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The Incompatibility Product of IncFII R Plasmid NR1 Controls Gene Expression in the Plasmid Replication Region

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The incompatibility properties of IncFII R plasmid NR1 were compared with those of two of its copy number mutants, pRR12 and pRR21. pRR12 produced an altered incompatibility product and also had an altered incompatibility target site. The target site appeared to be located within the incompatibility gene, which is located more than 1,200 base pairs from the plasmid origin of replication. The incompatibility properties of pRR21 were indistinguishable from those of NR1. Lambda phages have been constructed which contain the incompatibility region of NR1 or of one of its copy mutants fused to the *lacZ* gene. In lysogens constructed with these phages, β-galactosidase was produced under the control of a promoter located within the plasmid incompatibility region. Lysogens containing prophages with the incompatibility regions from pRR12 and pRR21 produced higher levels of β-galactosidase than did lysogens containing prophages with the incompatibility region from the wild-type NR1. The introduction into these *inc-lac* lysogens of pBR322 plasmids carrying the incompatibility regions of the wild-type or mutant plasmids resulted in decreased levels of β-galactosidase production. For a given lysogen, the decrease was greater when the pBR322 derivative expressed a stronger incompatibility toward the plasmid from which the fragment in the prophage was derived. This suggested that the incompatibility product acts on its target to repress gene expression in the plasmid replication region.

The control of DNA replication has been investigated by using a variety of bacterial plasmids as model systems. For each type of plasmid, there must be mechanisms which determine its copy number in host cells and which ensure its segregation into daughter cells at cell division. Two types of models have been proposed to account for maintenance of a fixed number of plasmid copies in host cells. In the replicon model, it is proposed that replicating genomes such as plasmids compete for a limiting amount of a positively acting replication initiator (12). According to the repressor dilution model, on the other hand, plasmid replication is controlled negatively by a replicon-specific repressor whose concentration in the cell determines the frequency of initiation of replication. It is proposed that this repressor is encoded on the plasmid DNA and binds at or near the origin of replication to control the number of rounds of DNA replication during the division cycle (27, 28).

Evidence to support both types of models has been obtained, although in different plasmid replication systems (5, 10, 14, 31, 35, 42). In many of these investigations, plasmid mutants which have increased copy numbers or altered incompatibility properties have been used to gain insight into the interaction of components involved in replication control. Plasmid incompatibility, the inability of two plasmids to coexist stably in descendants of a single cell, has been used as a measure of plasmid relatedness (9, 25). Incompatibility could be a manifestation of plasmid copy number control (27) or of competition for a shared segregation apparatus (12, 25).

The analysis of the incompatibility properties of a number of copy number mutants of IncFII plasmids has suggested that incompatibility and copy number control may be determined by a plasmid-coded repressor which acts on a receptor site on the plasmid molecule (20, 22, 31, 43). The characteristics of these copy mutants indicate that some of them are altered in the repressor and others are altered in its target site. Molin and Nordstrom (22) have shown that a 550-base-pair (bp) fragment which encodes a plasmid incompatibility gene also encodes a function which prevents an integrated IncFII R plasmid from suppressing a *dnaA*(Ts) mutation at the nonpermissive temperature. This indicates that

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the product of the incompatibility gene acts to switch off plasmid replication, which may be the means by which it brings about elimination of plasmids from the cell. Stougaard et al. (36) have identified a short untranslated RNA encoded within the 550-bp fragment that they suggest is the diffusible incompatibility product. A small RNA has also been shown to be involved in the expression of incompatibility of the ColE1 plasmid (6, 24, 42).

The IncFII R plasmid NR1 (also called R100 or R222) is a 90-kilobase (kb) self-transmissible drug resistance plasmid (32) which is similar in structure to the other IncFII R plasmids R6 and R1 (2, 33). NR1 is maintained at a copy number of about one per cell (32). The location on NR1 of the drug resistance genes (19) and cleavage sites for restriction endonucleases (2, 38) have been determined. We have previously described the generation of low-molecular-weight replicators from NR1 which consist of two adjacent *Pst*I fragments of 1.1 and 1.6 kb which are located within the 12.5-kb *Eco*RI fragment B (20). An origin of replication of small NR1 replicators has been located on the *Pst*I 1.6-kb fragment (26, 37; J. Kasner and R. Rownd, unpublished data). The *Pst*I 1.1-kb fragment alone can express plasmid incompatibility when cloned onto a plasmid vector, although neither this fragment nor the *Pst*I 1.6-kb fragment alone can support autonomous plasmid DNA replication (13, 20, 39, 41).

In this communication, we present a continuation of our earlier analysis (20) of the incompatibility properties of NR1 and its copy number mutants, pRR12 (23) and pRR21 (39, 40). These experiments clearly show that NR1 and pRR12 differ both in an incompatibility repressor and in a target site for its action. The pRR12 repressor and target site mutation(s) as well as the copy number control mutation were found to be located on the *Pst*I 1.1-kb fragment. Thus, at least one target site for the incompatibility product is distinct from the origin of DNA replication. Also, we have shown that the incompatibility target site in which NR1 and pRR12 differ is a site at which the incompatibility product regulates gene expression. Our data suggest that this gene expression control is the means by which the incompatibility product controls plasmid DNA replication.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. *Escherichia coli* K-12 strains KP245 *met trp his thy lac gal tsx* F and KP435 *trp ilv thy rpsL recA* were used for plasmid construction and incompatibility tests, respectively (20). Strain NK5031 *lacZ* M5275 *supF gyrA* (11) was used for the construction of lysogens. The phages λRS205 *cI857 nin5 fec lac* and λRS205 *lacP*$^+$ (a derivative of λRS205 which carries the lactose promoter upstream from the β-galactosidase gene) were constructed and provided by K. Bertrand and W. S. Reznikoff, University of Wisconsin, Madison. Most of the plasmids used are described below and in the tables. The plasmids pRR403 and pRR634 are derivatives of NR1 and pRR12, respectively, each of which has lost genes for resistance to tetracycline as the result of spontaneous deletion. The multicopy cloning vehicle pBR322 (4) was used in construction of recombinant plasmids. Some of the recombinant plasmids derived from pRR21 were provided by D. P. Taylor (39).

