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IncFII Plasmid Incompatibility Product and Its Target Are Both RNA Transcripts

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The region of DNA coding for incompatibility (*inc*) and copy number control (*cop*) of the IncFII plasmid NR1 is transcribed in both the rightward and leftward directions. The rightward transcripts serve as mRNA for the repA1 protein, which is required for replication. A small, 91-base leftward transcript is synthesized from the opposite DNA strand and is complementary to a portion of the rightward mRNA near its 5′ end. A 262-base-pair *Sau*3A restriction fragment that encodes the small leftward transcript, but does not include the rightward transcription promoters, was cloned into the vector pBR322 or pUC8. The same fragment was cloned from an Inc⁻ mutant of NR1 that does not make the small leftward transcript. Transcription through the cloned fragments in these derivatives was under control of the tetracycline resistance gene in pBR322 or the *lac* promoter-operator in pUC8. In one orientation of the inserted DNA, a hybrid transcript containing rightward NR1 RNA sequences was synthesized. In the other orientation, a hybrid transcript containing leftward NR1 RNA sequences was synthesized. These plasmids were used to vary the intracellular levels of the rightward or leftward NR1 RNA transcripts and to test their effects in *trans* on various coresident derivatives of NR1. An excess of rightward NR1 RNA in *trans* stimulated expression of the essential *repA1* gene and caused an increase in the copy number of a coresident NR1 plasmid. An excess of leftward NR1 RNA in *trans* inhibited the expression of the *repA1* gene and lowered the coresident NR1 copy number, thereby causing incompatibility. A pBR322 derivative with no transcription through the cloned NR1 DNA had no effect in *trans*. These results suggest that the small leftward transcript is the incompatibility inhibitor of NR1 and that its target is the complementary portion of the rightward mRNA.

The transmissible antibiotic resistance plasmid NR1 (30) belongs to the FII incompatibility group, which also includes plasmids R1 and R6 (6). NR1 has a size of approximately 90 kilobase pairs (kb) and a low copy number of about two per chromosome in *Escherichia coli* (31, 45). The region of DNA that codes for control of replication of NR1 consists of two contiguous *Pst*I restriction fragments of 1.1 and 1.6 kb. To form a functional replicon, these fragments must be joined in their native orientation (7, 22, 39). The structures of the replication control regions of R1 and R6 are similar to that of NR1 (18, 33). Most mutations of IncFII plasmids that affect incompatibility and copy number are located in the 1.1-kb (*inc/cop*) fragment (7, 22, 39, 40), whereas the origin of replication is in the 1.6-kb (*ori*) fragment (20, 22, 27, 38) (Fig. 1). In vitro, rightward transcription toward the origin is initiated from two sites in the 1.1-kb fragment, producing RNA-C and RNA-A (8). In vivo, transcription of RNA-C continues through its in vitro termination point to produce the elongated transcript RNA-CX (32). RNA-CX contains all of the sequences of RNA-C and RNA-A. Leftward transcription begins at one site in the *inc/cop* region, producing the 91-base RNA-E (8, 29, 36). RNA-E is completely complementary to sequences in the rightward transcripts RNA-CX and RNA-A. RNA-CX and RNA-A code for a 33,000-dalton protein, repA1, which is required for the initiation of replication at the FII origin (20, 22, 28, 33, 39). The 11,000-dalton repA2 protein (25, 28), which is a repressor of RNA-A transcription (15, 17), is also coded by RNA-CX or RNA-C. Although the repA2 protein of NR1 differs from the protein coded by the equivalent region of plasmid R1, called *copB*, the two proteins appear to serve similar functions. Additionally, RNA-CX and RNA-A might also serve as primers for the initiation of DNA replication at the origin. (7, 29).

Replication control and incompatibility of IncFII group plasmids are regulated by a plasmid-encoded *trans*-acting inhibitor (2, 6, 7, 14, 22, 24, 39, 40). The site of action of the inhibitor is also coded by the plasmid and is closely linked to the inhibitor gene (5, 7, 15, 22). Several mutations affecting incompatibility and copy number control have simultaneously altered the inhibitor and its target with a single base substitution (2, 5, 8, 22, 33, 37). Expression of the gene encoding the repA1 replication protein is regulated negatively by the incompatibility inhibitor (7, 8, 14, 16). In this communication, we present a continuation of our earlier analyses of NR1 incompatibility and copy number control (7, 8, 22). We show that the NR1 incompatibility inhibitor is the small, untranslated leftward RNA transcript and that its site of action is the complementary portion of the rightward mRNA, which codes for the required repA1 replication protein. This novel mechanism of gene regulation by the interaction of two RNA molecules results in negative regulation of plasmid replication by limiting the amount of repA1 protein that is synthetized. Experimentally altering the intracellular levels of the inhibitor RNA or its target RNA changes both the level of expression of the *repA1* replication gene and the copy number of a coresident NR1 plasmid. Although synthesis of rightward and leftward transcripts was not measured directly in their studies, Light and Molin (16) have come to similar conclusions based on gene fusions for the closely related FII plasmid, R1. The principle of RNA-RNA interaction may be similar to the mechanism that regulates in vitro replication the high-copy-number plasmid ColE1 (41).

