JOURNAL OF BACTERIOLOGY, May 1993, p. 3161–3173
0021-9193/93/103161-13$02.00/0

Vol. 175, No. 10

Suppression of Replication-Deficient Mutants of IncFII Plasmid NR1 Can Occur by Two Different Mechanisms That Increase Expression of the *repA1* Gene

RUPING WU, XUESONG WANG,† DAVID D. WOMBLE,* AND ROBERT H. ROWND
Center for Molecular Biology, Wayne State University, Detroit, Michigan 48202

Received 29 October 1992/Accepted 16 March 1993

Replication-proficient (Rep^+) revertants were isolated from mutants of IncFII plasmid NR1 that were replication defective (Rep^-). The parental Rep^- plasmids contained a mutation that inactivated promoter P_E for transcription of RNA-E, a *trans*-acting repressor of translation of the essential RepA1 replication initiation protein of NR1. The P_E mutation also introduced a nonsense codon into a leader peptide gene that precedes and slightly overlaps the *repA1* translation initiation site in the mRNA. This reduced the rate of synthesis of RepA1 by uncoupling its translation from that of the leader peptide. The reduced rate of RepA1 synthesis was responsible for the Rep^- phenotype. All Rep^+ revertants retained the P_E mutation and contained second-site mutations responsible for suppression of the Rep^- phenotype. One Rep^+ revertant contained a second mutation adjacent to the Shine-Dalgarno sequence of *repA1*. Another Rep^+ revertant contained a mutation in the *repA2* gene, which encodes the *trans*-acting repressor of transcription of *repA1*. By using translational *lacZ* gene fusions, it was found that both kinds of suppressor mutation increased the expression of *repA1* to a level sufficient to support replication. In both cases, the synthesis of RepA1 remained uncoupled from that of the leader peptide. The Shine-Dalgarno mutation increased the rate of leader peptide-independent translation of *repA1* mRNA and also reduced the sensitivity of *repA1* mRNA to inhibition by RNA-E. The *repA2* mutation inactivated the RepA2 repressor and increased the rate of transcription of *repA1* mRNA. The translational *lacZ* gene fusions were used to assess the range of regulation of expression of *repA1* provided by each of the RNA-E and RepA2 regulatory circuits. By constructing miniplasmids that contained various combinations of the mutations, the contributions of the RNA-E and RepA2 regulatory circuits were assessed with respect to control of plasmid copy number and stable inheritance. Plasmids that lacked either circuit were less stable than wild-type plasmids.

Regulation of plasmid copy number is equivalent to regulating the frequency of initiation of plasmid DNA replication during the cell division cycle. For IncFII plasmid NR1, a 95-kb self-transmissible antibiotic resistance plasmid that is stably maintained at a low copy number in *Escherichia coli* (43), replication control is accomplished by regulating the synthesis of the plasmid-specific replication initiation protein RepA1 (reviewed in references 26 and 42). The *cis*-acting RepA1 protein binds to the downstream plasmid replication origin (*ori*) after its synthesis to effect initiation of replication (10, 20, 25, 29). Negative regulation of expression of the *repA1* gene occurs by controlling both the transcription and translation of its mRNA by mechanisms that have been extensively studied (26, 42). Efficient synthesis of RepA1 requires translational coupling to the expression of an upstream 2.5-kDa leader peptide that is also encoded by *repA1* mRNA (3, 45). The coding sequence of the leader peptide overlaps the translation initiation site of the *repA1* gene in a different reading frame (Fig. 1 and 2).

In addition to the leader peptide, the *repA1* gene, and *ori*, the *repA* replicon of NR1 encodes *trans*-acting regulatory elements that control expression of *repA1* (Fig. 1). Transcription of *repA1* mRNA may be initiated from a constitutive promoter, P_C, and a regulated promoter, P_A, to synthesize RNA-CX and RNA-A, respectively (13, 31, 44). RNA-CX also serves as the mRNA for the *repA2* gene, whose product is a *trans*-acting repressor of transcription at P_A (8, 39, 44). At the normal plasmid copy number, RNA-A transcription is repressed approximately 95% by the RepA2 repressor, and constitutive RNA-CX transcription provides the majority of *repA1* mRNA (8, 44).

A third transcript, RNA-E, is initiated from promoter P_E and is transcribed in the direction opposite to that of RNA-CX and RNA-A (13, 31, 44). P_E is a strong constitutive promoter (44). The convergence of synthesis of *repA1* mRNA and RNA-E reduces the rate of transcription in both directions (44). RNA-E is a *trans*-acting repressor of translation of *repA1* mRNA that functions by binding to the complementary target sequences in RNA-CX or RNA-A to form an RNA-RNA duplex (4, 18, 28, 37, 41). RNA transcripts that contain the complementary target sequence for binding to RNA-E and the coding sequences for the leader peptide and *repA1* are generically referred to as *repA1* mRNA. Inhibition of translation of *repA1* mRNA by RNA-E is the primary basis for IncFII group plasmid incompatibility (18, 41). Translation of *repA2* from the 5′ end of RNA-CX is not affected by RNA-E.

We have previously described (45, 46) the isolation of mutants of NR1 that had simultaneously lost expression of IncFII plasmid incompatibility (Inc^-) and replication proficiency (Rep^-). Four independently isolated Inc^- Rep^- mutants contained the same single base pair substitution that inactivated promoter P_E. The absence of RNA-E transcription explained the Inc^- phenotype of the mutants. The P_E mutation also introduced a nonsense mutation in the leader peptide coding sequence, causing premature termination of

* Corresponding author.
† Present address: Department of Medicine, Northwestern University Medical School, Chicago, IL 60611.

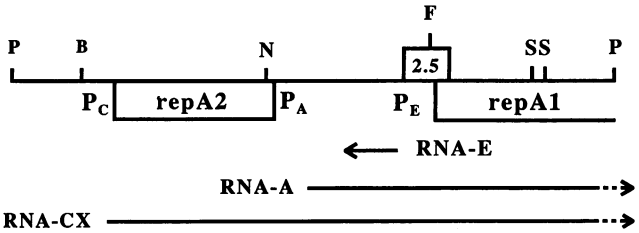

FIG. 1. Replication control region of NR1. The 1.1-kb *Pst*I fragment that contains the control elements and the 5′ end of *repA1* is shown. The 3′ end of *repA1* and the replication origin are contained in a contiguous 1.6-kb *Pst*I fragment that is not shown. Three transcription promoters, P_C, P_A, and P_E, for transcripts RNA-CX, RNA-A, and RNA-E, respectively (indicated by the arrows below the map), are indicated. RNA-CX and RNA-A continue through the remainder of *repA1* in the 1.6-kb fragment. From left to right, the coding regions for *repA2*, the 2.5-kDa leader peptide, and the 5′ end of *repA1* are shown boxed. Restriction endonuclease recognition sites: P, *Pst*I; B, *Bgl*II; N, *Nsi*I; F, *Fsp*I; S, *Sal*I. The right-hand *Pst*I site was used to construct *repA1-lacZ* translational fusions, whereas the *Fsp*I site was used to construct leader peptide-*lacZ* translational fusions. The left-hand *Sal*I site was used to construct transcriptional fusions.

its translation (Fig. 2). This reduced expression of the *repA1* gene at least 10-fold by uncoupling translation of its mRNA from that of the leader peptide (45). This explained the Rep$^-$ phenotype, since the mutants were unable to synthesize enough RepA1 to initiate plasmid replication. Translation of the leader peptide coding sequence and translation of *repA1* are both inhibited by RNA-E (3, 45). Regulation of translation of *repA1* mRNA by RNA-E may occur indirectly by uncoupling translation of *repA1* from that of the leader peptide (45). This mechanism has also been suggested for regulation of the closely related IncFII plasmid R1 (3) and for the seemingly otherwise unrelated plasmids of the IncIα and IncB groups (1, 15, 30).

Starting with the Inc$^-$ Rep$^-$ mutants that contain the P_E mutation, we have isolated Rep$^+$ revertants that in a *polA* host can rescue the replication of a pBR322 vector plasmid in a cointegrate with the *repA* replicon. The revertants contained different kinds of second-site mutations that increased expression of *repA1* by mechanisms that are independent of translation of the leader peptide. The properties of plasmids with various combinations of these mutations revealed fundamental aspects of the regulation of expression of the *repA1* gene and its relationship to the control of initiation of NR1 plasmid replication.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 strain JG112 *met thy rpsL polA* (23) and its *polA*$^+$ revertant JK17 (44) were used for replicon reconstitution experiments, for testing the *polA* dependence of chimeric plasmids composed of both pBR322 and NR1 replicons, and for plasmid copy number and stability assays. *E. coli* MC1061 *araD139* Δ(*ara-leu*)*697* Δ*lacX74 galU galK rpsL hsdR* (6) was used for construction of *lacZ* fusion plasmids. *E. coli* SE4006 *araD* Δ*lac169 relA thi rpsL recA56 srl*::Tn*10* (14) was used as the host for β-galactosidase assays. Strains KP435 *trp ilv thy rpsL recA* (12) and JK17 were used for plasmid incompatibility assays. Strains JM83 and JM101 were used for cloning with pUC8 and M13 vectors (21, 36). In most experiments, cells were cultured in 2YT medium (24) containing 16 g of

```
                    -35                 -10.
1   GATCTTCGTCACAATTCTCAAGTCGCTGATTTCAAAAAACTGTAGTATCCTCTGCGAAACGATCCCTGTTTGAGTATTGAGGAGGCGAGATGTCGCAGAC  100
                                                                                 S/D      fMetSerGlnTh

101 AGAAAATGCAGTGACTTCCTCATCTGGCGCAAAACGAGCATACAGAAAGGGGAATCCGCTTTCTGATGCAGAGAAACAAAGATTATCAGTGGCCCGTAAA  200
    rGluAsnAlaValThrSerSerSerGlyAlaLysArgAlaTyrArgLysGlyAsnProLeuSerAspAlaGluLysGlnArgLeuSerValAlaArgLys

                A
201 AGAGCTTCGTTCAAGGAAGTAAAAGTATTTCTTGAACCAAAGTATAAGGCCATGCTCATGCAAATGTGTCATGAAGATGGTCTGACTCAGGCTGAAGTTC  300
    ArgAlaSerPheLysGluValLysValPheLeuGluProLysTyrLysAlaMetLeuMetGlnMetCysHisGluAspGlyLeuThrGlnAlaGluValL

                        NsiI .                  -35                 -10 .
301 TGACCGCACTGATAAAAAGTGAAGCGCAAAAACGATGCATGTGATGATGGGCTTACATTCTTGAGTGTTCAGAAGATTAGTGCTAGATTACTGATCGTTT  400
    euThrAlaLeuIleLysSerGluAlaGlnLysArgCysMetEndEnd

                                                                    3′
401 AAGGAATTTTGTGGCTGGCCACGCCGTAAGGTGGCAAGGAACTGGTTCTGATGTGGATTTACAGGAGCCAGAAAAGCAAAAACCCCGATAATCTTCTTCA  500

                                                        5′      -10                 -35 T .
501 ACTTTGGCGAGTACGAAAAGATTACCGGGGCCCACTTAAACCGTATAGCCAACAATTCAGCTATGCGGGGAGTATAGTTATATGCCCGGAAAAGTTCAAG  600
                                                                                S/D      fMetProGlyLysValGlnA

                    FspI.           A.
601 ACTTCTTTCTGTGCTCGCTCCTTCTGCGCATTGTAAGTGCAGGATGGTGTGACTGATCTTCACCAAACGTATTACCGCCAGGTAAAGAACCCGAATCCGG  700
    spPheLeuCysSerLeuLeuLeuArgIleValSerAlaGlyTrpCysAspEnd
                                            S/D   fMetThrAspLeuHisGlnThrTyrTyrArgGlnValLysAsnProAsnProV

701 TGTTTACACCCCGTGAAGGTGCAGGAACGCTGAAGTTCTGCGAAAAACTGATGGAAAAGGCGGTGGGCTTCACTTCCCGTTTTGATTTCGCCATTCATGT  800
    alPheThrProArgGluGlyAlaGlyThrLeuLysPheCysGluLysLeuMetGluLysAlaValGlyPheThrSerArgPheAspPheAlaIleHisVa

                    SalI.                   SalI                PstI .
801 GGCGCATGCCCGTTCGCGTGGTCTGCGTCGACGCATGCCACCAGTGCTGCGTCGACGGGCTATTGATGCGCTCCTGCAGGGGGCTGTGTTTCCACTATGAC  900
    lAlaHisAlaArgSerArgGlyLeuArgArgArgMetProProValLeuArgArgArgAlaIleAspAlaLeuLeuGlnGlyLeuCysPheHisTyrAsp
```