Culture media. L broth (15), Penassay broth (Difco Laboratories), and nutrient broth (Difco Laboratories) were used for liquid culture media. Penassay agar plates were made by adding 1.5% Difco agar to Penassay broth. Nutrient agar plates were used for antibiotic selection. Thymine was added to culture media to a final concentration of 20 μg/ml. In solid media, antibiotics were used at the following concentrations (in micrograms per milliliter): tetracycline hydrochloride, 10; sodium ampicillin, 20; chloramphenicol, 10; spectinomycin sulfate, 10; kanamycin sulfate, 50; and nalidixic acid, 20. The medium used for phage plating contained (per liter): 10 g of tryptone (Difco), 5 g of NaCl, 11 g of agar (Difco), and 25 mg of 2,3,5-triphenyltetrazolium chloride (Calbiochem). After the medium was autoclaved, lactose was added to a final concentration of 1%. Top agar contained (per liter): 10 g of tryptone, 8 g of agar, and 8 g of NaCl. Glucose was added to top agar after autoclaving to a final concentration of 0.1%. On this medium, phages which could produce high levels of β-galactosidase formed plaques surrounded with dark red rings on lawns of Lac$^-$ bacteria. Growth of bacterial cultures in liquid medium was monitored at 600 nm, using a Gilford 240 spectrophotometer.

DNA isolation, analysis, and in vitro recombination. DNA isolation, restriction endonuclease digestion, gel electrophoresis, ligation of restriction fragments, and transformation of *E. coli* cells with plasmid DNA were performed as in previous work (20). Isolation of phage DNA used in recombinant DNA experiments, in vitro packaging of phage DNA, and restriction analysis of recombinant phage DNA from lysates have been described previously (3). In vitro packaging materials were generously provided by J. Slightom and E. Vanin.

Plasmid copy number determinations. Plasmid copy numbers were measured by two methods. (i) Ratios were determined between covalently closed circular DNA and chromosomal DNA in ethidium bromide-cesium chloride gradients made from lysates of exponentially growing cultures (44). The molecular weight of the *E. coli* chromosome was assumed to be 2.5×10^9 (3,800 kb). (ii) The copy numbers of plasmids which carry the gene for chloramphenicol acetyltransferase were estimated from gene dosage effects by measuring the amount of this enzyme in extracts of exponential-phase cells (optical density of 0.5 at 600 nm) harboring the plasmid. This was done by a modification of the method of Shaw (34). Cultures (5 ml) were grown in nutrient broth containing appropriate antibiotics (chloramphenicol, 25 μg/ml; tetracycline, 5 μg/ml) to select for the presence of the plasmids in all cells of the population. These cultures were harvested at 0 to 4°C, washed twice in cold 0.1 M Tris, pH 7.8,

and suspended in 1.0 ml of this buffer. The suspended cells were sonicated with a cell disrupter (Sonifier W140D) at 10 W for 20 s. Debris was removed by centrifugation for 2 min in an Eppendorf centrifuge. The supernatant fluids were tested for chloramphenicol acetyltransferase activity (34) and for total protein content (17).

Plasmid incompatibility measurements. Incompatibility tests were performed as previously described (20). Donor plasmid DNA was introduced by transformation into *recA* cells (KP435) harboring the resident plasmids. After recovery and growth for 90 min in drug-free L broth, the cells were spread on nutrient agar plates containing an antibiotic which selects only for the donor plasmid. After 36 h of incubation, 10 colonies were picked from the plates and streaked onto Penassay agar plates. From each of these streaks, 10 colonies were picked to drug-free plates. The patches of cells were tested by replica plating for resistance to single antibiotics to which donor and resident plasmids confer resistance. In this procedure, cells were examined for their drug resistance phenotype approximately 30 generations after the donor plasmid DNA was introduced into the cells harboring the resident plasmid.

Preparation of phage stocks and construction of lysogens. Single plaques formed on lawns of NK5031 cells at 37°C were picked into 1.0-ml sterile phage storage buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.10 M NaCl, 0.01 M $MgCl_2$, 0.05% gelatin) containing chloroform. These phage suspensions were used to infect a small culture of exponentially growing NK5031 cells. After overnight aeration at 37°C, the lysates were treated with chloroform, and the cell debris was pelleted. The phage suspension was used as a source of phage DNA as described before (3). To construct lysogens, 1 drop of the phage suspension was spotted onto plates seeded with NK5031 cells. These were incubated at 30°C until colonies appeared within the region of the phage spot. These cells were purified and tested for growth at 30°C and 42°C. Lysogens were those cells which did not survive at 42°C.

β-Galactosidase assays. β-Galactosidase assays were performed with some modifications by the method described before (21). NK5031 lysogens were grown at 30°C in 1× A medium to an absorbance (600 nm) of 0.4. When the cells harbored a plasmid conferring tetracycline resistance, 10 μg of tetracycline per ml was added to the culture medium. Samples of 0.05 ml, 0.1 ml, or 0.25 ml of the culture were mixed in Z buffer to a final volume of 1.0 ml. The cells were permeabilized by short Vortex mixing in the presence of sodium dodecyl sulfate and chloroform. After the *o*-nitrophenyl-β-D-galactopyranoside hydrolysis reaction was stopped by addition of 1.0 M Na_2CO_3, the cell debris was removed from the samples by centrifugation for 2 min in an Eppendorf centrifuge. The absorbance at 420 nm was measured.