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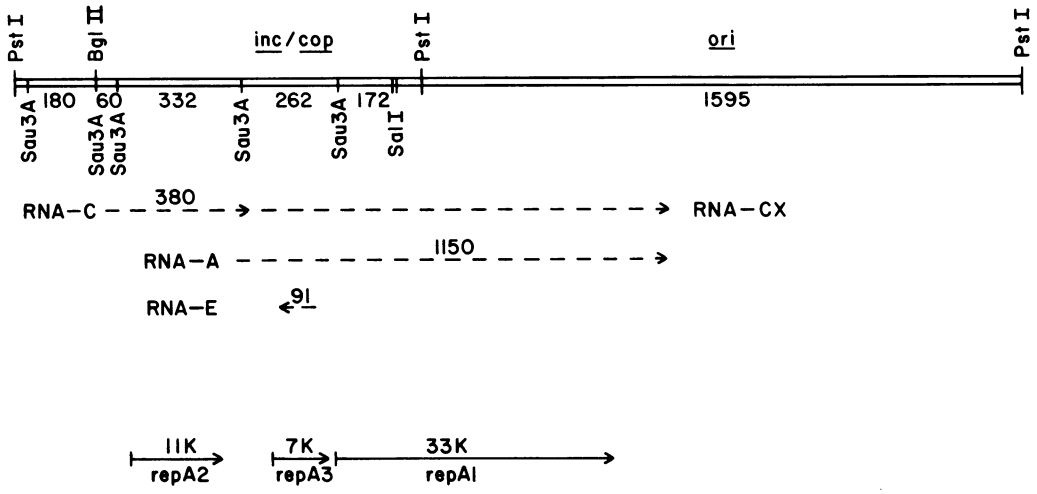

FIG. 1. Map of the replication control region of the IncFII R plasmid NR1. The location of restriction sites and fragment sizes (bp) were deduced from the published DNA sequence (28, 33) and confirmed by restriction enzyme analysis. The locations and directions of transcription of RNA-A, RNA-C, RNA-CX, and RNA-E (8, 29, 32) and the coding regions for *repA1*, *repA2*, and *repA3* (3, 5, 25, 28) are indicated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *E. coli* K-12 strains KP435 *recA* (7) and NK5031 *lacZM5275* (11) were used as hosts for copy number measurements and lysogen construction, respectively. Plasmid pRR12 is a spontaneous Inc$^-$ copy number mutant of NR1 (26). The cloning of *Pst*I restriction fragments from NR1 and pRR12 into the vector pBR322, the reconstruction of minireplicator derivatives composed of *Pst*I fragments, and the construction of the λ *lac* fusion phages, which place β-galactosidase synthesis coded by λRS205 under control of the rightward NR1 replication transcripts, have been described previously (7, 22).

Culture media. L broth (13) at 37°C was used for copy number measurements, whereas 1×A medium (23) at 30°C was used for labeling of RNA and β-galactosidase measurements. Antibiotics were included to select for cells carrying the plasmids: tetracycline hydrochloride, 5 μg/ml; sodium ampicillin, 25 μg/ml; and chloramphenicol, 20 μg/ml. Growth was monitored by turbidity at 600 nm with a Gilford model 260 spectrophotometer. When isopropyl β-D-thiogalactoside (IPTG) was used to induce the *lac* promoter-operator, sodium succinate (0.4%) replaced glucose as the carbon source in the 1×A medium.

Enzyme assays. The relative copy numbers of plasmids that carry the chloramphenicol acetyltransferase gene (*cat*) were estimated from gene dosage effects by measuring the enzyme-specific activity in cell extracts prepared from exponential-phase cultures (7, 34). The relative β-galactosidase activity of exponential-phase cultures was assayed by a modification of the method of Miller (23) as described previously (7).

RNA labeling and hybridization. Exponential-phase cells (NK5031 lysogenic for λRS205) cultured in 10 ml of 1×A medium were pulse-labeled for 2.0 min with [5-^{3}H]uridine at 20 μCi/ml (25 Ci/mmol) followed by the addition of a frozen mixture containing chloramphenicol and sodium azide (9). RNA was extracted (9) with a yield of about 250 μg with a specific activity greater than 10^5 cpm/μg. RNA (about 5 × 10^6 cpm) was quantitatively hybridized to a vast excess of single-stranded DNA fixed to nitrocellulose by published methods (9, 10). The DNA was from the single-stranded phage M13mp10 (21) carrying the 262-base-pair (bp) *Sau*3A fragment from the NR1 replication region (Fig. 1) inserted in either orientation relative to the viral strand (unpublished data). DNA from the + orientation hybridizes to rightward "RNA-A" sequences, whereas DNA from the − orientation hybridizes to leftward "RNA-E" sequences (Fig. 1). The filters were treated with RNase before measuring the bound counts per minute. Nonspecific binding (about 100 cpm per filter) was assayed for each RNA sample by using single-stranded M13mp10 DNA alone. The hybridization measured by this method is strand specific and directly proportional to RNA input (data not shown).

Construction of pBR322 and pUC8 plasmid derivatives. DNA isolation, restriction endonuclease digestion, gel electrophoresis, ligation of restriction fragments, and transformation of *E. coli* cells with plasmid DNA were performed as previously described (22). *Sau*3A restriction fragments from NR1 or its derivatives (Fig. 1) were purified by agarose gel electrophoresis (19) and then ligated to plasmid pBR322 (1) and plasmid pUC8 (42) that had been digested with *Bam*HI. The orientation of the inserted DNA was determined by analysis of the fragments produced by digestion with *Hae*III (data not shown). The structures of the pBR322 derivatives are illustrated in Fig. 2. Deletion mutant pDXRR5 was produced by *Hin*dIII digestion of pDXRR1 (Fig. 2) followed by treatment with S1 nuclease. Blunt-end ligation of this DNA in the presence of *Hin*dIII, which reduces the frequency of undeleted plasmids in the mixture, followed by transformation and selection for ampicillin resistance resulted in a series of plasmids with different size deletions. pDXRR5 has lost approximately 150 bp of DNA, including the *Eco*RI site and the *tet* promoter (data not shown).