FIG. 2. Partial nucleotide sequence of the NR1 replication control region (31). Base pair position 1 corresponds to the G in the *Bgl*II site in Fig. 1. The −10 and −35 sequences for promoters P_C, P_A, and P_E are underlined, as is the RNA-E-coding sequence (bp 564 to 474). RNA-E is transcribed from right to left, as indicated by "5′" and "3′" above the first and last nucleotides, respectively, in its coding sequence. Base substitutions in the mutants at positions 216 (*repA2*), 597 (P_E), and 639 (S/D) are indicated above or below the sequence. Translation start and stop signals for *repA2* (bp 90 to 341), the leader peptide (bp 582 to 653), and *repA1* (bp 649 to 1503) are shown in boldface type. The predicted amino acid sequences are indicated below the DNA sequence. The *repA2* mutation replaces a Glu codon (GAA) with a Lys codon (AAA). The P_E mutation replaces a Gln codon (CAA) with an ochre stop codon (UAA) in the leader peptide sequence. The S/D mutation replaces an Ala codon (GCA) with a Thr codon (ACA). Restriction endonuclease recognition sites are italicized.

tryptone, 10 g of yeast extract (both from Difco Laboratories), and 5 g of NaCl per liter or in Luria-Bertani medium (24) containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter. 2YT and Luria-Bertani plates contained 15 g of Bacto Agar (Difco) per liter. Clones were screened for β-galactosidase activity on Luria-Bertani or 2YT agar containing 40 mg of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (Research Organics, Inc.) per liter. For β-galactosidase assays, cells were cultured in 1× A minimal medium (24) plus $MgSO_4$ (1 mM), thiamine (20 mg/liter), glucose (4 g/liter), and 0.1% Casamino Acids (Difco). The following antibiotics (Sigma Chemical Co.) were included in the medium when appropriate to select for cells harboring various plasmids: tetracycline hydrochloride, sodium ampicillin, and chloramphenicol (10, 50, and 25 mg/liter, respectively). Cells were cultured at 37°C, and growth was monitored by turbidity at 600 nm with a Gilford model 260 spectrophotometer.

DNA isolation and manipulation. DNA isolation, restriction endonuclease digestion, gel electrophoresis, ligation of restriction fragments, and transformation of *E. coli* cells with plasmid or phage DNA were performed as described previously (12, 22). All enzymes were used as recommended by the suppliers. Restriction endonuclease fragments were purified from agarose gels with DEAE membranes (11) or from 0.8 or 1.4% low-melting-point agarose gels (33). The alkaline minilysate method (2) was used for screening of plasmid sizes and restriction endonuclease analysis of plasmid DNA.

Plasmids. The plasmids used in this study are listed in Table 1. The *repA* replicon of NR1 is contained within two contiguous 1.1- and 1.6-kb *Pst*I fragments (22). The 1.1-kb *Pst*I fragment encodes RepA2, RNA-E, the leader peptide, and the 5′ end of the *repA1* gene (Fig. 1). The remainder of *repA1* and the downstream *ori* are contained within the 1.6-kb *Pst*I fragment. Various derivatives of vector plasmid pBR322 contain one or both of the 1.1- and 1.6-kb *Pst*I fragments from the NR1 *repA* replicon inserted at the *Pst*I site in *bla*. In plasmid pRR945, the 1.1- and 1.6-kb *Pst*I fragments are in their native orientation such that the *repA* replicon is functional. The isolation and characterization of the Inc⁻ Rep⁻ mutants derived from pRR945 and containing the P_E mutation (Table 1) have been described previously (45, 46). The isolation of Inc⁻ Rep⁺ revertants pWN160-14 and pWN160-15 (Table 1) containing either *repA2* or Shine-Dalgarno (SD) mutations is described in Results.

RNA-E is encoded within a 262-bp *Sau*3A fragment (bp 393 to 654 in Fig. 2). Plasmid pWR130 was constructed by inserting the wild-type 262-bp *Sau*3A fragment from NR1 into the *Bam*HI site in the *tet* gene of the moderately high-copy-number vector plasmid pACYC184. The orientation of the inserted fragment in pWR130 was such that transcription from both *tetP* and P_E was in the same direction, referred to as the "−" orientation. Therefore, RNA-E is transcribed from pWR130; however, in the absence of a promoter for transcription in the opposite direction, *repA1* mRNA target sequences are not transcribed. Plasmid pWR119 was constructed by inserting the 262-bp *Sau*3A fragment containing the P_E mutation into the *Bam*HI site of pACYC184 in the + orientation, such that transcription from *tetP* would produce *repA1* mRNA target sequences complementary to RNA-E; because of the P_E mutation, RNA-E is not transcribed from pWR119. pWR151 is similar to pWR119, except that it produces *repA1* mRNA target sequences containing both the SD and P_E substitutions. pWR128 was constructed by inserting DNA that contains the wild-type *repA2* gene within a 332-bp *Sau*3A fragment into the *tet* gene of pACYC184, such that it produces RepA2 repressor protein.

pSKS105 is a high-copy-number pBR322-derived vector plasmid with a polylinker sequence in the amino-terminal coding region of *lacZ*, such that inserted genes can be fused in frame. pWR100 was constructed by deletion of the *Eco*RI fragment of pSKS105 that contains the *lacP* promoter and the first three codons of *lacZ*, such that the remaining *lac* sequences in the plasmid are not transcribed unless a promoter-containing fragment is inserted in the polylinker. pFZY1 is a low-copy-number vector plasmid with a polylinker sequence upstream from a promoterless *lac* operon that contains translational stop codons in all three reading frames between the polylinker and *lacZ*, for the purpose of constructing transcriptional *lacZ* fusions. For construction of low-copy-number translational fusions, vector plasmid pWR109 was constructed by replacing the upstream *lac* region of pFZY1 with the equivalent region from pWR100 by using the *Eco*RI and *Sac*I restriction sites in the polylinkers and *lacZ* genes, respectively. At the same time, the *Bam*HI-*Sac*I fragment from pWR100 was replaced with the equivalent region from pFZY1 to construct vector plasmid pWR127, which was used for construction of high-copy-number transcriptional fusions.

Translational *repA1-lacZ* fusions were constructed by inserting the *Bgl*II-*Pst*I fragment from NR1 (Fig. 1) between the polylinker *Bam*HI and *Pst*I sites of pWR100 and pWR109. High-copy-number translational 2.5-kDa leader-*lacZ* fusions were constructed by using the *Fsp*I site in the leader peptide coding region (Fig. 1) by taking the *Eco*RI-*Fsp*I fragments from the high-copy-number *repA1-lacZ* translational fusion plasmids (pWR100 derivatives) and inserting them between the polylinker *Eco*RI and *Sma*I sites of pWR100. Low-copy-number 2.5-kDa leader-*lacZ* translational fusions were then constructed by replacing the *Eco*RI-*Sac*I fragment of pFZY1 with the *Eco*RI-*Sac*I fragments from the high-copy-number 2.5-kDa leader-*lacZ* fusion plasmids. Low-copy-number transcriptional *repA-lacZ* fusions were constructed by inserting the *Bgl*II-*Sal*I fragment of NR1 (Fig. 1) between the polylinker *Bam*HI and *Sal*I sites of pFZY1. High-copy-number transcriptional *repA-lacZ* fusions were constructed by inserting the *Bgl*II-*Pst*I fragment of NR1 (Fig. 1) between the polylinker *Bam*HI and *Pst*I sites of pWR127. Equivalent fusions were constructed with both wild-type and mutant DNA fragments. Restriction fragment exchange was used to separate the *repA2* or SD mutation from the P_E mutation so that the effects of the individual mutations could be determined. Along with other appropriately chosen sites, the *Nsi*I site at bp 335 (Fig. 1 and 2) was used to separate the *repA2* mutation, whereas the *Fsp*I site at bp 625 was used to separate the SD mutation, resulting in plasmids pWW160-16 and pWW160-17, respectively (Table 1).