Curing of pBR322 plasmids from lysogenic cells. Tetracycline-sensitive cells were selected from a population of tetracycline-resistant cells, using D-cycloserine enrichment (7). Cultures of NK5031 lysogens harboring pBR322 derivatives were diluted into Penassay broth containing 2 μg of tetracycline per ml. After aeration for 1.5 h at 30°C, D-cycloserine was added to a final concentration of 2 mM. Cells were plated after 3 to 5 h of additional aeration. Tetracycline-sensitive colonies were identified by replica plating.

RESULTS

Incompatibility properties of recombinant plasmids derived from NR1 and two of its copy number mutants. The regions of NR1 plasmid DNA required for autonomous replication and for expression of incompatibility are shown in Fig. 1. Small plasmid derivatives were constructed which consisted of two contiguous *PstI* fragments of 1.1 kb (*inc*) and 1.6 kb (*ori*) ligated to a 2.1-kb *PstI* fragment which encodes a gene for resistance to chloramphenicol (*PstI cam*) (20). The structure of one of these plasmids is shown in Fig. 2. Such plasmids were constructed from the wild-type NR1 as well as from two copy number mutants (*cop*) of NR1 designated pRR12 (23) and pRR21 (39, 40). These plasmid derivatives were designated pRR933 (from NR1), pRR942 (from pRR12), and pRR1919 (from pRR21). The *PstI* replicator fragments from pRR21 were obtained from pDTP103 which is a derivative of pRR21 (39, 40).

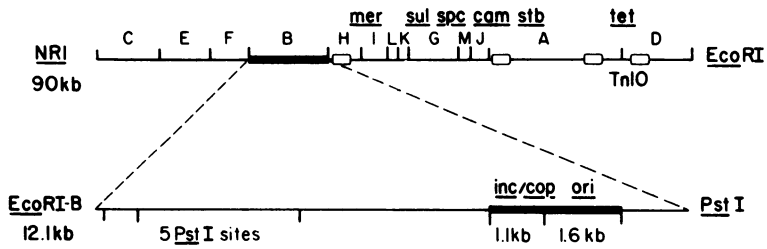

FIG. 1. Region of NR1 capable of autonomous replication. The fragments required for autonomous replication were identified previously (20). Boldface lines indicate fragments needed for replication. Genotype symbols indicate the location of genes for resistance to mercuric ions (*mer*), sulfonamides (*sul*), streptomycin and spectinomycin (*spc*), chloramphenicol (*cam*), and tetracycline (*tet*) (19). Other symbols indicate the location for plasmid stability locus (*stb*) (20), for plasmid incompatibility and copy number control (*inc/cop*) (20), and for the origin of vegetative replication (*ori*) (26, 37; Kasner and Rownd, unpublished data). The capital letters on the top line refer to the *Eco*RI fragments of NR1 (38).

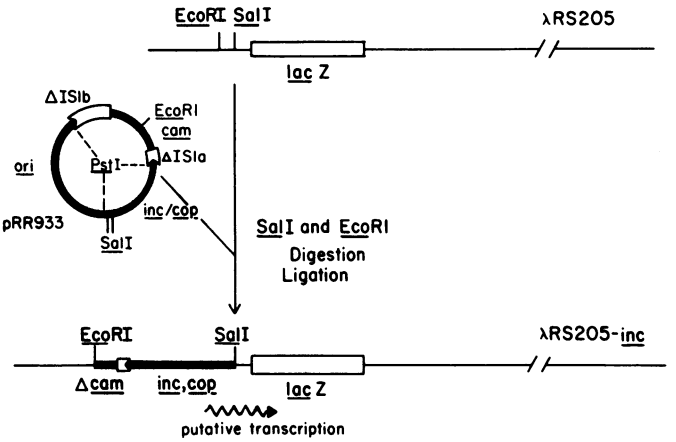

FIG. 2. Construction of lambda phages carrying the incompatibility region of NR1 adjacent to the *lacZ* gene. Recombinant DNA procedures are described in the text. Structures are not drawn to scale.

The incompatibility properties of the small autonomously replicating plasmids were found to be the same as those of the parent plasmids from which they were derived (20) (data not shown). All of the NR1 derivatives were incompatible with other NR1 derivatives and also with pRR21 derivatives. The plasmids derived from pRR21 were incompatible with NR1 and with other pRR21 derivatives as had been shown elsewhere (39). However, the small replicators from pRR12 were found to be compatible with replicators derived from NR1, pRR12, or pRR21 (data not shown) which is consistent with our previous results (20).

The *PstI* 1.1-kb incompatibility fragments of NR1, pRR12, and pRR21 were cloned onto the high-copy-number vector pBR322 (4). These recombinant plasmids were designated pRR935, pRR939, and pDPT251 (from D. P. Taylor), respectively. These three pBR322 derivatives maintained the incompatibility fragments of NR1, pRR12, and pRR21 at the same gene dosage when used as donor plasmids in incompatibility experiments. The resident plasmids in the cells in these experiments were either tetracycline-sensitive derivatives of NR1 (pRR403) and pRR12 (pRR634) or the *PstI*-generated replicators derived from NR1, pRR12, and pRR21. The results of these experiments are shown in Table 1. The donor plasmids carrying the *PstI* 1.1-kb fragments from either NR1 (pRR935) or from pRR21 (pDPT251) eliminated replicators derived from NR1 (pRR933 and pRR403) or from pRR21 (pRR1919) but did not eliminate those derived from pRR12 (pRR942 and pRR634). On the other hand, the donor pBR322 with the *PstI* 1.1-kb fragment from pRR12 (pRR939) completely eliminated the resident pRR12 derivatives (pRR942 and pRR634) but not the resident replicators derived from NR1 (pRR933 and pRR403) or from pRR21 (pRR1919). This specificity of elimination was maintained for derivatives of NR1 and pRR12 when coresident in the same host cells (data not shown). Donor plasmids carrying the *PstI* fragments in either orientation with respect to pBR322 behaved identically in these experiments.