In vitro transcription experiments. In vitro transcription experiments were carried out essentially as described by Winkler et al. (44). Reaction mixtures (25 μl) contained 36 mM Tris acetate (pH 7.8), 0.1 mM disodium EDTA, 0.1 mM dithiothreitol, 4 mM magnesium acetate, 150 mM KCl, 5% (vol/vol) glycerol, 150 μM ATP, 150 μM CTP, 150 μM UTP, approximately 0.2 μg of template DNA, and 0.036 μg (0.07 pmol) of RNA polymerase holoenzyme. Transcription was started by adding 20 μM [α-^{32}P]GTP (15 μCi) to each reaction tube. After 40 min at 37°C, transcription reactions

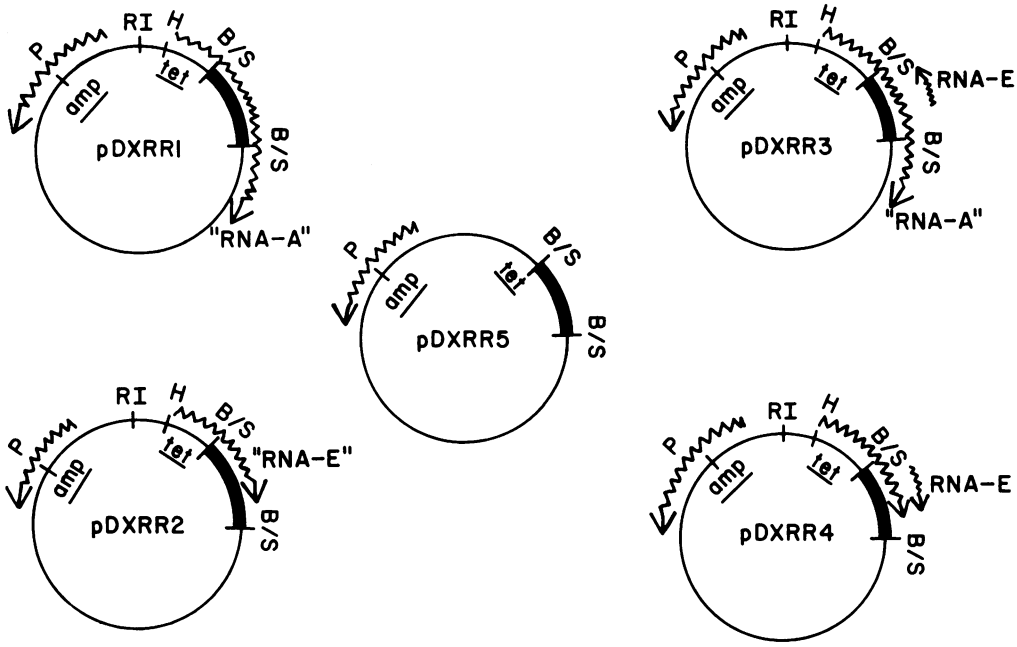

FIG. 2. Maps of pBR322 clones carrying the 262-bp *Sau*3A fragments (heavy lines) from the replication control region of pWNRR3 (pDXRR1, pDXRR2, and pDXRR5) or NR1 (pDXRR3 and pDXRR4). Insertions were in either orientation at the *Bam*HI site in the tetracycline resistance gene. Transcription products are indicated as wavy arrows. Restriction sites: P, *Pst*I; RI, *Eco*RI; H, *Hin*dIII; B/S, *Bam*HI-*Sau*3A fusion.

were stopped by the addition of 25 μl of 1× TBE (TBE is 0.09 M Tris borate, 2.5 mM EDTA, pH 8.3) containing 50% (wt/vol) urea, 4 mg of bromphenol blue per ml, 4 mg of xylene cyanol per ml, and 1 μg of sodium dodecyl sulfate per ml. The reaction mixtures were loaded onto 6% polyacrylamide–7 M urea gels containing 1× TBE and subjected to electrophoresis. Transcription products were detected by autoradiography.

RESULTS

Cloning of the *inc* region of NR1 into pBR322 and pUC8. The cloning vector pBR322 (1) can coexist stably with NR1. The recombinant plasmid pRR935 consists of the 1.1-kb *inc*/*cop* fragment of NR1 (Fig. 1) cloned into the *Pst*I site of pBR322 (22). pRR935 expresses strong incompatibility against NR1 and its Inc^+ derivatives (7, 22). Plasmid pWNRR3 is a mutant, obtained by in vitro mutagenesis of pRR935 DNA with hydroxalamine (23), that does not express incompatibility against NR1 or against other incompatibility mutants of NR1. When minireplicator derivatives of NR1 are present in the same cell as pWNRR3, the minireplicators have a copy number threefold higher than that of NR1. The small leftward transcript, RNA-E (Fig. 1), is not synthesized from pWNRR3 either in vitro (Fig. 3) or in vivo (see below), owing to a single-base substitution in the −35 promoter sequence for RNA-E (32). Figure 3 shows that RNA-E is transcribed in vitro from the cloned 1.1-kb *Pst*I fragment of NR1 and from the cloned 1.1-kb *Pst*I fragment of the Inc^- copy mutant, pRR12, but not from pWNRR3. The simultaneous loss of RNA-E synthesis and incompatibility by pWNRR3 suggests that RNA-E is the *trans*-acting NR1 incompatibility inhibitor.