Mutagenesis of plasmid DNA. Plasmid DNA (10 μg) from each of the Inc⁻ Rep⁻ mutants (Table 1) was mutagenized in vitro (16) with 0.12 M hydroxylamine in a volume of 0.2 ml (0.05 M sodium phosphate [pH 6.0], 0.1 M EDTA) at 75°C for 10 to 20 min, and then extensive dialysis against 20 mM $CaCl_2$ at 4°C was performed. The mutagenized plasmid DNA was then introduced into *E. coli* JG112 cells by transformation, with selection for tetracycline resistance. All of the 31 Rep⁺ revertants obtained by this procedure were produced in independent mutagenesis experiments.

Plasmid replicon reconstitution. Construction of 4.9-kb minireplicator plasmids composed of three *Pst*I fragments has been described previously (12, 46). These plasmids

TABLE 1. Plasmids used in this study

Plasmid	Description[a]	Reference or source
pBR322	pMB1-derived cloning vector; *bla tet*	5
pRR933	NR1 miniplasmid, wt 1.1-, 1.6-, and 2.2-kb *Pst*I fragments; *cat*	22
pRR935	pBR322 plus wt 1.1-kb *Pst*I fragment of NR1; *tet*	22
pRR936	pBR322 plus wt 1.6-kb *Pst*I fragment of NR1; *tet*	22
pRR714	pBR322 plus 2.2-kb *Pst*I fragment with *cat*; *tet cat*	22
pRR945	pBR322 plus wt 1.1- and 1.6-kb *Pst*I fragments of NR1, Inc^+ Rep^+; *tet*	22
pWNRR104	Inc^- Rep^- mutant of pRR945 (P_E); *tet*	46
pWNRR124	Inc^- Rep^- mutant of pRR945 (P_E); *tet*	46
pWNRR149	Inc^- Rep^- mutant of pRR945 (P_E); *tet*	46
pWNRR160	Inc^- Rep^- mutant of pRR945 (P_E); *tet*	46
pWN160-14	Inc^- Rep^+ revertant of pWNRR160 (P_E, *repA2*); *tet*	This study
pWN160-15	Inc^- Rep^+ revertant of pWNRR160 (P_E, SD); *tet*	This study
pWW160-16	pRR945 with *repA2* mutation; *tet*	This study
pWW160-17	pRR945 with SD mutation; *tet*	This study
pUC8	pBR322-derived cloning vector; *bla*	36
pRR775	pUC8 plus wt 1.1-kb *Pst*I fragment of NR1; *bla*	41
pACYC184	p15A-derived cloning vector; *cat tet*	7
pSKS105	pBR322-derived translational *lacZ* fusion vector; *bla*	6
pFZY1	Mini-F-derived transcriptional *lacZ* fusion vector; *bla*	17
pWR100	*Eco*RI deletion of pSKS105 to remove *lacP*; *bla*	45
pWR106	Low-copy-number *repA-lacZ* transcriptional fusion, wt; *bla*	45
pWR109	Mini-F-derived translational *lacZ* fusion vector; *bla*	45
pWR110	High-copy-number leader-*lacZ* translational fusion, wt; *bla*	45
pWR111	High-copy-number *repA1-lacZ* translational fusion, SD P_E; *bla*	This study
pWR112	High-copy-number *repA1-lacZ* translational fusion, *repA2* P_E; *bla*	This study
pWR113	High-copy-number *repA1-lacZ* translational fusion, P_E; *bla*	45
pWR114	High-copy-number *repA1-lacZ* translational fusion, *repA2*; *bla*	This study
pWR115	High-copy-number *repA1-lacZ* translational fusion, SD; *bla*	This study
pWR116	High-copy-number *repA1-lacZ* translational fusion, wt; *bla*	45
pWR119	pACYC184 plus P_E 262-bp *Sau*3A fragment, + orientation (target); *cat*	45
pWR121	Low-copy-number *repA1-lacZ* translational fusion, SD P_E; *bla*	This study
pWR122	Low-copy-number *repA1-lacZ* translational fusion, *repA2* P_E; *bla*	This study
pWR123	Low-copy-number *repA1-lacZ* translational fusion, P_E; *bla*	45
pWR124	Low-copy-number *repA1-lacZ* translational fusion, *repA2*; *bla*	This study
pWR125	Low-copy-number *repA1-lacZ* translational fusion, SD; *bla*	This study
pWR126	Low-copy-number *repA1-lacZ* translational fusion, wt; *bla*	45
pWR127	pBR322-derived transcriptional *lacZ* fusion vector; *bla*	45
pWR128	pACYC184 plus wt 332-bp *Sau*3A *repA2*$^+$ fragment (RepA2); *cat*	This study
pWR130	pACYC184 plus wt 262-bp *Sau*3A fragment, − orientation (RNA-E); *cat*	45
pWR136	High-copy-number *repA-lacZ* transcriptional fusion, wt; *bla*	45
pWR138	High-copy-number leader-*lacZ* translational fusion, *repA2* P_E; *bla*	This study
pWR139	High-copy-number leader-*lacZ* translational fusion, P_E; *bla*	45
pWR140	High-copy-number leader-*lacZ* translational fusion, *repA2*; *bla*	This study
pWR141	Low-copy-number leader-*lacZ* translational fusion, wt; *bla*	45
pWR142	Low-copy-number leader-*lacZ* translational fusion, P_E; *bla*	45
pWR143	Low-copy-number leader-*lacZ* translational fusion, *repA2*; *bla*	This study
pWR144	Low-copy-number leader-*lacZ* translational fusion, *repA2* P_E; *bla*	This study
pWR148	pRR933 with SD P_E; *cat*	This study
pWR149	pRR933 with *repA2* P_E; *cat*	This study
pWR150	pRR933 with *repA2*; *cat*	This study
pWR151	pACYC184 plus SD P_E 262-bp *Sau*3A fragment, + orientation (target); *cat*	This study
pWR153	pRR933 with SD; *cat*	This study

[a] *bla*, ampicillin resistance; *tet*, tetracycline resistance; *cat*, chloramphenicol resistance; wt, wild-type DNA sequence; P_E, mutant RNA-E promoter; SD, Rep^+ suppressor mutation near the SD sequence of *repA1*; *repA2*, Rep^+ suppressor mutation in *repA2*.

consist of the 1.1- and 1.6-kb *Pst*I fragments containing *repA* ligated to a 2.2-kb *Pst*I fragment that contains the *cat* gene. For replicon reconstitution, the individual 1.1-kb *Pst*I fragments from pRR945 or its mutant derivatives were first cloned into the *Pst*I site of pUC8 to produce plasmids like pRR775 (Table 1). DNA from the pUC8 clones with inserted 1.1-kb fragments was mixed with DNA from pRR936 (wild-type 1.6-kb fragment clone) and pRR714 (2.2-kb fragment clone), digested with *Pst*I, ligated, and introduced into JG112 (*polA*) by transformation, with selection for chloramphenicol resistance. Transformants that were not resistant to tetracycline (i.e., those that did not contain pBR322) were found to contain minireplicator plasmids composed of three *Pst*I fragments. Plasmids having the same relative orientations of the three *Pst*I fragments as pRR933, a wild-type derivative, were retained for further analysis.

Nucleotide sequence analysis. The 1.1-kb *Pst*I fragment of *repA* contains five sites for cleavage by *Sau*3A (44). The various *Sau*3A and *Pst*I-*Sau*3A fragments from the 1.1-kb *Pst*I fragments from pRR945 and its mutants were cloned

into M13 phage DNA according to the technique of Messing (21). DNA sequencing kits were purchased from New England Biolabs, and dideoxy sequencing was performed as described by Williams et al. (38) by using [α-^{35}S]dATP (New England Nuclear). The sequencing reactions of the DNA from the mutants were compared side by side with those from the wild type in order to locate the sites of mutation. Appropriately located synthetic oligonucleotides were obtained for use as primers to sequence double-stranded DNA clones to confirm the presence or absence of the mutations in various plasmid derivatives.

Plasmid incompatibility assay. Incompatibility tests were performed as described previously (12, 22). The donor plasmid DNA was introduced by transformation into recipient cells harboring a resident miniplasmid by selection for the resistance conferred by the donor plasmid only. Retention of the unselected resident plasmid and the donor plasmid was then tested by replica plating for resistance to each antibiotic.

Plasmid copy number measurements and stability assay. The relative copy numbers of miniplasmids that carry the *cat* gene were estimated from gene dosage effects by measuring chloramphenicol acetyltransferase specific activity in cell extracts prepared from exponentially growing cultures as described previously (12, 34), except that total protein concentrations were determined with Bio-Rad protein assay kits by following the instructions supplied with the kit. Estimates of copy numbers were relative to wild-type miniplasmid pRR933.

The relative copy numbers of the *lacZ* fusion plasmids that contained the *bla* gene were estimated from gene dosage by assaying their β-lactamase activities as described by Lupski et al. (19), by using cephaloridine (Sigma) as the colorigenic reagent. Protein concentrations were determined with Bio-Rad protein assay kits. Estimates of copy numbers were relative to low-copy-number vector plasmid pFZY1 or to high-copy-number vector plasmid pWR100.

The stability of plasmid inheritance was determined by measuring the decrease in the fraction of antibiotic-resistant cells during a period of nonselective growth according to the standard protocol (22). After the cells that harbored various miniplasmids were cultured in medium containing chloramphenicol, they were repeatedly subcultured by 10^6-fold dilution into drug-free medium and incubated overnight. After each subculture, appropriate dilutions of the cultures were spread onto drug-free 2YT agar plates, and the antibiotic resistance of at least 100 colonies was tested by replica plating by the toothpick method (24). Representative resistant and sensitive colonies were tested directly for the presence of plasmid DNA by the alkaline minilysate method (2).

β-Galactosidase assays. The β-galactosidase activities of cultures of cells harboring the various fusion plasmids were assayed by a modification of the method of Miller (24) as described previously (12). To obtain accurate measurements of cultures with low β-galactosidase activities, the reactions were carried out for extended periods. The low level of β-galactosidase produced in strains harboring only the appropriate vector plasmid without DNA inserts was considered to be background and was subtracted from the activities assayed for the fusion plasmids.

