cultured in Penassay broth at 25°C. The three cultures were diluted 10^4-fold into Penassay broth and then incubated at either 30, 34, 37, 40, or 42°C until they reached the stationary phase. After appropriate dilution, the cells were spread on drug-free Penassay agar plates and incubated at 25°C. 100 colonies from each plate were then analyzed for resistance to ampicillin, using replica-plating.

TABLE 3. *Inability of EcoRI fragment A of NR1 to rescue replication of RSF2124 in a polA(Am) mutant*[a]

Plasmid	Fragment	Vector	No. of transformants/ml per μg of DNA	
			KP245 *pol*$^+$	JG112 *polA*
pRR138	*Eco*RI-A	RSF2124	2.2×10^6	<10
pRR149	*Eco*RI-B	RSF2124	6.8×10^5	2.8×10^5

[a] *E. coli polA*(Am) mutant JG112 was transformed by using hybrid plasmids pRR138 and pRR149. The number of transformants was determined by spreading appropriate dilutions of the transformed cells on nutrient agar plates containing 20 μg of ampicillin per ml and incubating for 24 h at 37°C.

referred to as *Sal*I fragments B$^-$ and C$^-$, respectively) (Fig. 2). In our experiments with *Sal*I cloning, we used recombinant plasmid pRR163, which contained the entire *Eco*RI fragment B ligated to a pBR313 derivative (pRR160) containing the part of *Eco*RI fragment G of NR1 between the *Eco*RI site and *Hin*dIII site which flank the *str/spc* resistance gene. pRR163 was constructed as shown in Fig. 3. This recombinant plasmid was useful for the present study of *Sal*I cloning, since the *str/spc* gene on pRR163 was covalently joined to *Sal*I fragment C$^-$ and thus served as a marker for this fragment.

pRR163 DNA was digested with *Sal*I (Fig. 4a), ligated, and used to transform JG112 *polA* and KP245 *pol*$^+$ to Ap^r, Sp^r, or both. By this procedure, plasmids containing pBR313 as a vector and various combinations of *Sal*I fragments D, E, and C$^-$ were obtained (Fig. 4 and 5). *Sal*I fragment B$^-$ was present on all of these recombinant plasmids but will not be considered in the following discussion since this subfragment was not required for autonomous replication. pDW2 (Fig. 4b and 5) and pDW1 (Fig. 4c and 5), which carried the single *Sal*I fragments D and E, respectively, ligated to pBR313, were also used in our experiments. Only pRR163 and pRR174, which contained both *Sal*I C$^-$ and E, could transform JG112 *polA* at high frequency, whereas all of the recombinant plasmids could transform the *pol*$^+$ strain KP245 in which pBR313 could replicate. Thus, *Sal*I fragments C$^-$ and E were necessary to rescue the replication of pBR313 in a *polA* host.

After treatment with *Eco*RI, pRR174 DNA was cleaved into the pBR313 component and a component consisting of *Sal*I fragments B$^-$, E, and C$^-$ since *Sal*I fragment D had been eliminated in the construction of this plasmid. *Eco*RI treatment of pRR101 DNA produced the RSF2124 component and the *kan* fragment. A mixture of pRR174 DNA and pRR101 DNA was cleaved with *Eco*RI, ligated, and used to transform JG112 *polA* to Km^r. The plasmid DNA isolated from the Km^r transformants contained the *kan* fragment and *Sal*I fragments E and C$^-$ as the replicator fragments, showing that this region of NR1 could mediate autonomous replication even when not ligated to pBR313 as a vector (data not shown).

Inability of a "helper" plasmid to rescue the replication of cloned *Sal*I fragments. As described below, the *Eco*RI B replicator fragment from pRR12, a copy mutant of NR1, was found to be compatible with NR1 and its derivatives; both plasmids could coexist stably in the same host cell. This finding made it possible to test whether any of the individual cloned *Sal*I fragments could rescue the replication of pBR313 in the presence of a "helper" plasmid present in the same host cell, i.e., in the presence of the essential gene products which would be provided in *trans* by the helper plasmid. None of the plasmids that carried a single *Sal*I fragment could transform at high frequency JG112 *polA*, which harbored pRR114, a recombinant plasmid that carried the *Eco*RI B fragment of copy mutant pRR12 (Table 4). Although the frequency of transformation of JG112 *pol*A was somewhat higher when the recipient strain harbored pRR114, this may have been due to recombination between the transforming plasmid and the resident plasmid. This suggests either that some of the essential gene products from the R plasmid replicator region did not act in *trans* or that none of the individual *Sal*I fragments had a functional origin of replication when present alone on a recombinant plasmid.

***Pst*I cloning of replicator region of NR1.** There are 11 *Pst*I sites in *Eco*RI fragment B of NR1. The location of three of the *Pst*I sites in the *Sal*I fragment E-C$^-$ region of NR1 were mapped (Fig. 2). To identify the *Pst*I fragments required for autonomous replication, a small *Pst*I fragment (1.8 kb) cloned from a deletion mutant of NR1 which carries only the chloramphenicol (*cam*) and fusidic acid (*fus*) resistance genes of the r-determinants (*Pst*I *cam* fragment) was used in *Pst*I cloning experiments. The DNA of pRR177 (*Eco*RI fragments B and G of NR1) (Fig. 6 and 7a) and a pBR322 derivative that contained the *Pst*I *cam* fragment (pRR714) (Fig. 6 and 7b) were mixed, digested with *Pst*I, ligated, and used to transform JG112 *pol*A to Cm^r. pBR322 could not replicate in a *polA* host. All of the Cm^r transformants analyzed were found