In incompatibility experiments, low-copy-number NR1 derivatives such as pRR403 which harbored the *stb* function previously shown to be required in *cis* for stable plasmid inheritance (20) were not excluded at all by pBR322 with the *PstI* 1.1-kb fragment of pRR12 (pRR939) (Table 1). However, a low level of loss of low-copy-number NR1 derivatives such as pRR933 which lack the *stb* function was observed after transformation by the pBR322 vector, which probably reflects the spontaneous loss of the resident plasmids from the host cells. A slightly greater loss was found after transformation by pRR939, although, as mentioned previously, pRR939 did not exclude the Stb^+ NR1 derivative pRR403 (Table 1). The reason for this difference is presently under investigation.

The incompatibility of pBR322 derivatives carrying the *PstI* 1.1-kb fragment of pRR12 was expressed only toward the pRR12 replicators. This incompatibility was very strong (100% exclusion of resident pRR12 replicators), in contrast to the weak incompatibility expressed by pRR12 replicators against one another which is barely detectable by the assays employed in this laboratory (20). A possible explanation for this difference is presented in the Discussion.

Mapping the target site for the incompatibility product. Our observations suggest that NR1 and pRR12 produce different incompatibility prod-

TABLE 1. Properties of mutant and hybrid plasmid replicators

Plasmid no.	Resident plasmid[a]				% of transformants harboring the resident plasmid[a] after transformation by:			
	Source of:		Copy no.		pRR935[d]	pRR939[d]	pDPT251[d]	pBR322
	*Pst*I (1.1 kb)	*Pst*I (1.6 kb)	cat[b]	ccc/chr[c]				
pRR403	NR1	NR1	1.0	0.9	0	100	0	100
pRR933	NR1	NR1	1.0	ND[f]	0	46	0	90
pRR949[e]	NR1	NR1	1.1		0	41		84
pRR952[e]	NR1	pRR12	1.3	ND	0	60		96
pRR953[e]	pRR12	NR1	7.8	19	100	0		100
pRR957[e]	pRR12	pRR12	7.2		100	0		100
pRR942	pRR12	pRR12	7.0	23	100	0	100	100
pRR643	pRR12	pRR12	2.8		100	0		100
pRR1919	pRR21	pRR21	9.9		0	90	0	100

[a] Except for pRR403 and pRR634, all resident plasmids were composed of the *Pst*I 1.1-kb plus 1.6-kb fragments plus *Pst*I *cam*.

[b] Relative copy numbers were determined by measuring chloramphenicol acetyltransferase (cat) specific activities in extracts of KP245 cells harboring the resident plasmid. The values of change in absorbance at 412 nm/min per μg of protein in the sample were determined for each culture. These values were divided by that obtained for the strain harboring NR1.

[c] Calculated from ratios of covalently closed circular (ccc) DNA to chromosomal (chr) DNA in cesium chloride-ethidium bromide gradients (44).

[d] Donor plasmids consisted of pBR322 plus the *Pst*I 1.1-kb fragment of NR1 (pRR935), pRR12 (pRR939), or pRR21 (pDPT251).

[e] These resident plasmids were constructed from individual *Pst*I 1.1- or 1.6-kb fragments which had been cloned independently onto a pBR322 vector.

[f] Not detectable.

[g] Incompatibility tests were performed as described in the text.

ucts and also have different target sites. The NR1 incompatibility product acts preferentially on the NR1 target, whereas the altered pRR12 incompatibility product acts preferentially on the altered pRR12 target. The incompatibility products of both NR1 and pRR12 are specified by the *Pst*I 1.1-kb fragment (Table 1). The incompatibility target site in which NR1 and pRR12 differ must be within the *Pst*I 1.1- and 1.6-kb fragments, which define the replicator region.

To determine the location of the target more precisely, we constructed hybrid replicators consisting of one *Pst*I fragment from NR1 and one from pRR12. The plasmid DNA of pBR322 derivatives carrying the individual *Pst*I 1.1- and 1.6-kb fragments from NR1 and pRR12 were mixed in appropriate combinations with the DNA of a pBR322 derivative carrying the *Pst*I *cam* fragment. These plasmid DNAs were digested to completion with *Pst*I, ligated, and used to transform KP245 cells to chloramphenicol resistance. Tetracycline-sensitive transformants, which therefore did not contain pBR322, were chosen from among the transformants.

The plasmid DNAs in these cells contained the *Pst*I 1.1- and 1.6-kb fragments ligated to the *Pst*I *cam* fragment. In independently constructed recombinants, the *Pst*I *cam* fragment was found in either orientation with respect to the replicator fragments as determined by electrophoresis of plasmid DNAs cleaved with both *Bgl*II and *Eco*RI, which cleave asymmetrically within the *Pst*I 1.1-kb and *Pst*I *cam* fragments, respectively (data not shown). However, the *Pst*I 1.1- and 1.6-kb fragments were always found in native orientation with respect to each other as determined by electrophoresis of DNA digested with both *Sal*I and *Sma*I (data not shown). This indicated that the *Pst*I junction was required for autonomous replication (20, 39). In these experiments, both types of NR1-pRR12 hybrid replicators were constructed, and both wild-type and mutant replicators were also reconstituted from their individual *Pst*I fragments.