The transcription promoter and entire coding sequnce for RNA-E lie in a 262-bp *Sau*3A fragment from the NR1 replication region (Fig. 1) (8, 29). The transcription promoters for the rightward transcripts RNA-C and RNA-A are within the 60- and 332-bp *Sau*3A fragments, respectively. Therefore, the only promoter within the 262-bp *Sau*3A fragment is for leftward RNA-E transcription. That promot-

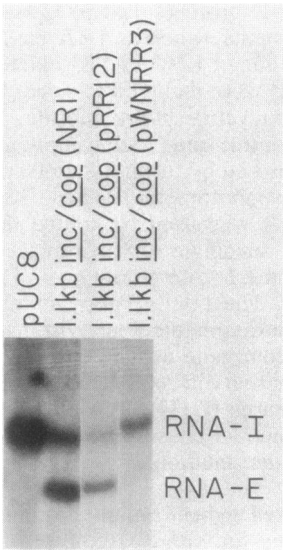

FIG. 3. Synthesis of RNA-E in vitro. Various DNA templates were used in the reactions as described in the text. From left to right, the templates were as follows: the pBR322 derivative pUC8, the pBR322 derivatives with the 1.1-kb *Pst*I fragments from NR1 and pRR12, and the Inc^- mutant pWNRR3. The larger transcript produced by the pBR322 derivatives is the 108-nucleotide RNA-I (41), whereas the smaller transcript is the 91 nucleotide RNA-E (Fig. 1).

TABLE 1. In vivo transcription rates from the 262-bp *Sau*3A fragments inserted into the pBR322 tetracycline resistance gene

Template[a]	Orientation[a]	Fraction bound ($\times 10^4$)[b]	
		"RNA-A"	"RNA-E"
pDXRR1	+	45.8 ± 7.6	0.42 ± 0.40
pDXRR2	−	0.23 ± 0.45	45.9 ± 0.0
pDXRR3	+	21.6 ± 3.3	57.2 ± 0.1
pDXRR4	−	0.00 ± 0.00	129 ± 21
pDXRR5	+	0.21 ± 0.24	0.72 ± 0.04
pBR322		0.04 ± 0.05	0.02 ± 0.04

[a] The plasmid structures are shown in Fig. 2. The orientation of the 262-bp *Sau*3A fragment in the *Bam*HI site is designated "+" if transcription from the *tet* promoter produces hybrid "RNA-A" sequences or "−" if transcription from the *tet* promoter produces hybrid "RNA-E" sequences.

[b] The fraction of total ^{3}H-labeled RNA that bound to specific single-stranded DNA probes was determined as described in the text.

er has been inactivated by the mutation in pWNRR3. The 262-bp *Sau*3A fragments from both pWNRR3 and NR1 were each cloned into the *Bam*HI site in the tetracycline resistance gene (*tet*) of plasmid pBR322. Both orientations were obtained for each fragment (Fig. 2). pDXRR1 and pDXRR3 contain the 262-bp *Sau*3A fragment from pWNRR3 and NR1, respectively, in the + orientation. RNA synthesis from pDXRR1 and pDXRR3 initiated at the *tet* promoter results in a hybrid transcript containing rightward RNA-A sequences (designated "RNA-A" in Fig. 2). RNA-E is also made from pDXRR3, which has the wild-type NR1 fragment, but not from pDXRR1, which has the mutant pWNRR3 fragment (Table 1). In the corresponding clones with the 262-bp *Sau*3A fragments in the − orientation, pDXRR2 and pDXRR4, transcription from the *tet* promoter produces a hybrid RNA containing RNA-E sequences ("RNA-E"). Wild-type RNA-E is also made from pDXRR4, which has the NR1 fragment. "RNA-A" sequences are not synthesized from pDXRR2 and pDXRR4 (Table 1) because there is no promoter for transcription in that direction in these two plasmids. A plasmid carrying the 262-bp fragment with no transcription in either direction was produced by deleting the *tet* promoter at the *Hin*dIII site of pDXRR1, producing pDXRR5 (Fig. 2, Table 1).

The 262-bp *Sau*3A fragment from pWNRR3 also was cloned into the *Bam*HI site of the cloning vector pUC8, which was derived from pBR322 (42). Again, both orientations of the inserted fragment were obtained. Transcription through the *Bam*HI site of pUC8 is under control of the *lac* promoter-operator. Therefore, when the 262-bp fragment is cloned in the + orientation (pVLRR5), a hybrid transcript containing "RNA-A" sequences is synthesized. For the clone with the fragment in the − orientation (pVLRR6), a hybrid transcript containing "RNA-E" sequences is synthesized. Neither pVLRR5 nor pVLRR6 can produce natural RNA-E, because they carry the pWNRR3 mutant 262-bp *Sau*3A fragment. Therefore transcription from pVLRR5 and pVLRR6 is analogous to transcription from pDXRR1 and pDXRR2, respectively, except that the transcription can be regulated by the *lac* repressor and an inducer such as IPTG. These sets of plasmids were then used to test the *trans* effects of extra "RNA-A" or RNA-E (or both) on the regulation of plasmid copy number for various derivatives of NR1 and its mutants.