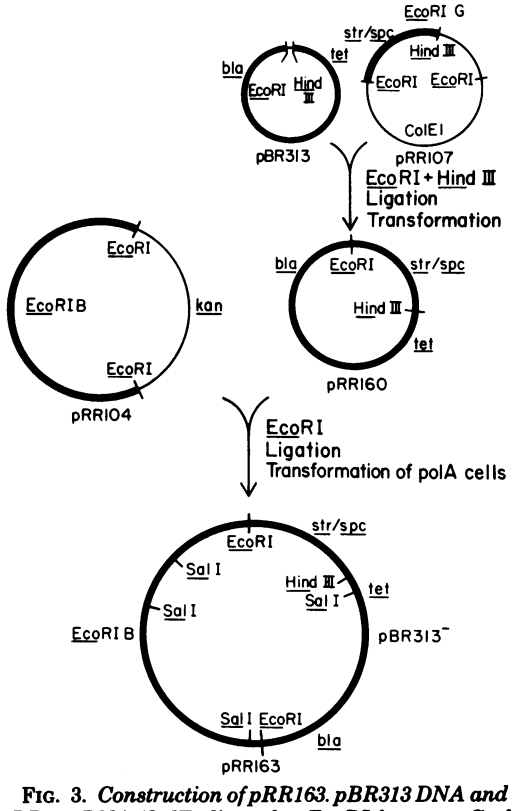

FIG. 3. *Construction of pRR163. pBR313 DNA and pRR107 DNA (ColE1 ligated to EcoRI fragment G of NR1) were digested to completion by using a mixture of EcoRI and HindIII, and the resulting DNA fragments were ligated and used to transform KP245 to Sp^r and Ap^r. One recombinant plasmid (pRR160) consisted of pBR313 lacking the small region of pBR313 between the EcoRI and HindIII sites ligated to the corresponding EcoRI and HindIII sites which flank the str/spc gene, which is located on EcoRI fragment G of NR1. pRR160 DNA and pRR104 DNA (EcoRI fragment B of NR1 plus kan fragment) were digested with EcoRI and ligated, and the DNA mixture was used to transform E. coli JG112 polA to Ap^r. Transformants which were also Sp^r and Tc^r contained all of the pRR160 drug resistance genes and had the ability to replicate in the absence of DNA polymerase I as a result of ligation to EcoRI fragment B. One such recombinant plasmid (pRR163) was shown to have the structure shown in this figure. The regions of pBR313, pRR107, pRR160, and pRR104 that are present in pRR163 are drawn as thick lines in each of the plasmids.*

to contain at least two *Pst*I fragments from the *Sal*I C$^-$-E region of *Eco*RI fragment B, in addition to the *Pst*I *cam* fragment used for selection of transformants (Fig. 6 and 7c). These two *Pst*I fragments had sizes of 1.6 and 1.1 kb and were contiguous on the R plasmid molecule (Fig. 6). Similar experiments with pRR109 (*Eco*RI fragments B and G from copy mutant pRR12) as a donor of the R plasmid replication fragments identified the corresponding fragments of the copy mutant as the two *Pst*I fragments capable of mediating autonomous replication (data not shown). These two *Pst*I fragments were always present in the same orientation in the recombinant plasmids as they were in *Eco*RI fragment B. In similar experiments involving *Pst*I cloning of pRR177 and pRR109, it was found that the same two *Pst*I fragments could support replication of the *Pst*I fragment from pRR109, which carried the spectinomycin resistance gene present on *Eco*RI fragment G of pRR12 (Fig. 7d). This suggests that the *Pst*I *cam* fragment was not necessary for autonomous replication of the small recombinant plasmids.

The 1.6- and 1.1-kb *Pst*I fragments were cloned individually onto pBR322 (Fig. 7e and f). pBR322 derivatives containing both *Pst*I frag-

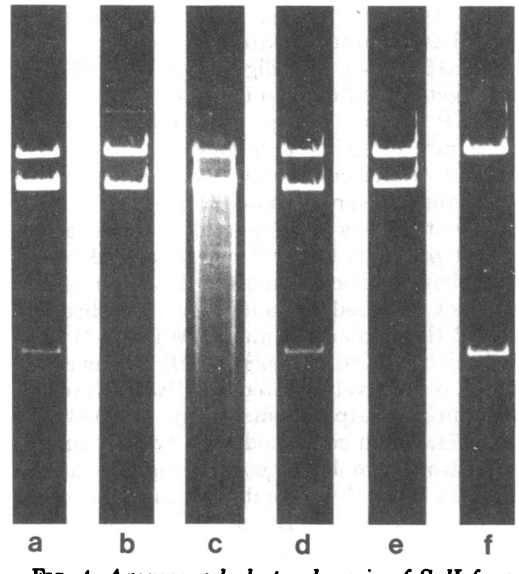

FIG. 4. *Agarose gel electrophoresis of SalI fragments of recombinant plasmids derived from pRR163. pRR163 DNA was digested with SalI, ligated, and then used to transform E. coli KP245 pol$^+$ to Ap^r. The DNAs of a number of recombinant plasmids isolated from the transformants were cleaved with SalI and examined by agarose gel electrophoresis. (a) pRR163 DNA; (b) pRR175 DNA; (c) pRR173 DNA; (d) pRR174 DNA; (e) pDW2 DNA; (f) pDW1 DNA. The structures of these recombinant plasmids are shown in Fig. 5. Recombinant plasmids pDW2 and pDW1, which consisted of pBR313 ligated to SalI fragments D and E, respectively, of NR1 (7), were also examined. Although SalI fragment D and the SalI fragment containing the str/spc gene (see Fig. 5) were not resolved on these gels, the two fragments can be separated if electrophoresis is carried out for a longer period of time.*