RESULTS

Isolation and characterization of Rep⁺ revertants. The isolation and characterization of the Inc⁻ Rep⁻ mutants pWNRR104, pWNRR124, pWNRR149, and pWNRR160 (Table 1), which contain the P_E mutation, have been described previously (45, 46). These mutants were isolated from Inc⁺ Rep⁺ plasmid pRR945, which consists of a cointegrate of pBR322 and the 1.1- and 1.6-kb *Pst*I fragments that contain the wild-type NR1 *repA* replication control region (Table 1). Unlike parental plasmid pRR945, the four Inc⁻ Rep⁻ mutants are unable to replicate in *polA* host JG112 (45, 46). DNA from each of the four mutants was treated with hydroxylamine, and then JG112 was transformated. Thirty-one independent Rep⁺ revertants that could transform JG112 at a frequency similar to that of pRR945 were obtained by this procedure. No Rep⁺ revertants were obtained in the absence of mutagenesis. The incompatibility phenotype of each of the mutants was tested by transformation of KP435 *recA*-containing resident plasmid pRR933, a wild-type miniplasmid. All 31 revertants remained Inc⁻, since they were compatible with pRR933, in contrast to parental plasmid pRR945, which excluded pRR933 in parallel experiments (data not shown). This suggested that the Inc⁻ Rep⁺ plasmids were not true revertants but were likely to contain second-site suppressor mutations that restored replication proficiency.

Two Rep⁺ revertants isolated from each of the four original Inc⁻ Rep⁻ mutants were analyzed further. The 1.1-kb *Pst*I fragments from each Rep⁺ revertant were used in replicon reconstitution experiments to construct miniplasmids that consist of three *Pst*I fragments. The 1.1-kb fragments from the revertants were combined with wild-type 1.6- and 2.2-kb fragments to construct plasmids similar to pRR933, which were then introduced into JG112 by transformation. Each of the revertant miniplasmids was capable of transforming JG112, whereas in parallel experiments with the 1.1-kb fragments from the Inc⁻ Rep⁻ mutants no transformants were obtained. This indicated that the mutations responsible for restoring the Rep⁺ phenotype were located within the 1.1-kb *Pst*I fragments of the revertants.

Promoter P_E is contained within a 262-bp *Sau*3A fragment (bp 393 to 654 in Fig. 2). The nucleotide sequences of the 262-bp *Sau*3A fragments from the eight Inc⁻ Rep⁺ revertants were determined. All eight revertants retained the P_E mutation, C-597 to T (Fig. 2). This explained the Inc⁻ phenotype of the revertants, since they were unable to transcribe RNA-E. Seven of the revertants contained no other mutations within the 262-bp *Sau*3A fragment. One revertant, pWN160-15, contained a transition, G-639 to A, adjacent to the SD sequence of *repA1* (Fig. 2). Because revertant pWN160-14 was obtained from the same parental plasmid as pWN160-15 and was clearly different from pWN160-15 by nucleotide sequence, these two revertants were subjected to further analysis. The nucleotide sequences of the entire 1.1-kb *Pst*I fragments from pWN160-14 and pWN160-15 were determined and compared with those of parental mutant pWNRR160 and the wild-type plasmid pRR945. The 1.1-kb fragment from pWN160-15 contained only the P_E and SD mutations. The 1.1-kb fragment from pWN160-14 contained the P_E mutation and one other transition, G-216 to A (Fig. 2). This second mutation in pWN160-14 replaced a Glu codon with a Lys codon in the *repA2* gene. Therefore, the revertants pWN160-14 and pWN160-15 each contained two mutations: the original P_E mutation responsible for the Inc⁻ and Rep⁻ phenotypes and a mutation either at the SD sequence of *repA1* or within the coding sequence of *repA2*, apparently responsible for suppressing the Rep⁻ phenotype.

To assess the effects of the individual mutations, the

TABLE 2. Expression of *repA1* and 2.5-kDa leader peptide in low-copy-number *lacZ* fusions

Plasmid	Fusion[a]	Mutation	β-Galactosidase activity (Miller units) in the presence of coresident plasmid[b]:			
			pACYC184	pWR128	pWR130	pWR119
pWR126	*repA1*	None	0.41	0.057	0.027	4.7
pWR123	*repA1*	P_E	0.041	0.016	0.030	0.041
pWR124	*repA1*	*repA2*	5.5	0.068	0.057	25
pWR125	*repA1*	SD	3.2	0.014	0.55	10
pWR122	*repA1*	*repA2*, P_E	0.25	0.019	0.096	0.25
pWR121	*repA1*	SD, P_E	0.15	0.076	0.25	0.16
pWR141	Leader	None	21	5.1	0.17	85
pWR142	Leader	P_E	0.002	0.001	0.001	0.003
pWR143	Leader	*repA2*	97	6.3	0.34	340
pWR144	Leader	*repA2*, P_E	0.013	0.009	0.008	0.017
pWR106	Transcriptional	None	19	5.3	14	16

[a] *repA* and leader, translational fusion at the *Pst*I site in *repA1* or the *Fsp*I site in the leader gene; transcriptional fusion is at the *Sal*I site in *repA1*.
[b] The data are averages of multiple measurements. Some data were presented previously (45). pWR128, pWR130, and pWR119 are vector pACYC184 derivatives that synthesize RepA2, RNA-E, and *repA1* mRNA target sequences, respectively.

repA2 mutation of pWN160-14 and the SD mutation of pWN160-15 were separated from the P_E mutation by using the *Nsi*I site at bp 335 or the *Fsp*I site at bp 625, respectively (Fig. 1 and 2), resulting in plasmids pWW160-16 (*repA2*) and pWW160-17 (SD). These two plasmids were found to be incompatible with pRR933, as expected, and were each capable of transforming JG112; i.e., they were Inc^+ Rep^+. In replicon reconstitution experiments, the 1.1-kb *Pst*I fragments from both pWW160-16 and pWW160-17 were capable of forming three-*Pst*I-fragment miniplasmids that could replicate in JG112. Therefore, the second-site suppressor mutations either in the SD of *repA1* or within the *repA2* gene by themselves did not have phenotypes revealed by these tests.

Effects of mutations on expression of *repA1* and the 2.5-kDa leader peptide. The Rep^- phenotype of the original mutants resulted from their inability to synthesize enough RepA1 protein for initiation of replication (45). To determine the effects of various combinations of mutations on the expression of the *repA1* gene, transcriptional and translational *lacZ* fusions were constructed. Low-copy-number fusion plasmids were constructed by using pFZY1, a mini-F-based vector plasmid, and high-copy-number fusion plasmids were constructed with pWR100, a pBR322-based vector plasmid. The *Pst*I site at bp 874 (Fig. 1 and 2) was used to construct *repA1-lacZ* translational fusions, the *Fsp*I site at bp 625 was used to construct leader peptide-*lacZ* translational fusions, and the *Sal*I site at bp 827 was used to construct *repA-lacZ* transcriptional fusions (see Materials and Methods) (Table 1). The fusion plasmids contained NR1 DNA between the *Bgl*II site at position −1 (Fig. 1 and 2) and either the *Fsp*I, *Sal*I, or *Pst*I site. Therefore, these fusion plasmids contain genes for the normal *trans*-acting regulatory elements, RepA2 and RNA-E, that control expression of *repA1*. Fusions were constructed with wild-type NR1 DNA and with DNA containing each of the P_E, *repA2*, and SD mutations, individually and in combination.

Data for the low-copy-number translational fusion plasmids are shown in Table 2. At the low copy number of the mini-F vector, which is about twofold lower than that of NR1 (42), the P_A promoter is expected to be partially repressed by RepA2 repressor and the translation of the mRNA for the leader peptide and *repA1* should be partially inhibited by RNA-E. Under these conditions, the leader peptide was expressed at a much higher level than was *repA1*. For example, the plasmid with the wild-type leader peptide-*lacZ* translational fusion produced about 50-fold (21/0.41) more β-galactosidase activity (measured in Miller units) than that with the wild-type *repA1-lacZ* translational fusion. The presence of the P_E mutation by itself caused a decrease in the expression of both *repA1* and the leader peptide. Compared with β-galactosidase activity for the wild-type translational fusion plasmids, the P_E mutation decreased expression of *repA1* about 10-fold (0.41/0.041) and decreased expression of the leader peptide about 10^4-fold (21/0.002), owing to the nonsense mutation introduced at the sixth codon of the leader peptide coding region (Fig. 2). This 10-fold decrease in *repA1* expression results in the Rep^- phenotype. By itself, the *repA2* mutation increased expression of both *repA1* and the leader peptide. The increase for *repA1* was about 13-fold (5.5/0.41), and that for the leader peptide was about 5-fold (97/21). The SD mutation alone increased expression of *repA1* about eightfold (3.2/0.41). The effect of the SD mutation on expression or the leader peptide could not be examined with these fusions, since the site of the SD mutation is downstream from the *Fsp*I site used to construct the leader peptide-*lacZ* fusions (Fig. 2). In combination with the P_E mutation, the *repA2* mutation increased expression of *repA1* above that for the translational fusion plasmid with the P_E mutation alone about sixfold (0.25/0.041) and increased leader peptide expression by about the same factor (0.013/0.002). The SD mutation in combination with the P_E mutation increased the expression of *repA1* about 3.5-fold (0.15/0.041). These results suggested that the mutations in either *repA2* or the SD sequence suppressed the Rep^- phenotype by increasing expression of *repA1*. Because the expression of the leader peptide remained about 20-fold lower than that of *repA1* in the translational fusions that contained the P_E mutation, this indicated that the increased expression of *repA1* caused by the suppressor mutations was independent of leader peptide translation.

Similar results were obtained with the high-copy-number translational-fusion plasmids (Table 3). However, at high copy number, the configuration of the regulatory elements is different, revealing different aspects of the control of expression of *repA1*. The high copy number results in high gene dosage of the regulatory genes. Therefore, it is expected that the P_A promoter will be fully repressed by the high concentration of RepA2 repressor protein and that translation of the RNA-CX transcripts will be strongly inhibited by the high concentration of RNA-E. Under these conditions, the plas-

TABLE 3. Expression of *repA1* and 2.5-kDa leader peptide in high-copy-number *lacZ* fusions

Plasmid	Fusion[a]	Mutation	β-Galactosidase activity (Miller units)[b]
pWR116	*repA1*	None	3.1
pWR113	*repA1*	P_E	7.3
pWR114	*repA1*	*repA2*	19
pWR115	*repA1*	SD	18
pWR112	*repA1*	*repA2*, P_E	42
pWR111	*repA1*	SD, P_E	18
pWR110	Leader	None	4.2
pWR139	Leader	P_E	0.0
pWR140	Leader	*repA2*	65
pWR138	Leader	*repA2*, P_E	1.4
pWR136	Transcriptional	None	470

[a] *repA1* and leader, translational fusion at the *PstI* site in *repA1* or the *FspI* site in the leader gene; transcriptional fusion is at the *SalI* site in *repA1*.