***Trans* effects of RNA-A and RNA-E.** Plasmid pRR12 is a mutant of NR1 that has an elevated copy number (22, 26, 45) and is compatible with NR1. pRR12 therefore is described as Inc$^-$ Cop$^-$ (7, 22). The copy number mutation in pRR12 has simultaneously altered both the incompatibility inhibitor and its target (7, 22). Minireplicator plasmids pRR933 and pRR942 were derived from NR1 and pRR12, respectively, by ligation of the 1.1- and 1.6-kb *Pst*I restriction fragments from their replication control regions (Fig. 1) to a 2.2-kb *Pst*I fragment coding for resistance to chloramphenicol (*cat*) (22). These minireplicators retain the copy number and incompatibility properties of their parents (7, 22). The DNA from the replication control regions of pRR933 and pRR942 was fused to the *lac* β-galactosidase-coding region of phage λRS205, using the left *Sal*I site in the *repA1* gene (Fig. 1) and the *Eco*RI site in *cat* to produce λ933 and λ942, respectively, (7, 8). As described previously (7), in strains lysogenic for these phages, β-galactosidase synthesis is under control of the rightward NR1 transcription from the promoters for RNA-CX and RNA-A, whereas RNA-E is transcribed in the opposite direction. The amount of β-galactosidase activity in the lysogens should reflect the ability to synthesize the repA1 replication protein coded by the rightward NR1 transcripts. λ *lac* derivatives with the wild-type and mutant plasmid DNA inserts can be compared, and the effects of introducing various plasmids into the lysogens in *trans* can be examined.

To test the effects of varying the *trans* RNA-A or RNA-E concentration on gene expression and copy number control of NR1, the pBR322 derivatives described above (Fig. 2, Table 1) were introduced into strains lysogenic for λ933 or λ942 or into strains harboring the autonomous minireplicator plasmids pRR933 or pRR942. β-Galactosidase activities from the lysogens and copy numbers of the minireplicators were then assayed and compared (Table 2). As previously reported (7), the lysogen with the mutant derivative λ942 produced more β-galactosidase than that with the wild-type λ933. The minireplicator pRR942 also had a higher copy number than pRR933, presumably reflecting the mutant's enhanced ability to synthesize the repA1 replication protein. The presence of plasmid pDXRR1 in *trans*, which produces only hybrid "RNA-A" sequences, stimulated β-galactosidase activity in the λ933 lysogen and raised the copy number of a coresident pRR933 minireplicator (Table 2). In contrast, pDXRR1 had minimal effect on the corresponding Inc$^-$ Cop$^-$ derivatives λ942 and pRR942. Although pDXRR1 synthesizes a hybrid "RNA-A," the hybrid transcript con-

TABLE 2. Gene expression and copy number of plasmid derivatives in the presence of coresident plasmids that produce RNA-A or RNA-E

Coresident plasmid[a]	*trans* NR1 RNA products[b]	Relative β-galactosidase activity in lysogen[c]		Relative copy number of minireplicator[c]	
		λ933	λ942	pRR933	pRR942
pBR322	None	1.0	2.6	1.0	13
pDXRR1	"A"	3.0	3.0	2.5	13
pDXRR2	"E"	0.3	2.1	0.7	11
pDXRR3	"A," E	0.3	1.5	0.0[d]	9.2
pDXRR4	E, "E"	0.3	1.0	0.0[d]	4.7
pDXRR5	None	1.2	2.3	1.1	10

[a] Coresident plasmid structures are shown in Fig. 2.

[b] The *trans* NR1 RNA products are from Table 1 and Fig. 2. "A" is a hybrid *tet*-RNA-A transcript, E is RNA-E, and "E" is a hybrid *tet*-RNA-E transcript.

[c] The results were normalized to the β-galactosidase level in the λ933 lysogen (161 U) or to the copy number of pRR933, as described in the text.

[d] The coresident plasmids expressed strong incompatibility against pRR933, effectively reducing its copy number to zero.

tains only the portion of RNA-A coded by the 262-bp *Sau*3A fragment (Fig. 1). The "RNA-A" of pDXRR1 therefore does not code for repA1 synthesis, but its nucleotide sequence is complementary to the RNA-E synthesized from the λ *lac* prophages or the minireplicators. The stimulation by pDXRR1 most likely results from titration of the incompatibility inhibitor produced by the lysogenic λ *lac* prophage or the minireplicator by the excess "RNA-A" transcripts produced from pDXRR1.

The presence of plasmid pDXRR2 in *trans*, which produces only hybrid "RNA-E" sequences, depressed β-galactosidase activity in the λ933 lysogen and lowered the copy number of the pRR933 minireplicator, but had minimal effect on the mutant derivatives λ942 or pRR942 (Table 2). pDXRR4, which produces wild-type RNA-E from its natural promoter in addition to hybrid "RNA-E," had the greatest overall inhibitory effects on both β-galactosidase synthesis and copy number. pDXRR3, which produces both "RNA-A" and RNA-E, but with RNA-E in excess (Table 1), was less inhibitory than pDXRR4. Probably the hybrid "RNA-A" from pDXRR3 titrated some of the RNA-E, reducing the effective inhibitor concentration compared with pDXRR4, which only makes RNA-E and hybrid "RNA-E." In each case where the pBR322 derivative produced RNA-E or hybrid "RNA-E," there was more inhibition toward the wild-type λ933 prophage or pRR933 minireplicator than toward the mutant λ942 or pRR942 (Table 2). Finally, plasmid pDXRR5, which has lost the *tet* promoter and has the pWNRR3 262-bp *Sau*3A fragment with little transcription from either strand, had little effect on either β-galactosidase activity or copy number.