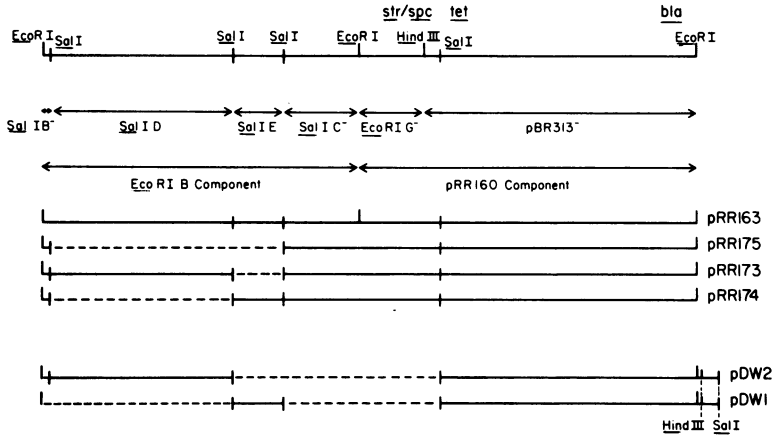

FIG. 5. *Structure of recombinant plasmids containing various combinations of SalI fragments from EcoRI fragment B of NR1. The map at the top shows the structure of pRR163 (Fig. 3), drawn in linear form. The structures of the recombinant plasmids derived from pRR163 (see Fig. 4) are shown below. The solid lines correspond to the SalI fragments of pRR163 which were present in these recombinant plasmids; the dashed lines indicate regions that were not present. Recombinant plasmids pDW2 and pDW1 consisted of pBR313 ligated to SalI fragments D and E, respectively, of NR1 (7). Note that pDW2 and pDW1 are not missing the small region between the EcoRI and HindIII sites of the vector pBR313 (see Fig. 3).*

TABLE 4. *Replication properties of cloned SalI fragments from EcoRI fragment B or R plasmid NR1*[a]

Plasmid	NR1 fragments			Vector	No. of transformants/μg of DNA		
	*Sal*I-D	*Sal*I-E	*Sal*I-C⁻		KP245 *pol*⁺	JG112 *polA*	JG112(pRR114)
pRR163	+	+	+	pBR313	2.3×10^5	3.8×10^5	2.1×10^5
pDW2	+	−	−	pBR313	8.2×10^5	<10	4.1×10^3
pDW1	−	+	−	pBR313	1.45×10^6	<10	5.6×10^2
pRR175	−	−	+	pBR313	2.5×10^5	2×10	1.65×10^2
pRR173	+	−	+	pBR313	3.7×10^4	<10	ND[b]
pRR174	−	+	+	pBR313	9.4×10^4	2.4×10^5	1.47×10^5

[a] Recombinant plasmids containing *Sal*I fragments from *Eco*RI fragment B of NR1 were constructed by using pBR313 as a vector as described in Fig. 4 and 5 and in the text. Recombinant plasmid DNA was used to transform the *E. coli* strain KP245 *pol*⁺, JG112 *polA*, or JG112(pRR114), using selection for ampicillin resistance, spectinomycin resistance, or both. pRR114 is a recombinant plasmid containing the *Eco*RI B replicator fragment of pRR12.

[b] ND, Not determined.

ments were also obtained in the cloning experiments (Fig. 7g). None of the pBR322 derivatives which carried a single *Pst*I fragment could transform JG112 *polA*, whereas all of these plasmids could transform KP245 *pol*⁺ at a high frequency. Similar results were obtained when JG112 *polA*, which harbored the compatible copy mutant plasmid, was used as the recipient strain in the transformation experiments. The pBR322 derivatives containing the 1.6- and 1.1-kb *Pst*I fragments in native orientation transformed both JG112 *polA* and KP245 *pol*⁺ at approximately the same frequency (data not shown).

Instability of plasmids cloned from NR1. Although *Eco*RI fragment B, *Sal*I fragments E and C⁻, or the 1.6- and 1.1-kb *Pst*I fragments of NR1 were capable of mediating autonomous replication, cloned plasmids containing these fragments as the functional replicators were not stably inherited by host cells, as reflected in the relatively high frequency of occurrence of drug-sensitive cells. Only a small percentage of the KP245 cells that carried pRR104 (*Eco*RI fragment B of NR1 plus the *kan* fragment) (Fig. 8a) or pRR177 (*Eco*RI fragments B and G of NR1) (Fig. 8b) were resistant to kanamycin or spectinomycin, respectively, after the cells were repeatedly subcultured in drug-free medium. Similar experiments with cloned plasmids containing *Sal*I fragments E and C⁻ or the 1.6- and 1.1-kb *Pst*I fragments as the replicators showed that these plasmids were unstable as well (data not shown). NR1 was completely stable in KP245 when the host cells were cultured under similar conditions (Fig. 8a). *Eco*RI fragment B which was cloned from the copy mutant pRR12 was

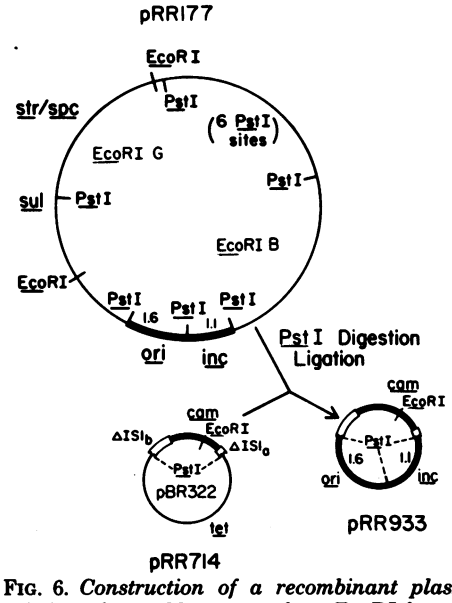

FIG. 6. *Construction of a recombinant plasmid consisting of a 2.7-kb segment from EcoRI fragment B of NR1. pRR177 DNA (EcoRI fragments B and G of NR1) and pRR714 DNA (cam fragment [1.8 kb] of the deletion mutant of NR1, ligated at the PstI site of pBR322) were mixed, digested with PstI, and ligated, and the DNA mixture was used to transform E. coli JG112 polA to Cm^r. The structure of one of the recombinant plasmids (pRR933) obtained is shown in this figure. The regions of pRR177 and pRR714 which were present in pRR933 are drawn as thicker lines in each of the plasmids.*

almost as stable as NR1 when ligated to the *kan* fragment (pRR114; Fig. 8a) or to *Eco*RI fragment G of NR1 (pRR109; Fig. 8b). These experiments suggest that there is a function required for stable replication or segregation of NR1 which was missing from the cloned plasmids containing the replication genes present on *Eco*RI fragment B. This function will be referred to as "*stb*" (for stability). This function does not appear to be as necessary for the stable inheritance of small cloned plasmids derived from copy mutant pRR12.