As residents in incompatibility experiments, the NR1-pRR12 hybrid replicators were eliminated from the cells with the specificity determined by the source of their *Pst*I 1.1-kb fragments (Table 1). The hybrid replicators were also found to be eliminated from the cells according to the source of their *Pst*I 1.1-kb fragment even when coresident with a compatible replicator containing the other type of *Pst*1 1.1-kb fragment (data not shown). Thus, the pRR12 incompatibility target site mutation is located on the *Pst*I 1.1-kb fragment. This shows that at least one target site for the incompatibility product is distinct from the origin of DNA replication, which is on the *Pst*I 1.6-kb fragment. The incompatibility specificity of the hybrid replica-

tors when used as donors in incompatibility tests was also determined by the source of their *Pst*I 1.1-kb fragment (data not shown).

Copy numbers of mutant and hybrid *Pst*I replicators. The copy number of plasmids containing the *Pst*I replicator fragments and the *Pst*I *cam* fragment was estimated by measuring the specific activity of chloramphenicol acetyltransferase in extracts of cells harboring these plasmids. The copy number of pRR933 was found to be the same as that of its parent NR1 (Table 1). The plasmids pRR942 and pRR1919 were maintained at even higher copy numbers than were the *cop* pRR12 and pRR21 plasmids from which they were derived (23, 40). An additional increase in the copy number of miniplasmids derived from copy mutant plasmids has been observed previously (40). The reason for this additional increase is not understood.

The NR1-pRR12 hybrid replicators pRR952 and pRR953 were maintained at the copy number of the plasmid from which their *Pst*I 1.1-kb fragment was derived. The replicators reconstituted entirely from the wild type (pRR949) or from pRR12 (pRR957) had the same copy number as did their parent *Pst*I replicators (Table 1). Thus, the mutation(s) which gave rise to the pRR12 phenotype are all located on the *Pst*I 1.1-kb fragment. These include changes in the specificity of the incompatibility product, in the specificity of the target site for its action, and in copy number control.

The copy number of some of these plasmids was also determined by measuring the ratios of covalently closed circular DNA to chromosomal DNA in cesium chloride-ethidium bromide gradients containing lysates of cells which harbor the plasmids (Table 1). Covalently closed circular (plasmid) peaks were not detectable in such gradients of DNA from cells harboring pRR933 (data not shown). This was not unexpected since such a small, low-copy-number plasmid would contain only about 0.1% of the radioactive counts of the chromosomal peak, which would be difficult to distinguish from the background level of counts in the gradients. The value for copy numbers for pRR942 and pRR953 were higher when determined by this method than when measured by the chloramphenicol acetyltransferase assay method. The reason for the discrepancy in the copy number values as measured by the two methods is not known. It is possible that the specific activity of chloramphenicol acetyltransferase is not directly proportional to gene dosage at very high plasmid copy numbers.

Construction of phages carrying a fusion between the incompatibility region and the *lacZ* gene. It seemed likely that the function of the incompatibility product may be to control the level of production of an RNA transcript or of the protein which it encodes. To examine this possibility, the incompatibility regions of NR1, pRR12, and pRR21 were ligated to the *lacZ* gene located on the phage λRS205 by the scheme shown in Fig. 2. This phage carries a copy of the *lacZ* gene which lacks it own promoter and operator but retains the ribosome binding site for translation of the β-galactosidase protein. The expression of β-galactosidase can be brought under the control of promoters on DNA inserted at the unique *Eco*RI and *Sal*I restriction sites located upstream from the ribosome binding site.

It was convenient to insert the incompatibility regions of NR1, pRR12, and pRR21 into λRS205, using the plasmids pRR933, pRR942, and pRR1919 as the sources of the fragments containing the incompatibility regions. The enzyme *Sal*I cleaves these plasmid DNAs twice within the *Pst*I 1.1-kb fragment 51 bp and 27 bp from the *Pst*I site which divides the *Pst*I 1.1- and 1.6-kb fragments (29). The *Eco*RI cleavage site is located in the gene for chloramphenicol resistance external to the replicator region (1, 19).

The DNAs of λRS205 and pRR933, pRR942, or pRR1919 were digested to completion with *Sal*I and *Eco*RI, ligated, and packaged in vitro, and the mixtures were used to infect cells of the strain NK5031. Individual phage plaques were picked into sterile buffer, and the phage DNA was examined for the presence of inserted fragments as described above. Phages were obtained which contained a *Sal*I-*Eco*RI fragment the same size as the smaller of the *Sal*I-*Eco*RI fragment from the plasmids pRR933, pRR942, and pRR1919 (Fig. 3). In all three cases, these phage DNA contained an extra recognition site for the restriction endonuclease *Bgl*II which cleaves within the *Sal*I-*Eco*RI fragment (20, 29). Therefore, the incompatibility regions of these plasmids had been inserted into the phage upstream from the *lacZ* gene such that expression of β-galactosidase should be under the control of the promoter of the large RNA which is transcribed toward the plasmid origin of replication (10, 30) (Fig. 2). Phages carrying the larger *Sal*I-*Eco*RI fragment of these three plasmids were not obtained in these experiments for reasons which are not understood.

The plaques produced on lawns of NK5031 cells on the lactose-tetrazolium medium by the phages which contained the incompatibility region of pRR933 or of pRR942 were no more red than those made by λRS205. However, the plaques produced by phages which contained the incompatibility region of pRR1919 were surrounded by dark red rings, indicating a higher level of β-galactosidase in the lysogens.