Variation of the levels of "RNA-A" or "RNA-E" in *trans*. The pBR322 derivatives shown in Fig. 2 produce "RNA-A" or "RNA-E" hybrid transcripts at fixed levels of synthesis from the *tet* promoter. The pUC8 clones pVLRR5 and pVLRR6 allow a more systematic variation in transcription rates by utilizing the *lac* regulatory system. The scheme for this set of experiments is illustrated in Fig. 4. Two plasmids are simultaneously introduced into strains lysogenic for the λ *lac* fusion phages. The plasmid pVLRR5 can synthesize "RNA-A" hybrid transcripts from the *lac* promoter of pUC8. This transcription is regulated by the *lac* repressor, synthesized from plasmid pVLRR10. The level of "RNA-A" transcription from pVLRR5 is increased by the addition of IPTG, which inactivates the *lac* repressor. Similarly, the level of hybrid "RNA-E" transcription will vary directly with IPTG concentration in strains harboring plasmid pVLRR6, which has the 262-bp *Sau*3A fragment in the opposite orientation. The results of these experiments are shown in Fig. 5A. As the level of *trans* hybrid "RNA-A" was varied in the lysogen with λ933 and pVLRR5, β-galactosidase increased with increasing IPTG concentration. In contrast, the λ942 lysogen was only slightly affected by *trans* hybrid "RNA-A." The highest level of β-galactosidase from λ933 was approximately equivalent to that from λ942. IPTG increased the *trans* hybrid "RNA-E" level in the lysogen with λ933 and pVLRR6, causing a reduction in β-galactosidase synthesis. The lowest level of β-galactosidase activity in the λ933 lysogen with pVLRR6 was about one-half the level in the λ933 lysogen with pBR322. It therefore appears that the hybrid "RNA-E" transcripts produced by pVLRR6 have less inhibitory activity than the "RNA-E"

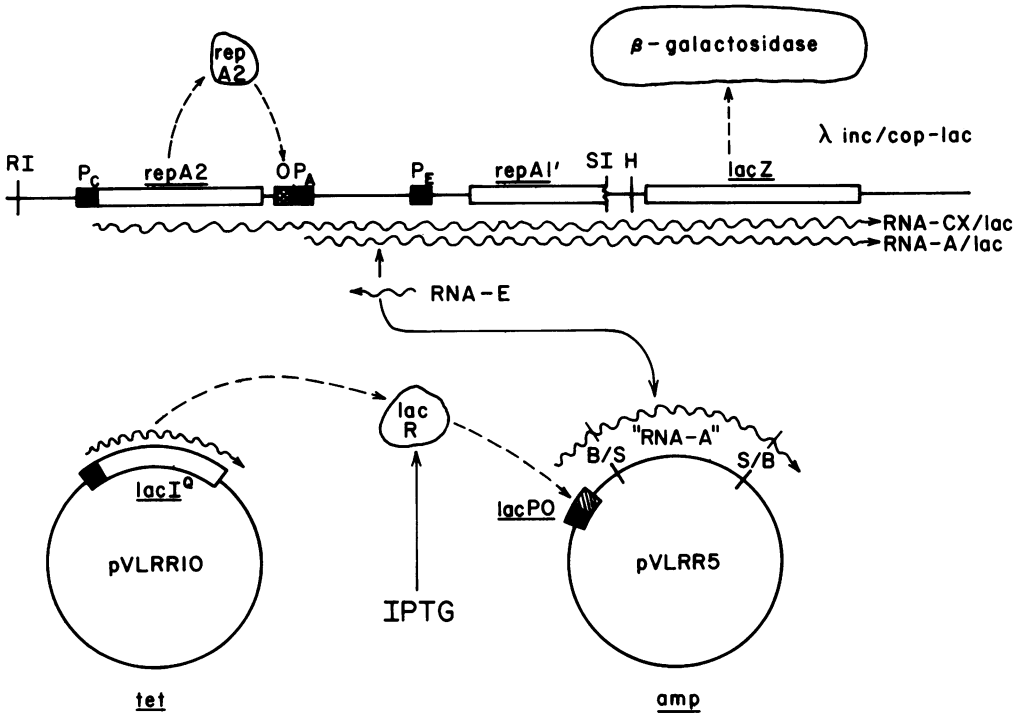

FIG. 4. Experimental scheme for variation of the level of *trans* "RNA-A" or "RNA-E" sequences. β-Galactosidase synthesis from λRS205 is driven by rightward transcription from the NR1 DNA inserted in front of the *lacZ* gene. The transcription promoters for RNA-C, RNA-A, and RNA-E are shown as filled boxes, whereas the truncated *repA1* gene and the *lacZ*-coding sequence are represented by the open boxes. Plasmid pVLRR10 has the *lacI*^{q} gene cloned into pACYC184 (4), which is compatible with pUC8. "RNA-A" transcription from the pUC8 derivative pVLRR5 is induced by IPTG. The restriction sites shown are for *Eco*RI (RI), *Sal*I (SI), *Hin*dIII (H), or fusions of *Bam*HI and *Sau*3A (B/S, S/B).