[b] The data are averages of multiple measurements; 0.0 indicates that β-galactosidase activity was indistinguishable from background. Some data were presented previously (45).

mid with the wild-type leader peptide-*lacZ* translational fusion produced only slightly more β-galactosidase activity than that with the wild-type *repA1-lacZ* translational fusion (4.2/3.1). Interestingly, although the P_E mutation severely reduced expression of the leader peptide, *repA1* expression was actually increased about twofold (7.3/3.1) by the mutation in the high-copy-number plasmids. This illustrated two points that were less obvious from examination of the data for the low-copy-number fusion plasmids. First, there was a residual, or basal, level of expression of *repA1* when leader peptide translation was inhibited either by the high concentration of RNA-E from the wild-type fusion plasmid or by the nonsense mutation in the leader peptide gene of the P_E mutant fusion plasmid. Second, the approximate twofold increase in the basal level of expression of *repA1* in the P_E mutant probably resulted from the twofold increased rate of transcription of *repA1* mRNA in the absence of interference from convergent RNA-E transcription, as previously observed (44). In a finding consistent with the pattern for low-copy-number translational-fusion plasmids, the *repA2* mutation, by itself or in combination with the P_E mutation, increased the expression of both the leader peptide and *repA1* (Table 3). The SD mutation, either by itself or in combination with the P_E mutation, also increased expression of *repA1* from the high-copy-number translational fusion plasmids (Table 3). These results suggested that the Rep⁻ phenotype conferred by the P_E mutation could be suppressed either by increasing the rate of *repA1* mRNA transcription (e.g., by the mutation in the *repA2* repressor gene) or by increasing the basal level of translation of *repA1* mRNA (e.g., by the SD mutation). For comparison, the level of β-galactosidase activity for the high-copy-number plasmid with the transcriptional *repA-lacZ* fusion was more than 100-fold higher than that for the translational fusions (Table 3), indicating that the high gene dosage of RNA-E had little effect on the transcription of *repA1* mRNA.

Effects of *trans*-acting regulatory elements on expression of *repA1* and the 2.5-kDa leader peptide. To explore further the relationships between expression of the leader peptide and *repA1* and the effects of the various mutations, compatible plasmids that encode the individual *trans*-acting regulatory elements of the *repA* replicon were introduced into the same cell with the low-copy-number translational-fusion plasmids (Table 2). Additional RepA2 repressor protein provided in *trans* by plasmid pWR128 reduced the level of β-galactosidase activity from every fusion plasmid tested. This reflected that at the low copy number of the mini-F-derived fusion plasmids, the P_A promoter was only partially repressed by the endogenously synthesized RepA2 repressor. The additional RepA2 provided in *trans* probably resulted in full repression of transcription at P_A, with the residual levels of expression of *repA1* and leader peptide coming from constitutive RNA-CX transcription. The repression by additional RepA2 was about 7-fold (0.41/0.057) for the wild-type *repA1-lacZ* translational-fusion plasmid but was about 80-fold (5.5/0.068) for the *repA1-lacZ* translational-fusion plasmid with the *repA2* mutation. The repressed level was approximately the same for both plasmids. This confirmed that the lesion in *repA2* inactivated the repressor protein, resulting in elevated transcription of the genes for the leader peptide and RepA1. In addition, the increased rate of transcription of *repA1* mRNA from the *repA2* mutant probably interfered with convergent RNA-E transcription, also contributing to a reduced ratio of RNA-E to mRNA. This most likely contributed to the 80-fold range in the overall regulation of *repA1* expression exhibited by the *repA2* mutant. In the absence of RNA-E transcription, as in the case of the *repA1-lacZ* translational-fusion plasmid with the both *repA2* and P_E mutations, the range of regulation of transcription by RepA2 was only about 13-fold (0.25/0.019). This reflects how intricately interrelated the mechanisms regulating transcription and translation of *repA1* mRNA are.

In similar experiments, additional RNA-E provided in *trans* by plasmid pWR130 reduced the level of β-galactosidase activity from most of the low-copy-number translational-fusion plasmids (Table 2). This reflected that, at low copy number, translation of *repA1* mRNA was only partially repressed by the endogenously synthesized RNA-E. However, differences in the quantitative effects among the various types of fusion plasmids were instructive. Additional RNA-E reduced *repA1* expression from the wild-type translational-fusion plasmid about 15-fold (0.41/0.027). The reduction for the *repA1-lacZ* translational-fusion plasmid that contained the SD mutation was only about 6-fold (3.2/0.55), and the actual level of *repA1* expression in the presence of additional RNA-E was about 20-fold higher (0.55/0.027) for the SD mutant than for the wild-type fusion plasmid. This suggested that the SD mutation reduced the sensitivity of *repA1* mRNA to inhibition of translation by RNA-E. Additional RNA-E had only minimal effects on β-galactosidase activity from the translational-fusion plasmids that contained the P_E mutation. Because the translation of the leader peptide is prematurely terminated in these mutants, this suggests that most of the inhibitory effect of RNA-E on *repA1* expression is mediated through translational coupling with leader peptide expression, and that effect is absent in these mutants.

The effects of providing additional *repA1* mRNA target sequences in *trans* were examined by introducing plasmid pWR119 into the same cell with the low-copy-number translational-fusion plasmids (Table 2). Plasmid pWR119 produces the part of *repA1* mRNA that is complementary to RNA-E and, therefore, titrates the RNA-E synthesized endogenously by the fusion plasmids. Additional target RNA sequences stimulated expression of *repA1* from the wild-type translational-fusion plasmid about 12-fold (4.7/0.41). Additional target RNA sequences had little effect on β-galactosidase activity from the fusion plasmids that contained the P_E mutation, reflecting the absence of endogenous

RNA-E synthesized by those mutant plasmids. The differences between the titrated level of expression in the presence of additional target RNA sequences and the inhibited level of expression in the presence of additional RNA-E were about 175-fold (4.7/0.027) for the wild-type *repA1-lacZ* translational-fusion plasmid and about 440-fold (25/0.057) for the equivalent fusion plasmid that contained the *repA2* mutation. These ratios should reflect the regulatory range of *repA1* expression contributed by the RNA-E translational inhibitory circuit. The higher value for the *repA2* mutant may result from the effects of convergent transcription when P_A is derepressed. For the wild-type plasmid and the *repA2* mutant leader peptide-*lacZ* translational-fusion plasmid, the differences between the titrated level of expression in the presence of additional target RNA sequences and the inhibited level of expression in the presence of additional RNA-E were about 500-fold (85/0.17) and about 1,000-fold (340/0.34), respectively. For the *repA1-lacZ* translational-fusion plasmid that contained the SD mutation, additional target RNA sequences stimulated *repA1* expression only about threefold (10/3.2) and the difference between titrated and inhibited levels of expression was only about 19-fold (10/0.55), a finding consistent with a reduced sensitivity to RNA-E regulation by SD mutant *repA1* mRNA. Under conditions of titration by additional target RNA sequences, the *repA1* mRNA with the SD mutation was translated about 2-fold more efficiently than the wild type (10/4.7), whereas that from the P_E mutant was translated about 115-fold less efficiently than the wild type (4.7/0.041). If the 2-fold-increased rate of *repA1* mRNA synthesis resulting from absence of interference from convergent transcription in the P_E mutant is taken into account, its *repA1* mRNA must be translated more than 200-fold less efficiently than that of the wild type.

repA1 mRNA that contains the SD mutation might be less sensitive to inhibition by RNA-E either because it does not bind RNA-E as well or because, even when bound by RNA-E, it can still be translated efficiently. Plasmid pWR151 has the same structure as pWR119, except that it produces *repA1* mRNA target sequences that contain the SD mutation. pWR151 was also introduced into the same cell with wild-type *repA1-lacZ* translational-fusion plasmid pWR126 to see whether the target sequences it provides could titrate the endogenously synthesized RNA-E. In parallel experiments, pWR119 stimulated *repA1* expression from 0.40 to 4.6 Miller units (11.5-fold), whereas pWR151 stimulated expression from 0.40 to 4.7 Miller units (also about 11.5-fold). Therefore, the target RNA sequences that contained the SD mutation bound RNA-E as well as those that did not contain the mutation. This suggested that *repA1* mRNA that contains the SD mutation is less sensitive to inhibition by RNA-E because it can be translated more efficiently even when bound by RNA-E.

The effects of the *repA2* mutation on the level of transcription of *repA1* mRNA could also be assessed in the presence of additional target RNA sequences. Under those conditions, the effects of RNA-E on translation are eliminated by titration, leaving only the effects of transcription control. In the presence of additional target RNA sequences, the *repA2* mutation increased expression of *repA1* about fivefold (25/4.7) and increased expression of the leader peptide about fourfold (340/85).

For comparison, additional RNA-E or target RNA sequences had little effect on expression of β-galactosidase activity from a *repA-lacZ* transcriptional fusion plasmid (Table 2), whereas additional RepA2 repressed transcription about 3.5-fold (19/5.3). This confirmed that the effects of RNA-E were mediated through translation of the *repA1* mRNA, rather than transcription. The effects of introducing plasmids pWR128, pWR130, and pWR119 into the same cell with the high-copy-number translational-fusion plasmids were minimal, presumably because of the already high concentrations of the regulatory components resulting from high gene dosage (data not shown). Finally, the copy numbers of the various fusion plasmids were monitored by measuring the gene dosages of their *bla* genes in the presence of the various coresident plasmids. No significant differences in copy number that would explain the differences in the levels of β-galactosidase activity reported in Tables 2 and 3 were observed (data not shown).