When *Eco*RI fragment B of NR1 was ligated to RSF2124, the hybrid plasmid was also found to be unstable (Table 2). RSF2124 was not unstable when present as a separate plasmid in the same host cell with plasmids containing the replicator fragments from NR1. Thus, *Eco*RI fragment B of NR1 conferred instability on RSF2124 only when joined in *cis*.

If the *stb* function is present on an *Eco*RI fragment of NR1, it should be possible to identify this fragment by selection for stabilization of the inheritance of *Eco*RI fragment B. A tetracycline resistance transfer factor (RTF-Tc) derivative of NR1 was used in these experiments, since it has less than one-half the number of *Eco*RI fragments and tetracycline resistance is stably inherited after deletion of the r-determinants component. This RTF-Tc (pRR250) contained ampicillin transposon Tn*3* on *Eco*RI fragment B, so that ampicillin resistance could be used to select directly for the replicator fragment. pRR250 DNA was digested with *Eco*RI, ligated, and used to transform KP245 *pol*$^+$ to Ap^r. A number of the Ap^r transformants were screened for stability of inheritance of drug resistance. Although most of the transformants were unstable as previously described, a few were completely stable (Fig. 9). The covalently closed circular plasmid DNA from one of the stable transformants was analyzed and found to contain both *Eco*RI fragments B and A$^+$ (data not shown). (The largest fragment of an RTF derivative of NR1 is slightly larger than *Eco*RI fragment A of NR1, since it contains *Eco*RI fragment A plus the part of *Eco*RI fragment H [ΔH_2] which is a part of the RTF component [28]. Other experiments in this laboratory have shown that subfragment ΔH_2 does not stabilize the inheritance of the replicator region on *Eco*RI fragment B.) Thus, these experiments suggest that the *stb* function was on *Eco*RI fragment A of NR1.

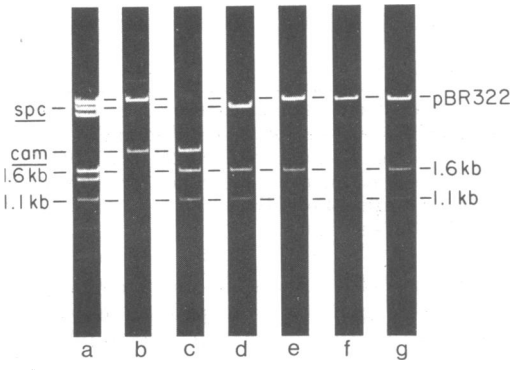

FIG. 7. *Agarose gel electrophoresis of PstI fragments of recombinant plasmids containing various combinations of PstI replicator fragments from EcoRI fragment B. Recombinant plasmids obtained in cloning experiments similar to the one described in the legend to Fig. 6 were digested with PstI and examined by agarose gel electrophoresis. (a) pRR177 DNA (EcoRI fragments B and G of NR1); (b) pRR714 DNA (cam fragment of NR1 cloned to pBR322 at the PstI site); (c) pRR933 (cam fragment plus the 1.6- and 1.1-kb PstI fragments); (d) pRR947 (Sp^r PstI fragment from pRR177 plus the 1.6- and 1.1-kb PstI fragments); (e) pRR936 (pBR322 plus the 1.6-kb PstI fragment); (f) pRR935 (pBR322 plus the 1.1-kb fragment); (g) pRR945 (pBR322 plus the 1.6- and 1.1-kb PstI fragments).*

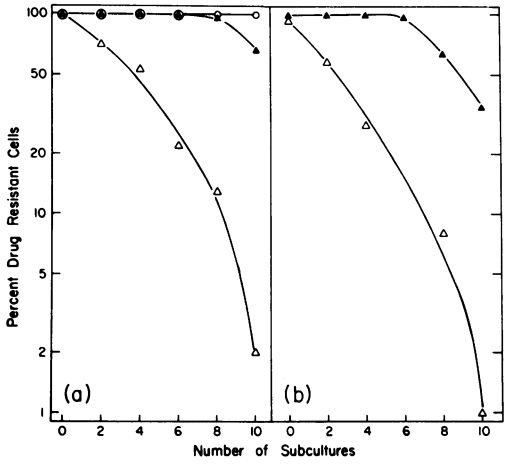

FIG. 8. *Instability of cloned EcoRI B fragment of NR1. E. coli KP245 harboring NR1 or recombinant plasmids derived from NR1 or its copy mutant pRR12 was cultured in Penassay broth containing either 5 μg of spectinomycin per ml or 10 μg of neomycin per ml. These starting cultures were then repeatedly subcultured for 10 successive cycles by diluting the cells 10^6-fold into drug-free nutrient broth and incubating overnight at 37°C until the cells reached the stationary phase. Each of the stationary-phase cultures was diluted appropriately and spread on drug-free Penassay agar plates. The percentage of drug-resistant cells was then determined by replica plating. The drug resistances conferred by the various recombinant plasmids are described in the text. Symbols: (a) ○, NR1; ▲, pRR114; △, pRR104; (b) ▲, pRR109; △, pRR177.*

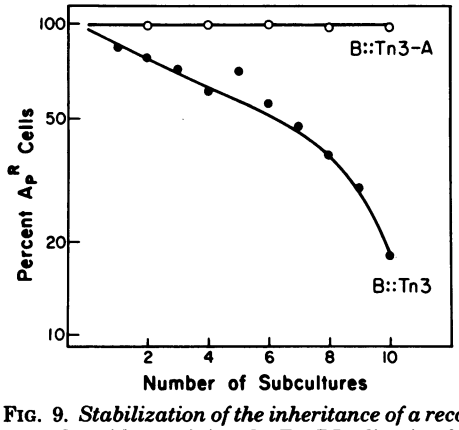

FIG. 9. *Stabilization of the inheritance of a recombinant plasmid containing the EcoRI relication fragment B by EcoRI fragment A.*

When pRR138 (RSF2124 plus *Eco*RI fragment A) and an unstable plasmid containing the *Eco*RI B replicator fragment from NR1 were present simultaneously in the same host cell, the unstable plasmid was still lost from the cells at relatively high frequency. This suggests that *stb* function did not confer stability in *trans*.