Measurement of β-galactosidase from lysogens

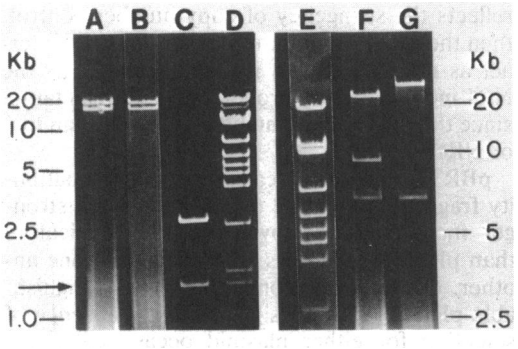

FIG. 3. Agarose gel electrophoresis of recombinant phage DNA treated with restriction enzymes. Lanes A through D are a 1.0% agarose gel for which the leftward kb scale applies. Lanes E through G are a 0.6% agarose gel for which the rightward kb scale applies. (A) λRS205 DNA carrying the *inc/cop* fragment of pRR933 treated with *Sal*I and *Eco*RI; (B) λRS205 DNA treated with *Sal*I and *Eco*RI; (C) pRR933 DNA treated with *Sal*I and *Eco*RI; (D and E) pRR12 DNA treated with *Eco*RI; (F) λRS205 DNA carrying the *inc/cop* fragment of pRR933 treated with *Bgl*II; (G) λRS205 DNA treated with *Bgl*II. The arrow on the left indicates the position of the *Sal*I-*Eco*RI incompatibility fragment.

of *inc-lac* phages. Lysogens of the strain NK5031 were made by using the three types of *inc-lac* phages as well as λRS205 and λRS205 carrying the lactose promoter inserted into the phage at the *Sal*I and *Eco*RI sites upstream from the *lacZ* gene. The levels of β-galactosidase produced in these lysogens are presented in Table 2. The lysogens containing λRS205 with no DNA inserted adjacent to the *lacZ* gene produced approximately 60 units of β-galactosidase, presumably due to readthrough transcription from chromosomal or phage genes.

The presence of inserts of the incompatibility region in the prophages resulted in increased production of β-galactosidase due to transcription from the incompatibility region into the *lacZ* gene. Each of the plasmid replication phenotypes was associated with a characteristic level of β-galactosidase production (Table 2). Phages containing the incompatibility region of the copy mutants pRR12 and pRR21 produced considerably higher levels of β-galactosidase than phages containing the incompatibility region of the wild type NR1. These β-galactosidase levels are consistent with the colors of plaques formed on the lactose-tetrazolium medium.

To test the effects of the incompatibility product on the levels of β-galactosidase production, the pBR322 derivatives carrying the *Pst*I 1.1-kb (*inc*) fragments from NR1, pRR12, or pRR21 were introduced into the lysogens. The presence of these plasmids in the cells resulted in a decrease in the level of β-galactosidase in all of the *inc-lac* lysogens (Table 2). Neither the vector pBR322 nor the pBR322 derivatives carrying the *Pst*I 1.6-kb (*ori*) fragments had any effects on the β-galactosidase levels in the *inc-lac* lysogens (Table 2). The plasmids carrying the incompatibility fragment did not have an effect on β-galactosidase levels in lysogens of λRS205 or λRS205 (*lacP*$^+$) (Table 2). When the *inc-lac* lysogens were cured of the pRB322 plasmids carrying the *Pst*I 1.1-kb fragments, the levels of β-galactosidase returned to those characteristic

TABLE 2. β-Galactosidase units produced in lysogens of λ*inc/cop-lacZ* phages

Plasmid in lysogen	Prophage in lysogen[a]				
	λRS205	λRS205 *inc*-NR1	λRS205 *inc*-pRR12	λRS205 *inc*-pRR21	λRS205 *lacP*$^+$
None	66	220 ± 10	500 ± 7	1,600 ± 117	1,768
pRR935 (pBR322 + *Pst*I [1.1 kb] NR1)	54	56 ± 9	140 ± 20	260 ± 38	1,433
pRR939 (pBR322 + *Pst*I [1.1 kb] pRR12)	55	95 ± 7	80 ± 19	410 ± 18	1,830
pDPT251 (pBR322 + *Pst*I [1.1 kb] pRR21)	77	59	147	230 ± 40	1,679
pRR936 (pBR322 + *Pst*I [1.6 kb] NR1)		216	408	1,567	
pRR937 (pBR322 + *Pst*I [1.6 kb] pRR12)		214	485	1,404	
pDPT266 (pBR322 + *Pst*I [1.6 kb] pRR21)		269	530	1,473	
pBR322	57	228	611	1,600 ± 145	

[a] Numbers are β-galactosidase units produced from lysogens ± the standard error. The strain used was NK5031, which produces no β-galactosidase. The values quoted with standard deviations are the average of the values taken in one experiment from three or four independently constructed strains. For each lysogen except λRS205 and λRS205 *lacP*$^+$, the differences between the values obtained with pRR935 in the cell and those with pRR939 in the cell have $P < 0.01$. The background levels of activity seen in strains containing λRS205 were not subtracted from the values listed.

of the lysogen (data not shown). For a given *inc-lac* lysogen, the *Pst*I 1.1-kb fragments which caused the greater reduction in the β-galactosidase level were those which expressed the stronger incompatibility toward the plasmid from which the fragment in the prophage was derived (compare Table 1 with Table 2).