TABLE 4. Copy number of minireplicator plasmids[a]

Plasmid	Mutation	Copy number[b]
pRR933	None	1.0 ± 0.16
pWR150	*repA2*	7.2 ± 2.9
pWR153	SD	2.1 ± 0.78
pWR149	*repA2*, P_E	1.1 ± 0.90[c]
pWR148	SD, P_E	1.6 ± 0.30

[a] Composed of 1.1-kb *inc*, 1.6-kb *ori*, and 2.2-kb *cat PstI* fragments.
[b] Relative to wild-type plasmid pRR933 in strain JG112 on the basis of *cat* gene dosage. The data are the averages of four experiments.
[c] The copy number of pWR149 was highly variable.

Effects of mutations on copy number, stable inheritance, and incompatibility of reconstituted minireplicon plasmids. The effects of the various mutations on plasmid replication were tested directly by using the miniplasmids that were constructed during the replicon reconstitution experiments. Unlike the cases for the *lacZ* fusion plasmids described above, in which the copy numbers of the *repA1* gene and its regulatory elements were fixed by the mini-F- and pBR322-derived vector plasmids, the copy numbers of the minireplicon plasmids will reflect the balance of the adjusted levels of transcription and translation of *repA1* mRNA that are feedback regulated by gene dosage. The relative copy numbers of the reconstituted miniplasmids were compared with that of wild-type miniplasmid pRR933 (Table 4). By itself, the *repA2* mutation increased the copy number of miniplasmid pWR150 about sevenfold, and this increase was observed consistently from experiment to experiment. The SD mutation by itself also increased the copy number of miniplasmid pWR153 about twofold, and again, an increased copy number for pWR153 was observed consistently from experiment to experiment. In contrast, the copy number of the miniplasmid that contained both *repA2* and P_E mutations, pWR149, was unpredictable and varied from much lower than that of the wild type to much higher. The copy number of the miniplasmid that contained both SD and P_E mutations, pWR148, was consistently slightly higher than that of the wild type. Plasmid copy numbers were also estimated by inspection of yields of plasmid DNA present in minilysates. Results consistent with those reported in Table 4 were obtained, with the yield of pWR149 plasmid DNA being highly variable (data not shown).

Inheritance of low-copy-number miniplasmids such as pRR933, which lacks the stability (*stb*) locus of NR1, is unstable in the absence of continuous antibiotic selection (22, 35). Mutations that affect plasmid copy number often alter the stability phenotypes of miniplasmids by changing the number of independently segregating plasmid copies.

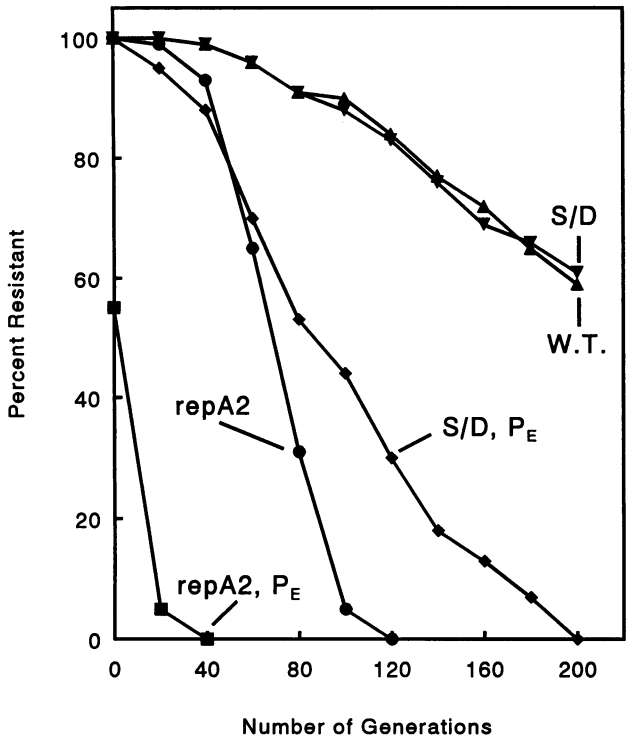

FIG. 3. Stability of inheritance of minireplicator plasmids. The percentages of JG112 cells that were resistant to chloramphenicol after various periods of nonselective growth in drug-free medium were determined by replica plating. The data are the averages of three experiments. Plasmids: W.T., pRR933; S/D P_E, pWR148; *repA2* P_E, pWR149; *repA2*, pWR150; S/D, pWR153.

The stabilities of the miniplasmids with the various combinations of Rep⁻ and suppressor mutations were compared with that of wild-type miniplasmid pRR933 (Fig. 3). The stability of pWR153, which contains only the SD mutation, was indistinguishable from that of pRR933. The plasmid with only the *repA2* mutation, pWR150, was considerably less stable than pRR933, even though it had a higher copy number in Table 4. The miniplasmid with both SD and P_E mutations, pWR148, also was less stable than pRR933. This suggests that miniplasmids that lack one or the other of the regulatory circuits that control expression of *repA1* are less stable than wild-type miniplasmids, which contain both the RNA-E and RepA2 circuits. The plasmid that contained both *repA2* and P_E mutations, pWR149, was extremely unstable. This plasmid lacks both the RNA-E and RepA2 regulatory circuits and, most likely, is unable to respond to fluctuations in plasmid copy number that would be expected to occur by chance. This was probably also reflected in the wide variability in copy number observed from experiment to experiment for pWR149 in Table 4.

Plasmid incompatibility is primarily a manifestation of shared replication control elements (27). The incompatibility properties of the various mutants were tested as both donor and resident plasmids (Table 5). The reconstituted miniplasmids were used as residents, and the donor plasmids were cointegrates with vector plasmid pBR322. Miniplasmid pWR153, which contains only the SD mutation, behaved indistinguishably from wild-type miniplasmid pRR933. Both were lost in the presence of donor plasmids that could provide RNA-E and RepA2 in *trans* (pRR935 and pWW160-17). Miniplasmid pWR148, which contains both SD and P_E mutations, was also lost in the presence of those two donor plasmids. In contrast, all resident plasmids tested were compatible with donor plasmid pWW160-15, which is incapable of supplying RNA-E in *trans*, owing to the presence of the P_E mutation. What is of interest about these results is that the miniplasmids that contain the SD mutation were still lost in the presence of another plasmid that could provide RNA-E in *trans*, even though the expression of *repA1* was found to be much less sensitive to inhibition by RNA-E when the SD mutation was present (Table 2). The miniplasmid with both *repA2* and P_E mutations, pWR149, was so unstable in the absence of continuous direct selection that it was not possible to test its incompatibility properties as a resident plasmid (data not shown).

TABLE 5. Incompatibility tests with minireplicator plasmids

Donor plasmid[a]	% Retention of resident plasmid[b]:		
	pRR933	pWR153	pWR148
None	100	100	72
pBR322	100	100	67
pRR935	0	0	0
pWW160-17	0	0	0
pWW160-15	100	100	70

[a] pBR322, vector; pRR935, wild type; pWW160-17, SD; pWW160-15, P_E SD.

[b] Tested after transformation of JK17 cells by indicated donor plasmid with selection for donor plasmid only. pRR933, wild type; pWR153, SD; pWR148, P_E SD.

Computer predictions of *repA1* mRNA secondary structure. The potential changes in *repA1* mRNA secondary structure that might be effected by the P_E and SD mutations and that might affect expression of *repA1* were examined by using a computer program (47) to predict the most stable RNA secondary structures on the basis of the nucleotide sequences of the transcripts (Fig. 4). The structures were predicted starting with bp 565, which is immediately downstream from the region of RNA-E complementarity, through bp 682, which is within the *repA1* gene (Fig. 2). We have previously reported the predicted structure of wild-type *repA1* mRNA between these coordinates (9, 40). Because the mRNA sequences that are complementary to RNA-E are not included in the predictions and, therefore, do not participate in formation of secondary structure, the predicted structures are formally equivalent to those for which RNA-E has formed a duplex with *repA1* mRNA (9, 40). Figure 4 shows stem-loop structures that contain the SD sequence and the GUG start codon for *repA1* that are present in the overall predicted structures of the transcripts. Predicted structures of RNA sequences that contained the P_E mutation were not significantly different from those that contained the wild-type sequence at bp 597 (data not shown). However, the presence of the SD mutation at bp 639 resulted in a very different predicted structure. For the wild-type RNA sequence, the G at bp 639 stabilized the structure shown on the left in Fig. 4 by producing a GC base pair in the stem. This resulted in base pairing of both the SD sequence and the GUG codon of *repA1*, as previously predicted (9, 40). For the mutant with an A at bp 639, the CA base pair destabilized the stem of the wild-type structure but stabilized the stem of the alternative structure predicted for the SD mutant by providing AU pairing. In this structure, both the SD and GUG sequences of *repA1* are more accessible in single-stranded regions. This could contribute to the increased

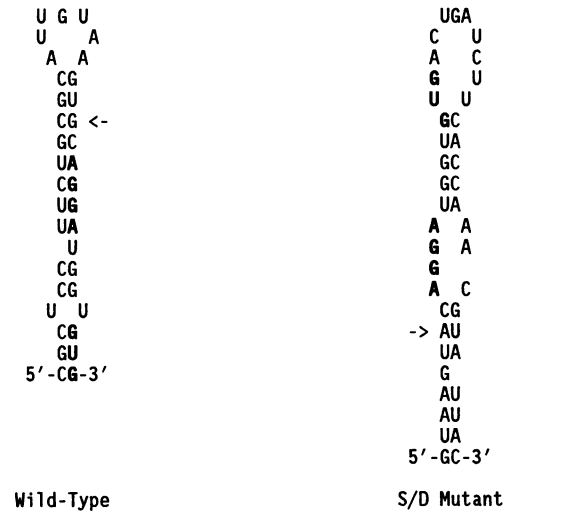

FIG. 4. Predicted secondary structures near the translation initiation site of *repA1* mRNA. The most stable secondary structure of *repA1* mRNA from nucleotides 565 through 682 (Fig. 2) was predicted with the computer program of Zuker and Stiegler (47) by using Salser's rules (32) for base stacking and loop-destabilizing free energies (in kilocalories). Predicted stem-loop structures that contain the SD and GUG sequences for initiation of *repA1* translation are shown. The structure for wild-type *repA1* mRNA is shown on the left (nucleotides 616 to 651), and the structure for *repA1* mRNA containing the SD mutation is shown on the right (nucleotides 633 to 675). The site of the base substitution in the SD mutant (bp 639) is indicated (arrows).

expression of *repA1* from the SD mutant and to the reduced sensitivity to inhibition by RNA-E, since the *repA1* translation initiation site of the SD mutant is predicted to be open independently of binding by RNA-E to its complementary target sequences upstream from bp 565 (Fig. 2).