Mapping of the incompatibility gene of NR1. NR1 is a member of the FII incompatibility group. The location of the region of NR1 responsible for incompatibility was mapped by transforming *recA* recipient cells harboring a resident plasmid with a distinguishable drug resistance pattern with the plasmids containing cloned fragments from the replicator region of NR1. Transformants were selected on nutrient plates containing a drug to which resistance was conferred only by the donor (transforming) plasmid. Ten or 20 well-isolated transformant colonies were suspended in dilution buffer and streaked on drug-free Penassay agar plates to obtain single colonies. Ten colonies from each individual streak were picked onto Penassay agar plates and tested for the presence of the donor and resident plasmids by examination of the drug resistance pattern of the colonies. The results were expressed as the percentage of cells carrying both the donor and resident plasmids. Using this procedure, we analyzed cells which had been incubated for approximately 30 generations after the donor plasmid was introduced into a cell harboring the resident plasmid.

In control experiments in which pRR104 DNA (*Eco*RI fragment B of NR1 plus the *kan* fragment of R6-5) was transformed into a *recA* strain carrying NR1, only about 1% of the colonies which were tested carried both plasmids. If a pair of plasmids gave a higher frequency of cells harboring both plasmids, it was concluded that they were compatible or that one of them was Inc^-.

Only the plasmids that carried *SalI* fragment E were incompatible with NR1 (Table 5). More than 90% of the transformants with plasmids lacking *SalI* fragment E carried both donor and resident plasmids. In similar experiments with the pBR322 derivatives carrying either the 1.6-kb or the 1.1-kb *PstI* fragment, only the plasmid that carried the 1.1-kb *PstI* fragment conferred incompatibility (data not shown). Thus, the incompatibility gene of NR1 must reside in the region of overlap between *SalI* fragment E and the 1.1-kb *PstI* fragment (Fig. 2). It should be noted, however, that although this experiment demonstrated the existence of an *inc* gene in this region, it did not prove that there were no other *inc* genes outside of this region which may not have been expressed in the cloned fragments.

Copy mutation and incompatibility. Uhlin and Nordström (34) have found that all of the copy mutants of FII incompatibility group R plasmid R1 have altered incompatibilities: some copy mutants have increased incompatibility, whereas others have decreased incompatibility. To examine whether copy mutant pRR12 had

altered incompatibility, plasmids containing the *kan* fragment of R6-5 ligated to the *Eco*RI B replicator fragment of NR1 (pRR104) or of pRR12 (pRR114) were transformed into a *recA* strain carrying NR1 or pRR12. The transformants obtained were analyzed for the presence of donor and resident plasmids. The *Eco*RI B replicator fragment of pRR12 was compatible with resident plasmid NR1 whereas the corresponding derivative of NR1 was incompatible with NR1 (Table 6). When used as donors in transformation experiments, both of these plasmids were compatible with resident copy mutant plasmid pRR12 (Table 6). In similar experiments, pBR322 ligated to the 1.1-kb *Pst*I fragment of pRR12 was also found to be compatible with NR1, whereas the corresponding *Pst*I fragment of NR1 conferred incompatibility when cloned onto pBR322.

TABLE 5. *Mapping of the incompatibility gene of NR1*[a]

Donor plasmid	% of transformed cells with:			
	$D^+ R^+$	$D^+ R^-$	$D^- R^+$	$D^- R^-$
pRR163	0	83	0	17[b]
pDW2	100	0	0	0
pDW1	0	100	0	0
pRR175	90	0	10	0
pRR173	100	0	0	0
pRR174	0	81	3	16[b]

[a] The recombinant plasmids described in the legends to Fig. 4 and 5 and footnote *a* of Table 4 were tested for their incompatibility with resident (R) NR1 as described in the text. D^+ and D^-, Presence and absence of the donor plasmid, respectively. R^+ and R^-, Presence and absence of the resident plasmid, respectively.

[b] These $D^- R^-$ cells most likely resulted from exclusion of the resident NR1 plasmid by the donor plasmid owing to incompatibility and the subsequent loss of the donor recombinant plasmid after the original transformant colonies had been streaked onto drug-free Penassay agar plates to obtain single colonies as described in the text. As discussed in the text, recombinant plasmids formed by the ligation of the unstable replicator region of NR1 to a ColE1 derivative were also unstable.

***inc* mutation of pRR12 is *cis* dominant.** pDW1 (pBR313 plus fragment *Sal*I E of NR1) was transformed into a *recA* recipient strain carrying either NR1 or pRR114 (*Eco*RI fragment B of pRR12 plus the *kan* fragment of R6-5) or both NR1 and pRR114. Selection was for ampicillin resistance conferred by the donor (transforming) plasmid, and then transformants were analyzed for the presence of the donor and the two resident plasmids as in the previous experiments. pDW1 was incompatible with NR1 and compatible with pRR114 when only a single type of resident plasmid was present in the recipient cells (Table 7). When both NR1 and pRR114 were present simultaneously in the recipient cells, only NR1 was excluded (incompatible) by pDW1, indicating that the *inc* mutation of pRR114 was *cis* dominant.