The differences in the level of expression of β-galactosidase in these experiments were not due to gene dosage effects. The pRB322 derivatives carrying the *Pst*I 1.1-kb fragment of either NR1 or pRR12 were each maintained at approximately the same copy number (23 to 25 per chromosome) in the NK5031 *inc*(NR1)-*lac* lysogens grown under conditions used for the β-galactosidase assays (data not shown). These values were determined from covalently closed circular to chromosome DNA ratios in ethidium bromide-cesium chloride gradients (44). In these experiments, more than 96% of the cells in the lysogenic cultures retained the pBR322 *Pst*I 1.1-kb derivative plasmids during growth before the β-galactosidase assays.

DISCUSSION

The phenotypic differences between the IncFII R plasmid NR1 and its copy number mutant pRR12 have provided a means to locate a target site for the incompatibility product and to show that this product functions at this target site by regulating gene expression. Since the incompatibility product has been shown to switch off plasmid replication (22), we presume that this gene regulation is relevant to the control of plasmid replication.

The copy number mutant pRR12 differs from the wild-type NR1 both in the incompatibility product and in a target site for its action. This conclusion seemed likely from our previous studies on the incompatibility properties of low-molecular-weight replicators derived from NR1 and pRR12 (20). However, differences between NR1 and pRR12 were shown more clearly by the incompatibility behavior of pBR322 derivatives which carry the *Pst*I 1.1-kb fragment from either NR1 or from pRR12 as described in this communication. These data also revealed that the NR1 and pRR12 incompatibility products act preferentially at their own incompatibility target sites. It is interesting to note that if NR1 and pRR12 had been isolated independently from nature, these two plasmids would have been classified in different incompatibility groups.

It is not surprising that an alteration in the incompatibility repressor would also require a simultaneous change in its target site since the interaction between these two control elements is most likely involved in the regulation of the frequency of initiation of plasmid replication. If the strength of the repressor-target interaction reflects the stringency of copy number control, then the pRR12 mutant repressor does not interact as effectively with its mutant target as the NR1 incompatibility product does with its target since the pRR12 copy number is higher than that of NR1.

pBR322 derivatives carrying the incompatibility fragment of pRR12 displayed a much stronger incompatibility toward pRR12 replicators than pRR12 replicators showed toward one another. Cell cultures containing two distinguishable pRR12 replicators grown in the absence of selection for either plasmid occasionally produced cells which spontaneously lost one of the two types of plasmids (18, 40; J. Greenberg and R. Rownd, unpublished data). Therefore, pRR12 replicators do express weak incompatibility toward one another, although this was difficult to detect by using the assays used in this laboratory (20). It is unlikely that the increased strength of the incompatibility expressed by the plasmids consisting of pBR322 and the *Pst*I 1.1-kb fragment of pRR12 is due either to a mutation in the PstI 1.1-kb fragment of pRR12 carried on pBR322 or to the orientation of the fragments on the vector. Strong incompatibility was expressed by four independently constructed pBR322 derivatives containing the *Pst*I 1.1-kb fragment of pRR12 in either orientation. The fact that the *Pst*I 1.1-kb fragment of pRR939 (pBR322 plus the *Pst*I 1.1-kb fragment of pRR12) was not altered from that of pRR12 was confirmed by using this *Pst*I 1.1-kb fragment to reconstruct replicators. The incompatibility and copy number properties of these plasmids were found to be the same as those of plasmids derived directly from pRR12. It is also unlikely that gene dosage differences of the *Pst*I 1.1-kb fragments are the explanation for the increased incompatibility expression of the pRR322 derivatives. The copy numbers per chromosome of pRR942 (*Pst*I replicator of pRR12) and of pRR939 were about the same as determined by the ethidium bromide-cesium chloride gradient method (44).

It seems likely that pRR12 replicators, when used as donors, are not effective at causing elimination of resident pRR12 replicators because the pRR12 donor plasmids are sensitive to the replication control exerted by the resident pRR12 replicators. After introduction of the donor pRR12 replicators into cells harboring a resident pRR12-derived plasmid, the cells would contain a multicopy mixed pool of pRR12 donor and resident plasmids from which complete loss of either type would be rare. The plasmid pRR939, on the other hand, is presumably immune to pRR12 replication control since it replicates under pBR322 control. Therefore, after transformation of cells containing pRR12 repli-

cators, pRR939 would replicate until its copy number reached that characteristic of the pBR322 vector. The number of copies of the incompatibility fragment on the pBR322 derivatives are presumably sufficient in number to cause the switch off of replication of the resident pRR12 replicators and thus would eliminate them from the cells.

The properties of the hybrid NR1-pRR12 replicators have shown that the pRR12 copy number mutation as well as the mutation in the incompatibility product lie within the *Pst*I 1.1-kb fragment. Moreover, the dramatic difference between NR1 and pRR12 in sensitivity to the incompatibility expressed by plasmids consisting of pBR322 and the *Pst*I 1.1-kb fragments of these plasmids has made possible the mapping of the pRR12 target site mutation to the *Pst*I 1.1-kb fragment. Recent experiments have shown that the incompatibility target site lies within the 490 bp of the *Pst*I 1.1-kb fragment proximal to the *Pst*I 1.6-kb fragment (Easton, unpublished data). Therefore, the incompatibility product can act at a target site which is located more than 1 kb from the DNA replication origin which is on the *Pst*I 1.6-kb fragment. Presumably, it acts before the initiation of replication to control the number of rounds of plasmid replication.