DISCUSSION

The *repA* replicon of IncFII plasmid NR1 is regulated by controlling the synthesis of the RepA1 initiation protein by two independent control circuits that together regulate both transcription and translation of *repA1* mRNA. The RepA2 control circuit regulates transcription of *repA1* by repressing promoter P_A (8). When P_A is repressed, there is a low level of constitutive transcription of *repA1* that is initiated at promoter P_C (8, 44). Direct measurements of the rates of RNA transcription indicated that P_A is about six times stronger than P_C, so that derepression of P_A causes a significant increase in the rate of transcription of *repA1* mRNA (44). The *repA2* gene is transcribed constitutively and provides RepA2 repressor protein in proportion to plasmid copy number (8). The active form of the RepA2 repressor is a tetramer that forms cooperatively and is in equilibrium with inactive monomers (8). That equilibrium allows active repressor concentration to change more rapidly than overall repressor protein concentration and provides for finely tuned regulation of *repA1* mRNA transcription (8, 42).

The RNA-E control circuit regulates translation of *repA1* mRNA. RNA-E binds to and forms a duplex with its complementary sequence in the *repA1* mRNA (4, 18, 37, 41). The binding of RNA-E to *repA1* mRNA inhibits translation of a 24-amino-acid leader peptide, whose translation promotes that of *repA1* (3, 45). The inhibition of *repA1* synthesis by RNA-E, therefore, may be indirect, by uncoupling *repA1* and leader peptide translation (3, 45). Formation of a duplex between RNA-E and its target may directly interfere in the binding of ribosomes to the initiation site of the leader peptide. Alternatively, the secondary structure of the leader mRNA might be affected in a way that inhibits ribosome binding, by a mechanism similar to that proposed earlier for *repA1* mRNA (9, 40). RNA-E is transcribed from promoter P_E, which is a constitutive promoter about 20 times stronger than P_C (44). Therefore, RNA-E is provided in proportion to plasmid copy number and is in excess compared with its target sequences in *repA1* mRNA (44). RNA-E is unstable, and therefore, its concentration can change rapidly in response to changes in plasmid copy number (42, 44). The convergence of RNA-E and *repA1* mRNA causes interference in the rate of transcription in both directions, which is influenced by derepression of transcription at P_A (44). This, too, contributes to the overall balance of RNA-E and its target sequences in *repA1* mRNA.

Examination of the Rep^- mutants and Rep^+ revertants presented here provides insight into the details of the complex interactions of the RepA2 and RNA-E control circuits and how they work together to regulate replication of plasmid NR1. Working with the various mutations that were identified, both individually and in combination, allowed us to carry out a comprehensive analysis of the IncFII plasmid replication control mechanism. The replication-defective phenotype conferred by the P_E mutation revealed the nature of the translational coupling between the leader peptide and *repA1*. The mutation introduced a stop codon in the leader peptide gene (Fig. 2) and caused premature termination of its translation (Tables 2 and 3). This uncoupled translation of *repA1* from the leader sequence and reduced the synthesis of RepA1 protein (Tables 2 and 3). When translation of *repA1* was uncoupled from that of the leader peptide, there was a basal level of RepA1 synthesis that was independent of coupling (Tables 2 and 3). However, that low rate of synthesis of RepA1 was apparently insufficient for initiation of replication, resulting in the Rep^- phenotype. The Rep^+ revertants overcame this deficit by increasing the rate of uncoupled RepA1 synthesis by two different mechanisms.

One Rep^+ suppressor mutation altered the sequence near the SD sequence of *repA1* (Fig. 2). The SD mutation increased the rate of leader peptide-independent translation of *repA1* mRNA (Tables 2 and 3). The SD mutation also reduced the sensitivity of *repA1* mRNA to inhibition of its translation by RNA-E (Table 2). In the presence of additional RNA-E provided in *trans*, the *repA1-lacZ* translational fusion plasmid that contained the *repA1* SD mutation produced 20-fold more β-galactosidase activity than the equivalent wild-type fusion plasmid. The increase in leader peptide-independent translation of *repA1* mRNA might result from the alteration of secondary structure around the mutated *repA1* SD sequence (Fig. 4). A comparison of the predicted secondary structures of wild-type and mutant RNAs suggested that the mutant SD sequence of *repA1* would be single stranded and more accessible to ribosomes. The predicted structure of mRNA with the wild-type *repA1* SD sequence suggests that it may be sequestered, which might explain the requirement for coupling of *repA1* translation to that of the leader peptide.

A second Rep^+ suppressor mutation was located in the *repA2* gene (Fig. 2). The mutation substituted a lysine residue in place of a glutamic acid residue in RepA2 and inactivated the transcription repressor. The resulting dere-

pression of P_A increased the rate of transcription of *repA1* mRNA (Tables 2 and 3). Even though the mRNA transcripts contain the P_E mutation and, therefore, are translated poorly in the absence of coupling (Table 2), the increased rate of transcription was apparently sufficient to supply enough RepA1 protein for initiation of replication.

The dynamic ranges for the two regulatory circuits can be estimated from the data in Table 2. By providing exogenously supplied regulatory components to the various fusion plasmids, the minimum and maximum levels of expression of the leader peptide and *repA1* could be analyzed. This provided a much better understanding of the relative contributions of the two circuits to the regulation of expression of *repA1* than was previously available. The wild-type *repA1-lacZ* translational fusion was regulated over a 175-fold (4.7/0.027) range by RNA-E, and the wild-type leader peptide-*lacZ* translational fusion was regulated over a 500-fold (85/0.17) range by RNA-E. Under conditions of titration of RNA-E by additional target RNA sequences provided in *trans*, the level of β-galactosidase activity from the fusion plasmids that contain the *repA2* mutation was about fivefold (25/4.7) higher than that of the wild type for the *repA1-lacZ* translational fusion and about fourfold (340/85) higher for the leader peptide-*lacZ* translational fusion. Because RNA-E was titrated, those differences should reflect a four- to fivefold increase in the rate of transcription of *repA1* mRNA caused by the mutation in *repA2*, compared with the partially repressed level resulting from the RepA2 synthesized endogenously by the wild-type fusion plasmid. A similar comparison of the *repA1-lacZ* fusion plasmid that contains only the P_E mutation with that containing both *repA2* and P_E mutations indicates a sixfold (0.25/0.041) increase in the rate of transcription caused by the *repA2* mutation. Slight differences in these estimates may reflect interference from convergent transcription, which certainly must contribute to the results observed for those fusion plasmids that synthesize RNA-E endogenously. The dynamic range for regulation of transcription by RepA2 alone can be estimated from the *repA1-lacZ* fusion plasmid that contains both *repA2* and P_E mutations, whose activity was repressed 13-fold (0.25/0.019) by additional RepA2 provided in *trans*. Therefore, RepA2 and RNA-E together can regulate expression of *repA1* over at least a 2,000-fold range.

The basal level of expression of the leader peptide was either extremely low or nonexistent, whereas that of *repA1* was significant (Tables 2 and 3). Therefore, the overall observed level of expression of *repA1* is the sum of its regulated (i.e., coupled to that of the leader peptide) and basal (uncoupled) levels. This, most likely, explains some of the differences between expression of the leader peptide and *repA1* in response to the various mutations or regulatory elements supplied in *trans*, since they may differentially affect the coupled and uncoupled modes of *repA1* expression.

The behavior of the reconstituted minireplicator plasmids that contained various combinations of the mutations was not predicted directly from the changes in the expression of *repA1* documented in Table 2. For example, the *repA2* mutation by itself increased expression from the low-copy-number *repA1-lacZ* translational-fusion plasmid by a factor of 13 (5.5/0.41) but increased the copy number of the equivalent miniplasmid only by a factor of 7 (Table 4). This may reflect uncertainties in the precision of the measurements. However, the differences could also reflect the dynamic state of the regulatory elements, which will reach a balance as the copy number of the miniplasmids and corresponding gene dosage of the regulatory elements increase. The adjusted levels of transcription and translation of *repA1* mRNA determine the final regulated copy number achieved. The SD mutation by itself increased expression from the low-copy-number *repA1-lacZ* translational-fusion plasmid by a factor of 8 (3.22/0.407) but increased miniplasmid copy number only by a factor of 2. Both the *repA2* and SD mutations increased the level of *repA1* expression from the low-copy-number fusion plasmids that contained the P_E mutation, but in neither case was the level of expression brought back up to that of the wild type (Table 2). However, the copy number of the SD P_E double mutant was not much different from that of the wild type, and that of the *repA2* P_E double mutant was highly variable (Table 4). These differences might indicate a level of complexity between the expression of *repA1* and plasmid replication not previously observed. One possible explanation is that a certain quantity of the *cis*-acting RepA1 protein may be needed to bind to the origin to effect initiation of replication. Any additional RepA1 protein beyond that required for initiation might not be utilized. An mRNA transcript that contains the SD mutation may provide much more *cis*-acting RepA1 protein than a wild-type transcript, but not all of the protein may be needed and used for initiation according to this explanation. This could explain the modest twofold increase in copy number for the SD mutant miniplasmid compared with the eightfold increase in expression of *repA1* from the low-copy-number *lacZ* translational-fusion plasmid. In the case of the *repA2* mutant, individual *repA1* mRNA transcripts are wild type, and those transcripts that are translated would provide the normal amount of RepA1 for initiation. The increased rate of transcription caused by the *repA2* mutation would simply increase the frequency with which such transcripts could provide RepA1 to the origin, causing an increase in copy number. Even so, if such transcription events occurred close together in time by chance, not all of the RepA1 might be utilized for initiation. The results for the *repA2* P_E double mutant suggest that RepA1 can be accumulated on the origin during successive transcription events until enough is bound to effect initiation. The presence of the P_E mutation uncouples *repA1* translation from that of the leader peptide, such that individual transcripts are translated only at the low basal level. However, increasing the frequency of transcription events by inactivating the RepA2 repressor allows for accumulation of RepA1 at the origin and replication proficiency for the *repA2* P_E double mutant. The time required for the RepA1 to accumulate at the origin could contribute to the instability observed for the double-mutant miniplasmid.