DISCUSSION

Analyses of several plasmids from different incompatibility groups have revealed that the genes and functional sites required for autonomous replication are clustered on a relatively small segment of the plasmid genome (5, 8, 15, 16, 30, 31). Our cloning experiments with the IncFII R plasmid NR1 localized the replication functions to a small segment of approximately 2.7 kb corresponding to the 1.6- and 1.1-kb *Pst*I fragments within *Eco*RI fragment B. The 1.1-kb *Pst*I fragment conferred the incompatibility characteristic of IncFII plasmids. Our results are in agreement with those reported recently by Taylor and Cohen (29), who analyzed a copy mutant of NR1. Similar findings have been reported for the closely related IncFII R plasmids R1 (13) and R6-5 (32, 33).

Our functional characterization of the cloned replicator regions from NR1 and its copy mutant, pRR12, revealed several interesting new findings. Cloned plasmids whose replicator region consisted of *Eco*RI fragment B of NR1, or

TABLE 6. *Loss of incompatibility of copy mutant pRR12*[a]

Donor		Plasmid in recipient cells		% of transformed cells			
Plasmid	Mutation	Plasmid	Mutation	$D^+ R^+$	$D^+ R^-$	$D^- R^+$	$D^- R^-$
pRR104	*cop*$^+$	NR1	*cop*$^+$	1	94	1.5	3.5
pRR114	*cop-12*	NR1	*cop*$^+$	95.5	4.5	0	0
pRR104	*cop*$^+$	pRR12	*cop-12*	79.5	0	20.5	0
pRR114	*cop-12*	pRR12	*cop-12*	92	4	4	0

[a] pRR104 DNA (*Eco*RI fragment B of NR1 plus *kan* fragment of R6-5) or pRR114 DNA (*Eco*RI fragment B of pRR12 plus *kan* fragment of R6-5) was used to transform to kanamycin resistance *E. coli* KP435 *recA* harboring either NR1 or its copy mutant pRR12. The ability of the donor plasmid to exclude the resident plasmid owing to incompatibility was determined as described in the text. The designations D^+, D^-, R^+, and R^- are the same as for Table 5.

TABLE 7. *cis dominance of the inc mutation of copy mutant pRR12*[a]

Donor plasmid	Plasmid(s) in recipient cells	% of transformed cells with:							
		$D^+ R^+$	$D^+ R_{NR1}^+ R_{114}^+$	$D^+ R^-$	$D^+ R_{NR1}^+ R_{114}^-$	$D^- R^+$	$D^+ R_{NR1}^- R_{114}^+$	$D^- R^-$	$D^+ R_{NR1}^- R_{114}^-$
pDW1	NR1	0		100		0		0	
pDW1	pRR114	100		0		0		0	
pDW1	NR1 + pRR114		0		0		100		0

[a] pDW1 (pBR313 plus *SalI* fragment E of NR1) DNA was used to transform to ampicillin resistant *E. coli* KP345 *recA* which harbored either NR1 or pRR114 (*Eco*RI fragment B of pRR12 plus *kan* fragment of R6-5) or both NR1 and pRR114. The ability of the donor plasmid (D) to exclude the resident plasmid(s) (R) owing to incompatibility was determined as described in the text. The designations D^+, D^-, R^+, and R^- are the same as for Table 5. R_{NR1}^+, Presence of resident plasmid NR1; R_{NR1}^-, absence of NR1. R_{114}^+ and R_{114}^-, Presence and absence of resident plasmid pRR114, respectively.

*Sal*I fragments E and C^-, or the 1.6- and 1.1-kb *Pst*I fragments were not stably inherited when the host cells were cultured under conditions which did not select for the drug resistance conferred by the plasmid. Thus, the replicator region of NR1 within *Eco*RI fragment B was missing a function (*stb*) required for either stable plasmid replication or segregation at cell division. Plasmids containing both *Eco*RI fragment A and *Eco*RI fragment B were stably inherited, so that the *stb* function appeared to be located on *Eco*RI fragment A, which is separated from *Eco*RI fragment B by the r-determinants component.

Hashimoto and Mitsuhashi (9), Yoshikawa (36), and Dempsey and Willetts (6) have previously described mutants of NR1 or related R plasmids which are not stably maintained by host cells. These unstable plasmids were only characterized by genetic experiments, so that the missing function has not been located on the R plasmid physical map. In all three cases, the unstable plasmids had deleted either the *tet* or the *cam* resistance genes. Since these resistance genes are located at the ends of *Eco*RI fragment A (Fig. 2), it is likely that the region of this fragment which confers stable inheritance in *cis* was also deleted. An unstable mutant of NR1 isolated in this laboratory has been found to have deleted the region between *Eco*RI fragments I and D, which would include *Eco*RI fragment A (T. Miki, V. Luckow, and R. Rownd, unpublished data). Taken together, these experiments also indicate that a function located on *Eco*RI fragment A is required for stable replication or segregation of NR1. Yoshikawa has referred to this function as *repB*. Since there is no evidence that this function is required for replication per se, we suggest that it be designated *stb* (for stability), as originally suggested by Hashimoto and Mitsuhashi (9), since this is the phenotype by which this function has been recognized. Although its mode of action is presently not known, it is interesting that the *stb* function is brought into close proximity to the replication and incompatibility genes on *Eco*RI fragment B by deletion of the r-determinants component of NR1. Thus, the *stb* function and the replication genes of NR1 may have been physically separated on the plasmid genome during evolution of the plasmid by the insertion of the r-determinants between them.

Unstable plasmids containing the replicator region within *Eco*RI fragment B were still lost from the cells even when *Eco*RI fragment A was present on a compatible hybrid plasmid such as pRR138 (RSF2124 plus *Eco*RI fragment A). This suggests that the *stb* function did not confer stability in *trans*. If the *stb* function can only stablize the *Eco*RI fragment B replication region when physically joined to it, then it seems likely that it may play a structural role in stable plasmid maintenance rather than providing a diffusible gene product which could act in *trans*.