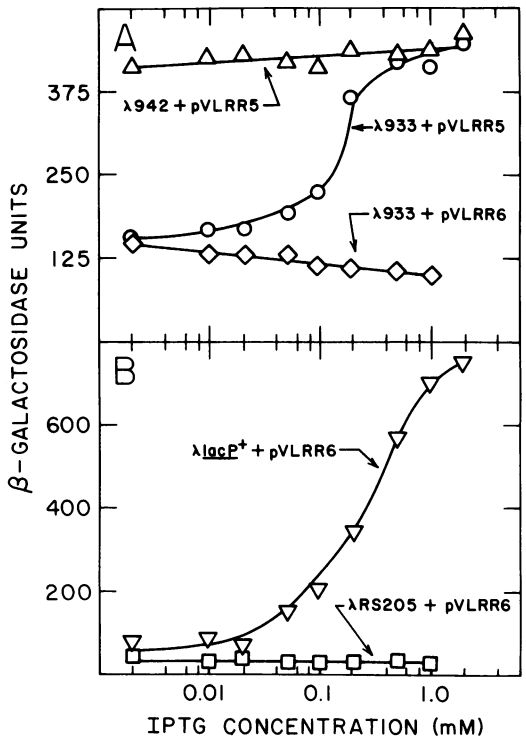

FIG. 5. β-Galactosidase activity in the λ *lac* fusion lysogens as a function of IPTG concentration. The scheme for the experiments is shown in Fig. 4. In A, the lysogens contained λ933 and pVLRR5 (○), λ933 and pVLRR6 (◇), or λ942 and pVLRR5 (△). In B, the lysogens contained λRS205 and pVLRR6 (□), or λ*lac*P$^+$, which has the *lac* promoter-operator inserted into λRS205, and pVLRR6 (▽).

hybrid from pDXRR2, which reduced β-galactosidase to 0.3 times the original value in the λ933 lysogen (Table 2). The presence of pVLRR6 had no effect on β-galactosidase synthesis in the λ942 lysogen (data not shown). As experimental controls, Fig. 5B shows that the *lac* promoter inserted into λRS205 was inducible by IPTG, whereas the background level of β-galactosidase activity from a lysogen with λRS205 alone was unaffected.

DISCUSSION

The plasmid pWNRR3 has a mutation in the 1.1-kb *Pst*I *inc/cop* fragment, which inactivated the strong incompatibility expressed by the parental plasmid, pRR935 (7, 22). pWNRR3 has a single base substitution in the −35 transcription promoter sequence for the leftward transcript RNA-E (32), which inactivates the promoter both in vitro (Fig. 3) and in vivo (Table 1). The mutation, which is in the 262-bp *Sau*3A fragment (Fig. 1), does not alter the actual nucleotide sequence of RNA-E or the complementary portion of the rightward NR1 transcripts. Interestingly, viable minireplicators could not be constructed in experiments in which we attempted to join the mutant 1.1-kb *Pst*I fragment from pWNRR3 to the 1.6-kb *Pst*I *ori* fragment. Such minireplicators were obtained routinely with the wild-type fragment from NR1 or with the Inc$^-$ Cop$^-$ fragment from pRR12 to produce minireplicators such as pRR933 or pRR942, respectively. This suggests that loss of incompatibility as a result of inactivation of the RNA-E promoter is lethal to the NR1 replication system, possibly because replication would be uncontrolled.

The Inc$^-$ Cop$^-$ phenotype of pRR12 is also the result of a single-base substitution in the 262-bp *Sau*3A fragment (32, 33). However, RNA-E is still synthesized from pRR12 (Fig. 3). The mutation changes the nucleotide sequence of both RNA-E and the rightward transcripts in a complementary way. The site of the mutation is within the 6-base single-stranded loops of the proposed secondary structures for these transcripts (29, 32, 33). pRR12 derivatives express incompatibility toward themselves, but less strongly than wild-type NR1 derivatives exclude each other (7, 22). However, pRR12 and NR1 derivatives are compatible. Although the loop sequence of the mutant pRR12 RNA-E is complementary to the loop of the mutant pRR12 rightward transcripts, there would be a mispairing of the sequences if wild-type transcripts were matched with those of pRR12.

Increasing the intracellular level of leftward "RNA-E" sequences, either natural RNA-E or a hybrid transcript initiated at some other promoter, inhibited the rightward expression of the essential *repA1* gene from the NR1 replication control region, as measured by β-galactosidase activity (Table 2, Fig. 5). This resulted in a lowering of the copy numbers of the minireplicators pRR933 and pRR942, but to different extents. In these experiments, the nucleotide sequence of the RNA-E portion of the hybrid transcripts was wild type, and the inhibition was greater against the wild-type derivatives, λ933 and pRR933, than against the derivatives of the Inc$^-$ Cop$^-$ pRR12, λ942 and pRR942. These results indicate that RNA-E is the negative incompatibility inhibitor, consistent with the Inc$^-$ phenotype of pWNRR3, which fails to make RNA-E. RNA-E inhibits synthesis of the repA1 replication protein, which results in a reduced frequency of initiation of plasmid replication and a lower copy number. The wild-type RNA-E has a greater affinity for the wild-type target than for the mutant pRR12 target. Other experiments have shown that the mutant pRR12 inhibitor has a greater affinity for the mutant target than for the wild-type NR1 target (7, 22; unpublished data). It is reasonable to assume that the difference results from the change in the nucleotide sequences of the single-stranded 6-base loops of the transcripts.