The insertion of plasmid incompatibility regions upstream of the *lacZ* gene on the phage λRS205 resulted in increased levels of β-galactosidase production in lysogens constructed with these phages. This indicated that there is an in vivo transcript which initiates on the *Pst*I 1.1-kb fragment and is read toward the origin of DNA replication. A transcript which corresponds in position and direction to this one is produced in vitro by RNA polymerase acting on templates containing the replication region (10, 30; P. Sampathkumar and R. Rownd, manuscript in preparation). This transcript is at least 1,200 bases in length (10) and could encode two replication proteins designated RepA3 and RepA1 which were predicted from the DNA sequence of the pRR12 replication region (29). A smaller RNA 91 bases in length has also been observed in both in vivo and in vitro experiments (10, 30, 36). This RNA species is synthesized from the opposite strand of the putative mRNA for RepA3 and RepA1 near its 5′ end. There is increasing evidence that this smaller complementary RNA is the incompatibility product of IncFII plasmids (36). A small RNA which can be processed to form a primer for the initiation of plasmid ColE1 replication has also been shown to be involved in the expression of ColE1 incompatibility (6, 24, 42). It is presently not known whether the large RNA which corresponds to the in vivo transcript monitored in our experiments on β-galactosidase levels is used as a primer for the initiation of IncFII plasmid replication.

Higher levels of β-galactosidase were produced from lysogens which carry prophages with incompatibility region inserts derived from copy mutant plasmids. This strongly suggests that an increased level of gene expression in the replication region may lead to an increased frequency of replication and, hence, to an increased plasmid copy number. The relationship between the level of gene expression and the plasmid copy number is presently not known. Increased gene expression in the plasmid replication region may also result in an increased availability of RNA primers for the initiation of plasmid replication.

The presence of plasmids carrying the incompatibility region decrease the levels of β-galactosidase produced in the *inc-lac* lysogens. The incompatibility target site and the target site for gene expression control as well as the repressors for both of these sites were found to be located in the same DNA region. The *Pst*I 1.1-kb fragment expresses both incompatibility and the depression of β-galactosidase levels in the *inc-lac* lysogens. The *Sal*I-*Eco*RI fragment which contains a site for replication gene expression control contains all of the DNA sequences of the *Pst*I 1.1-kb fragment except about 50 bp of DNA near the *Pst*I (1.1 kb)-*Pst*I (1.6 kb) junction, which the *Sal*I-*Eco*RI fragment lacks (29). The correlation between the expression of incompatibility and the control of β-galactosidase expression is made stronger by the observation that the diffusible repressors produced by NR1 and by pRR12 interact more strongly with their own target sites in both the incompatibility tests and in the gene expression control assays. This suggests that the incompatibility target site and the gene expression control site are the same and that it is the incompatibility product that acts to control gene expression. This last conclusion has recently also been reached in studies on the IncFII R plasmid R1 (16).

The mechanism by which the incompatibility product affects the level of expression of β-galactosidase in the *inc-lac* lysogens is not known. The small incompatibility RNA might directly repress the amount or the extension of the transcript which proceeds through the *Sal*I site toward the origin of replication. Alternatively, the incompatibility RNA could block the translation of proteins encoded on the large message which could render the message more susceptible to degradation or premature termination.

The plasmid fragment present in the prophage must also produce the incompatibility substance since NR1 replicators were seen to be rapidly lost from the lysogens which carry the incom-

patibility fragment of NR1 in their chromosome after removal of selection for the plasmid (data not shown). The repressor made from the copy of the *inc* fragment in the lysogen would also have some effect on the level of expression of the downstream β-galactosidase gene. For each of the plasmids used in our experiments, the strength of the interaction between the repressor and its target site would be reflected in the level of expression of the *lacZ* gene in the prophage in the absence of any autonomous plasmid in the cells.

There appears to be some degree of residual interaction between the incompatibility products of NR1 and pRR12 and the target site of the other plasmid. The interaction of the repressor of NR1 with the target of pRR12 and the interaction of the repressor of pRR12 with the target of NR1 appear to be stronger when measured in the transcription assay than in the incompatibility tests (compare Table 1 with Table 2). The assays of transcript levels are probably more sensitive to weak interactions than is the measure of loss of plasmids from the cells in incompatibility tests.

The number and location of mutational changes between NR1, pRR12, and pRR21 are presently being determined in this laboratory. The *Pst*I 1.1-kb fragment of pRR21 has been shown to contain a copy number mutation as determined by the copy numbers of *Pst*I-generated pRR21-NR1 hybrid replicators (D. P. Taylor, personal communication). A comparison of the DNA sequences of the entire *Pst*I 1.1-kb fragments of NR1 and pRR21 to that of pRR12 (29) should help to explain the phenotypes of these copy mutant plasmids. Since the pRR12 repressor and target site mutations are located in the same small region of DNA, it is possible that the target and the structural gene sequences overlap and that a single mutation may have altered both components simultaneously. Alternatively, a mutation in either the target or the repressor could have been accompanied by a compensating mutation in the other one to form a plasmid capable of autonomous replication since some affinity between the repressor and its target site may be essential for plasmid replication control.

Danbara et al. (8) concluded that an incompatibility target site is located on the *Pst*I 1.1-kb fragment of the IncFII plasmid R6-5. The nucleotide sequences of the replicator regions of R6-5 and NR1 are virtually identical (8, 29), so it seems certain that these two plasmids use the same mechanism for regulation of their replication. However, the data of Danbara et al. are incomplete since incompatibility tests were not performed on the hybrid *Pst*I replicators which were constructed by using the wild-type R6-5, and the plasmids mutated in their incompatibility targets. Only partial DNA sequences of the mutant *Pst*I 1.1-kb fragments were determined (8), which does not rule out the possibility that additional mutations affecting either the incompatibility repressor or its target site might be present in their copy number mutants. A mutation in one component of a replication control system may require a compensating mutation in another control element to form a mutant capable of autonomous replication. Sequencing data will determine the number and location of the alteration(s) which gave rise to the pRR12 and pRR21 phenotypes and may suggest the means by which the incompatibility product controls gene expression.

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