Although pRR104 and pRR177, which use *Eco*RI fragment B from the wild-type (low-copy-number) NR1 R plasmid, required *stb* for stable inheritance, the corresponding plasmids from the copy mutant pRR12 were much more stable (Fig. 8). Miniplasmids derived from the replicator region of the copy mutant pRR12 have about the same stability as the cloned replicator fragments from pRR12 (J. Greenberg and R. Rownd, unpublished data). Thus, the copy number mutation within the replicator region on *Eco*RI fragment B in some way bypasses the requirement for the *stb* function for stable inheritance. On the other hand, a hybrid plasmid formed by ligating *Eco*RI fragment B of NR1 to the ColE1 derivatives RSF2124 is unstable, even though RSF2124, a high-copy-number plasmid, is stably inherited by the host cells.

Timmis et al. (32) have reported that *Eco*RI fragments A and D of R plasmid R6-5 can rescue the replication of ColE1 in a *polA*(Ts) host at the restrictive temperature. This was not the case with *Eco*RI fragment A of NR1. pRR135 (RSF2124 plus *Eco*RI fragment A) could not

transform the *E. coli* JG112 *polA*, nor was there any significant difference in the degree of loss of RSF2124 and pRR138 after a period of growth of a *polA* (Ts) host at the restrictive temperature. There may be basic differences in the number and function of the replicator genes of R6 and NR1.

The replicator fragments cloned from copy mutant pRR12 showed a much lower degree of incompatibility with NR1 than the replicator fragments cloned from NR1. Moreover, the various plasmids which used the pRR12 replicator showed much less incompatibility with each other than the corresponding NR1 derivatives. Thus, the *cop* mutant of pRR12 simultaneously affected copy number, incompatibility, and dependence on the *stb* function for stability. It is not known whether all of these phenotypes are due to the same mutation.

Pritchard et al. (24) have suggested that plasmid replication is controlled by a plasmid-encoded repressor molecule which acts negatively to inhibit replication by binding to a specific site on the plasmid molecule. The repressor structural gene is located near the origin of replication and is expressed soon after the initiation of plasmid replication. This results in an increase in repressor concentration which inhibits further initiation of plasmid replication until the repressor concentration is lowered below a critical threshold value by cell growth. According to this repressor dilution model, incompatibility is basically a manifestation of the control of plasmid copy number. If the plasmid copy number is stringently controlled, then the introduction of a second plasmid which shares the same replication control mechanism in a host cell would lead to the inhibition of the replication and subsequent exclusion of one or the other of the two plasmids from the descendant cells. Plasmid copy number mutants could be due to either a decrease in repressor activity or an alteration in the repressor binding site which lowers its affinity for the wild-type repressor. Uhlin and Nordström (34) have reported that a number of copy mutants of the IncFII R plasmid R1 have altered incompatibility properties and suggested that their phenotypes could be explained by either an altered repressor or its binding site.

The behavior of the copy mutant used in our experiments, however, cannot be explained simply by the repressor dilution model if this copy mutant is the result of a single mutation. Our findings that the pRR12 Inc$^-$ phenotype was *cis* dominant is consistent with the view that there is an alteration in the binding site for the repressor on the copy mutant plasmid such that its affinity for the repressor is diminished. Thus, when both NR1 and pRR12 are present simultaneously in the same host cell, pRR12 would not be excluded when the NR1 incompatibility gene is introduced by transformation. Only NR1 would be excluded from the cells since it has the wild-type receptor site. An altered receptor site for pRR12 would also explain its increased copy number, since the wild-type repressor would bind to the altered site with a lower affinity. However, an altered pRR12 receptor site is not consistent with the observation that the cloned replicator fragments from NR1 and pRR12 are compatible. If pRR12 has only an altered receptor site and continues to specify the wild-type repressor, NR1 should not be able to coexist stably with pRR12 derivatives since it contains the wild-type receptor site. Indeed, pRR12 might have been expected to exclude NR1 more strongly than NR1 excluded itself owing to gene dosage effects. This, however, was not true. To explain our observations according to the repressor dilution model, it would be necessary to postulate that pRR12 had both an altered repressor to which NR1 was no longer sensitive and an altered receptor site which did not interact with the NR1 wild-type repressor. In addition, it would seem that the pRR12-altered repressor must have been able to interact with the pRR12-altered receptor site to set the pRR12 copy number. Even though there was an increased number of copies of pRR12 per cell, the increase was only fourfold, so that the pRR12 copy number must still have been regulated. Taken together, these findings suggest that the pRR12 receptor site may lie within the repressor structural gene such that both are affected simultaneously by the same mutation or that there must have been two separate mutations to form copy mutant pRR12.

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LITERATURE CITED

1. **Barton, C. R., R. L. Warren, P. Jezo, A. M. Easton, and R. Rownd.** 1979. *SalI* restriction endonuclease map of FII incompatibility group R plasmids. Plasmid 2:150–154.
2. **Bolivar, F., R. L. Rodriguez, M. C. Betlach, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene 2:75–93.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
4. **Clowes, R.** 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. 36:361–405.