The stabilities of inheritance of the miniplasmids with various combinations of mutation revealed the contributions of each of the two control circuits to the overall stable maintenance of NR1. These control circuits increase stability by responding to chance fluctuations in copy number. If copy number has drifted lower than average, there is a possibility of segregating plasmid-free cells at cell division. Both the RepA2 and RNA-E circuits respond to such a situation by increasing expression of *repA1* to bring the copy number back toward average (42). At low copy number, the reduced concentration of active RepA2 repressor causes an increase in transcription of *repA1* mRNA. Likewise, the low concentration of RNA-E resulting from the low copy number causes an increase in the frequency of translation of *repA1* mRNA transcripts. Miniplasmids that had lost either control circuit were less stable than their wild-type counterpart (Fig. 3). The *repA2* P_E double-mutant miniplasmid, pWR149, which lacks both circuits, was highly unstable. pWR149 has

essentially constitutive replication control, since it lacks both the RepA2 and RNA-E control elements and cannot respond to changes in plasmid copy number. Although this plasmid replicates and is maintained by continuous antibiotic selection, plasmids with constitutive replication control would not survive long in the wild.

In total, 31 Rep^+ revertant plasmids were obtained, but only two have been examined in detail. Those two have revealed two different mechanisms by which the Rep^- phenotype of the P_E mutation can be suppressed. Both of those second-site mutations increased the synthesis of RepA1. One can surmise that a third mechanism of suppression might involve mutations that alter RepA1 itself. Since the P_E mutants do synthesize RepA1, but not in great enough quantity for initiation of replication, it might be possible to obtain *repA1* mutants that can initiate replication with smaller amounts of protein. This kind of mutant might provide further insights into the process of initiation of replication. Such mutants, having a more active initiation protein, are presently being sought.

ACKNOWLEDGMENTS

We thank Kim Wilber for excellent technical assistance.

This work was supported by Public Health Service research grant GM-30731 from the National Institute of General Medical Sciences.

REFERENCES

1. **Asano, K., A. Kato, H. Moriwaki, C. Hama, K. Shiba, and K. Mizobuchi.** 1991. Positive and negative regulation of plasmid ColIb-P9 *repZ* gene expression at the translational level. J. Biol. Chem. **266:**3774–3781.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Blomberg, P., K. Nordstrom, and E. G. H. Wagner.** 1992. Replication control of plasmid R1: RepA synthesis is regulated by CopA RNA through inhibition of leader peptide translation. EMBO J. **11:**2675–2683.
4. **Blomberg, P., E. G. H. Wagner, and K. Nordstrom.** 1990. Control of replication of plasmid R1: the duplex between the antisense RNA, CopA, and its target, CopT, is processed specifically in vivo and in vitro by RNase III. EMBO J. **9:**2331–2340.
5. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multi-purpose cloning system. Gene **2:**95–113.
6. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou.** 1983. β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100:**293–307.
7. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
8. **Dong, X., D. D. Womble, V. A. Luckow, and R. H. Rownd.** 1985. Regulation of transcription of the *repA1* gene in the replication control region of IncFII plasmid NR1 by gene dosage of the *repA2* transcription repressor protein. J. Bacteriol. **161:**544–551.
9. **Dong, X., D. D. Womble, and R. H. Rownd.** 1987. Transcriptional pausing in a region important for plasmid NR1 replication control. J. Bacteriol. **169:**5353–5363.
10. **Dong, X., D. D. Womble, and R. H. Rownd.** 1988. In vivo studies of the cis-acting replication initiator protein of IncFII plasmid NR1. J. Mol. Biol. **202:**495–509.
11. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Biochemistry **112:**295–298.
12. **Easton, A. M., and R. H. Rownd.** 1982. The incompatibility product of IncFII plasmid NR1 controls gene expression in the plasmid replication region. J. Bacteriol. **152:**829–839.
13. **Easton, A. M., P. Sampathkumar, and R. H. Rownd.** 1981. Incompatibility of IncFII R plasmid NR1, p. 125–141. *In* D. S. Ray (ed.), The initiation of DNA replication. Academic Press, Inc., New York.
14. **Friedman, S. A., and S. J. Austin.** 1988. The P1 plasmid-partition system synthesizes two essential proteins from an autoregulated operon. Plasmid **19:**103–112.
15. **Hama, C., T. Takizawa, H. Moriwaki, and K. Mizobuchi.** 1990. Role of leader peptide synthesis in *repZ* gene expression of the ColIb-P9 plasmid. J. Biol. Chem. **265:**10666–10673.
16. **Humphreys, G. D., G. A. Willshaw, H. R. Smith, and E. S. Anderson.** 1976. Mutagenesis of plasmid DNA with hydroxylamine: isolation of mutants of multi-copy plasmids. Mol. Gen. Genet. **145:**101–108.
17. **Koop, A. H., M. E. Hartley, and S. Bourgeois.** 1987. A low-copy-number vector utilizing β-galactosidase for the analysis of gene control elements. Gene **52:**245–256.
18. **Light, J., and S. Molin.** 1983. Post-transcriptional control of expression of the *repA* gene of plasmid R1 mediated by a small RNA molecule. EMBO J. **2:**93–98.
19. **Lupski, J. R., A. A. Ruiz, and G. N. Godson.** 1984. Promotion, termination, and antitermination in the *rpsU-dnaG-rpoD* macromolecular synthesis operon of *E. coli* K-12. Mol. Gen. Genet. **195:**391–401.
20. **Masai, H., and K. Arai.** 1987. RepA and DnaA proteins are required for initiation of R1 plasmid replication in vitro and interact with the *oriR* sequence. Proc. Natl. Acad. Sci. USA **84:**4781–4785.
21. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–79.
22. **Miki, T., A. M. Easton, and R. H. Rownd.** 1980. Cloning of replication, incompatibility, and stability functions of R plasmid NR1. J. Bacteriol. **141:**87–99.
23. **Miller, J., J. Manis, B. Kline, and A. Bishop.** 1978. Nonintegrated plasmid-folded chromosome complexes. Plasmid **1:**273–283.
24. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Miyazaki, C., Y. Kawai, H. Ohtsubo, and E. Ohtsubo.** 1988. Unidirectional replication of plasmid R100. J. Mol. Biol. **204:**331–343.
26. **Nordstrom, K., S. Molin, and J. Light.** 1984. Control of replication of bacterial plasmids: genetics, molecular biology, and physiology of the plasmid R1 system. Plasmid **12:**71–90.
27. **Novick, R. P.** 1987. Plasmid incompatibility. Microbiol. Rev. **51:**381–395.
28. **Ohman, M., and E. G. H. Wagner.** 1989. Secondary structure analysis of the RepA mRNA leader transcript involved in control of replication of plasmid R1. Nucleic Acids Res. **17:**2557–2579.
29. **Ortega, S., E. Lanka, and R. Diaz.** 1986. The involvement of host replication proteins and of specific origin sequences in the in vitro replication of miniplasmid R1 DNA. Nucleic Acids Res. **14:**4865–4879.
30. **Praszkier, J., I. W. Wilson, and A. J. Pittard.** 1992. Mutations affecting translational coupling between the *rep* genes of an IncB miniplasmid. J. Bacteriol. **174:**2376–2383.
31. **Rosen, J., T. Ryder, H. Inokuchi, H. Ohtsubo, and E. Ohtsubo.** 1980. Genes and sites involved in replication and incompatibility of an R100 plasmid derivative based on nucleotide sequence analysis. Mol. Gen. Genet. **179:**527–537.
32. **Salser, W.** 1977. Globin mRNA sequences: analysis of base pairing and evolutionary implications. Cold Spring Harbor Symp. Quant. Biol. **42:**985–1102.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43:**737–755.
35. **Tabuchi, A., Y. N. Min, C. K. Kim, Y. L. Fan, D. D. Womble, and R. H. Rownd.** 1988. Genetic organization and nucleotide

sequence of the stability locus of IncFII plasmid NR1. J. Mol. Biol. **202:**511–525.

36. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
37. **Wagner, E. G. H., P. Blomberg, and K. Nordstrom.** 1992. Replication control in plasmid R1: duplex formation between the antisense RNA, CopA, and its target, CopT, is not required for inhibition of RepA synthesis. EMBO J. **11:**1195–1203.
38. **Williams, S. A., B. E. Slatko, L. S. Moran, and S. M. DeSimone.** 1986. Sequencing in the fast lane: a rapid protocol for [α-^{35}S] dATP dideoxy DNA sequencing. BioTechniques **4:**138–147.
39. **Womble, D. D., X. Dong, V. A. Luckow, R. P. Wu, and R. H. Rownd.** 1985. Analysis of the individual regulatory components of the IncFII plasmid replication control system. J. Bacteriol. **161:**534–543.
40. **Womble, D. D., X. Dong, and R. H. Rownd.** 1987. Changes in RNA secondary structure may mediate the regulation of IncFII plasmid gene expression and DNA replication, p. 225–247. *In* M. Inouye and B. S. Dudock (ed.), New perspectives on the molecular biology of RNA. Academic Press, Inc., New York.
41. **Womble, D. D., X. Dong, R. P. Wu, V. A. Luckow, A. F. Martinez, and R. H. Rownd.** 1984. IncFII plasmid incompatibility product and its target are both RNA transcripts. J. Bacteriol. **160:**28–35.
42. **Womble, D. D., and R. H. Rownd.** 1986. Regulation of IncFII plasmid DNA replication. A quantitative model for control of plasmid NR1 replication in the bacterial cell division cycle. J. Mol. Biol. **192:**529–548.
43. **Womble, D. D., and R. H. Rownd.** 1988. Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. **52:**433–451.
44. **Womble, D. D., P. Sampathkumar, A. M. Easton, V. A. Luckow, and R. H. Rownd.** 1985. Transcription of the replication control region of the IncFII R-plasmid NR1 in vitro and in vivo. J. Mol. Biol. **181:**395–410.
45. **Wu, R., X. Wang, D. D. Womble, and R. H. Rownd.** 1992. Expression of the *repA1* gene of IncFII plasmid NR1 is translationally coupled to expression of an overlapping leader peptide. J. Bacteriol. **174:**7620–7628.
46. **Wu, R. P., D. D. Womble, and R. H. Rownd.** 1985. Incompatibility mutants of the IncFII plasmid NR1 and their effect on replication control. J. Bacteriol. **163:**973–982.
47. **Zuker, M., and P. Stiegler.** 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. **9:**133–148.