Increasing the intracellular level of rightward "RNA-A" sequences stimulated in *trans* the expression of the essential *repA1* gene and raised the copy number of the coresident wild-type pRR933 minireplicator (Table 2, Fig. 5). When the 262-bp *Sau*3A fragment was cloned into pBR322 or pUC8, the presence of the DNA sequences in *trans* at high copy number had no stimulatory effect unless "RNA-A" sequences were being transcribed. This can be seen by comparing the effects of plasmids pDXRR1 and pDXRR5 (Table 2) or by comparing the effects of plasmid pVLRR5 in the presence of low or high IPTG inducer concentrations (Fig. 5). For the later case, the amount of stimulation observed was proportional to the amount of "RNA-A" transcripts produced (Fig. 5). The hybrid "RNA-A" transcripts synthesized from pDXRR1 or pVLRR5 have the wild-type nucleotide sequence in the portion of the transcript that is complementary to RNA-E. The stimulation by *trans* "RNA-A" sequences was greater for the wild-type derivatives, λ933 and pRR933, than for the mutant pRR12 derivatives, λ942 and pRR942. Together, these results indicate that the target of the incompatibility inhibitor, RNA-E, is the complementary portion of the rightward mRNA, which codes for the essential repA1 protein, and that the DNA sequences alone do not titrate the inhibitor. In functional replicons derived from NR1, both of the rightward transcripts RNA-CX and RNA-A could serve as mRNA for repA1 (Fig. 1). The wild-

type target transcripts have a greater affinity for the wild-type RNA-E than for the mutant pRR12 RNA-E. This suggests that RNA-E inhibits repA1 protein synthesis by pairing directly with the repA1 mRNA, and that this pairing is weaker for the Inc$^-$ Cop$^-$ mutant, pRR12. In other experiments, the corresponding DNA from the pWNRR3 mutant was inserted into λRS205, producing a structure similar to those in the λ933 and λ942 lysogens. This new prophage differs from λ933 and λ942 in that no RNA-E is synthesized in its lysogens. The new lysogen had a high level of β-galactosidase activity similar to that of λ942, and this level was not stimulated in *trans* by "RNA-A" transcription (data not shown), because there was no RNA-E to titrate. β-Galactosidase synthesis was inhibited in *trans* by wild-type RNA-E, as expected (data not shown).

In the lysogens with the λ *lac* derivatives, the DNA templates all have the same low copy number of one per chromosome. The RNA-E synthesized from the *inc/cop* region of the wild-type λ933 prophage inhibits synthesis of β-galactosidase from the fused *repA1* gene. The inhibition can be relieved by titrating the RNA-E by *trans* hybrid "RNA-A" transcripts (Fig. 5). The titration curve suggests that the highest level of β-galactosidase synthesis obtained from λ933 is approximately equivalent to the level from the mutant λ942 lysogen. This suggests that at the low copy number of λ942 in the lysogen, the pRR12 mutant RNA-E has little inhibitory activity. pRR12-derived minireplicators such as pRR942 do not exhibit incompatibility against each other unless the *inc* region is cloned into a high-copy vector plasmid, such as pBR322 (7, 22). This indicates that a high dose of mutant RNA-E is required before significant inhibition occurs.

It has previously been suggested that the NR1 incompatibility inhibitor interacted with the replication origin (29) or with an "operator" in the DNA sequence (5) to regulate the initiation of plasmid replication. Previous results from this laboratory (7, 8, 22) clearly showed that both the inhibitor and its target were altered simultaneously by the mutation in pRR12. The results presented here definitively demonstrate that the NR1 incompatibility inhibitor is the small leftward transcript, RNA-E, and that RNA-E inhibits synthesis of the required repA1 replication protein by interacting directly with its mRNA. Light and Molin (16) have come to similar conclusions for the closely related FII group plasmid, R1. It has also been suggested that RNA-RNA interactions are involved in control of plasmid ColE1 replication (41). For ColE1-like plasmids, the small RNA-I (Fig. 3) is synthesized from the opposite strand from that for the larger RNA-II. In vitro, RNA-II is prevented from acting as a primer for DNA replication by the presence of excess RNA-I (41). For NR1, it is not known whether the rightward transcripts can serve as primers for replication in vivo, but the in vitro termination point for RNA-A is very close to the replication origin (Fig. 1) (8). Masai et al. have shown that in vitro replication of R1 plasmid DNA does not require that transcription be initiated at the promoters for RNA-A or RNA-CX (20). However, the possibility that rightward transcription was initiated at promoters in the vectors used to clone the "origin" was not examined in their experiments. It is therefore possible that RNA-E inhibits both repA1 protein synthesis and primer formation, but this requires further study.

At the wild-type copy number of NR1, there is a sufficient amount of repA2 repressor protein for nearly complete represion of RNA-A transcription (32). Therefore, RNA-CX transcription provides the mRNA for the repA1 initiator protein, and the interaction of RNA-E with RNA-CX provides the primary means of regulating NR1 replication. The mechanism by which RNA-E inhibits synthesis of the repA1 initiator is unclear. The translation start site for repA1 is downstream from the part of the rightward mRNA that is complementary to RNA-E (Fig. 1). A computer analysis of possible secondary structures of the rightward transcripts has suggested that binding of RNA-E to the messenger may alter the downstream secondary structure in the region of repA1 translation initiation (32). If true, this would mean that RNA-E is a translational inhibitor of repA1 protein synthesis, and this could explain the polar effect on β-galactosidase synthesis in the λ *lac* lysogens. Similar mechanisms of translational repression may be involved in regulating ribosomal protein synthesis (46), phage T4 gene 32 protein synthesis (43), Tn*10* "transposase" synthesis (35), and staphylococcal antibiotic resistance (12). The mode of action of repA1 as an initiator protein also is not understood. However, it is a *cis*-acting protein both in vivo and in vitro (20, 22). One possible explanation is that the repA1 protein interacts with its own mRNA to convert it into a replication primer on its own template. By inhibiting repA1 translation, RNA-E would limit this activity to a fraction of the rightward transcripts, such that initiation of replication took place on average once per plasmid per cell generation. In principle, such a mechanism of replication control could regulate replicons of any copy number, including chromosomal DNA replication.

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