5. **Crosa, J. H., L. K. Luttropp, M. F. Thomas, and S. Falkow.** 1978. Replication of R6K and its derivatives in *Escherichia coli* K-12, p. 110–111. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
6. **Dempsey, W. B., and N. S. Willetts.** 1976. Plasmid cointegrates of prophage lambda and R factor R100. J. Bacteriol. **126**:166–176.
7. **DeWilde, M., J. E. Davies, and F. J. Schmidt.** 1978. Low-molecular-weight RNA species coded by plasmid NR1, p. 188–191. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
8. **Figurski, D., R. Kolter, R. Meyer, M. Kahn, R. Eichenlaub, and D. R. Helinski.** 1978. Replication regions of plasmids ColE1, F, R6K, and RK2, p. 105–109. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
9. **Hashimoto, H., and S. Mitsuhashi.** 1970. Segregation of R factors p. 545–551. *In* H. Umezawa (ed.), Progress in antimicrobial and anticancer chemotherapy. Proceedings of the 6th International Congress of Chemotherapy. University of Tokyo Press, Tokyo.
10. **Helinski, D. R.** 1973. Plasmid determined resistance to antibiotics: molecular properties of R factors. Annu. Rev. Microbiol. **27**:437–470.
11. **Hershfield, V., H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski.** 1974. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. Proc. Natl. Acad. Sci. U.S.A. **71**:3455–3459.
12. **Kingsbury, D. T., and D. R. Helinski.** 1973. Temperature-sensitive mutants for the replication of plasmids in *Escherichia coli*: requirement for deoxyribonucleic acid polymerase I in the replication of the plasmid ColE1. J. Bacteriol. **114**:1116–1124.
13. **Kolleck, R., W. Oertel, and W. Goebel.** 1978. Isolation and characterization of the minimal fragment required for autonomous replication ("basic replicon") of a copy mutant (pKN102) of the antibiotic resistance factor R1. Mol. Gen. Genet. **162**:51–57.
14. **Kupersztoch-Portnoy, Y. M., M. A. Lovett, and D. R. Helinski.** 1974. Strand and site specificity of the relaxation event for the relaxation complex of the antibiotic resistance plasmid R6K. Biochemistry **13**:5484–5490.
15. **Lovett, M. A., and D. R. Helinski.** 1976. Method for the isolation of the replication region of a bacterial replicon: construction of a mini F'*Km* plasmid. J. Bacteriol. **127**: 982–987.
16. **Manis, J. J., and B. C. Kline.** 1978. F plasmid incompatibility and copy number genes: their map location and interactions. Plasmid **1**:429–507.
17. **Mickel, S., and W. Bauer.** 1976. Isolation by tetracycline selection of small plasmids derived from R factor R12 in *Escherichia coli* K-12. J. Bacteriol. **127**:644–655.
18. **Miki, T., A. M. Easton, and R. H. Rownd.** 1978. Mapping of the resistance genes of the R plasmid *NR1*. Mol. Gen. Genet. **158**:217–224.
19. **Miller, J., J. Manis, B. Kline, and A. Bishop.** 1978. Nonintegrated plasmid-folded chromosome complexes. Plasmid **1**:273–283.
20. **Morris, C. F., H. Hashimoto, S. Mickel, and R. Rownd.** 1974. Round of replication mutant of a drug resistance factor. J. Bacteriol. **118**:855–866.
21. **Nakaya, R., and R. H. Rownd.** 1971. Transduction of R factors by a *Proteus mirabilis* bacteriophage. J. Bacteriol. **106**:773–783.
22. **Ohtsubo, E., J. Feingold, H. Ohtsubo, S. Mickel, and W. Bauer.** 1977. Unidirectional replication in *Escherichia coli* of three small plasmids derived from R factor R12. Plasmid **1**:8–18.
23. **Pritchard, R. H.** 1978. Control of DNA replication in bacteria, p. 1–26. *In* I. Molineux and M. Kuhiyama (ed.), DNA synthesis: present and future. Plenum Publishing Corp., New York.
24. **Pritchard, R. H., P. T. Barth, and J. Collins.** 1969. Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. **19**:263–297.
25. **Rownd, R. H.** 1978. Plasmid replication, p. 751–772. *In* I. Molineux and M. Kohiyama (ed.), DNA synthesis: present and future. Plenum Publishing Corp., New York.
26. **Rownd, R. H., and D. D. Womble.** 1978. Molecular nature and replication of R factors, p. 161–193. *In* S. Mitsuhashi (ed.), R factor, drug resistance plasmid. University of Tokyo Press, Tokyo.
27. **So, M., R. Gill, and S. Falkow.** 1975. The generation of a ColE1 Ap^r cloning vehicle which allows detection of inserted DNA. Mol. Gen. Genet. **142**:239–249.
28. **Tanaka, N., J. H. Cramer, and R. H. Rownd.** 1976. *Eco*RI restriction endonuclease map of the composite R plasmid NR1. J. Bacteriol. **127**:619–636.
29. **Taylor, D. P., and S. N. Cohen.** 1979. Structural and functional analysis of cloned DNA segments containing the replication and incompatibility regions of a miniplasmid derived from a copy mutant NR1. J. Bacteriol. **137**:92–104.
30. **Taylor, D. P., J. Greenberg, and R. H. Rownd.** 1977. Generation of miniplasmids from copy number mutants of the R plasmid NR1. J. Bacteriol. **132**:986–995.
31. **Timmis, K., F. Cabello, and S. N. Cohen.** 1975. Cloning, isolation, and characterization of replication regions of complex plasmid genomes. Proc. Natl. Acad. Sci. U.S.A. **72**:2242–2246.
32. **Timmis, K., P. Cabello, and S. N. Cohen.** 1978. Cloning and characterization of *Eco*RI and *Hind*III restriction endonuclease-generated fragments of antibiotic resistance plasmids R6-5 and R-6. Mol. Gen. Genet. **162**:121–137.
33. **Timmis, K. N., I. Andres, and P. M. Slocombe.** 1978. Plasmid incompatibility cloning analysis of an *inc*FII determinant of R6-5. Nature (London) **273**:27–32.
34. **Uhlin, B. E., and K. Nordström.** 1975. Plasmid incompatibility and control of replication: copy mutants of the R-factor R1 in *Escherichia coli* K-12. J. Bacteriol. **124**:641–649.
35. **Womble, D. D., D. P. Taylor, and R. H. Rownd.** 1977. A method for obtaining more-accurate covalently closed circular plasmid-to-chromosome ratios from bacterial lysates by dye-buoyant density centrifugation. J. Bacteriol. **130**:148–153.
36. **Yoshikawa, M.** 1974. Identification and mapping of the replication genes of an R factor, R100-1, integrated into the chromosome of *Escherichia coli* K-12. J. Bacteriol. **118**:1123–1131.