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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

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Transcription of the *repA1* gene of the IncFII plasmid NR1 is initiated at two promoters in the replication control region. Transcription from the upstream promoter is constitutive at a low level, whereas transcription from the downstream promoter is regulated. The 5′ end of the constitutively synthesized transcript also encodes the transcription repressor protein for the regulated downstream promoter. Therefore, the level of the repressor protein in the cell is gene dosage dependent. Using both *lac* gene fusions and quantitative hybridization methods, we have determined the in vivo relationship between the rate of transcription from the regulated promoter and the repressor protein concentration as a function of gene dosage. At the wild-type copy number of NR1, transcription from the regulated promoter is 96% repressed, but substantial derepression occurs when the copy number falls below the normal value. At or above the normal plasmid copy number, the basal level of *repA1* mRNA is provided by transcription from the constitutive upstream promoter.

The transmissible antibiotic resistance plasmid NR1 has a size of ca. 90 kilobase pairs (kb) and a copy number of about two per chromosome in *Escherichia coli* (25, 35). NR1 belongs to the FII incompatibility group, which also includes plasmids R1 and R6 (4). The replication control region of NR1 is contained within two *Pst*I restriction fragments of sizes 1.1 and 1.6 kb, which must be joined in their native orientation to form a functional replicon (20, 29). Minireplicator plasmids composed of these two *Pst*I fragments plus a 2.2-kb *Pst*I fragment encoding chloramphenicol resistance (Fig. 1) retain the copy number and incompatibility characteristics of their 90-kb parent plasmids (5, 33).

The *repA1* protein encoded by the NR1 replication control region (Fig. 1) is required for the initiation of DNA replication at the plasmid origin (17, 24). Plasmid replication is controlled by regulating the synthesis of the *repA1* initiation protein (5, 13, 26). Transcription of the *repA1* gene of NR1 is initiated at two promoters in the 1.1-kb *Pst*I fragment (Fig. 1) (6, 26). Transcription from the promoter for RNA-CX is constitutive at a low level (26, 33), whereas transcription from the promoter for RNA-A is regulated by the *repA2* repressor protein, encoded by the 5′ end of RNA-CX (15, 26). The *copB* protein of plasmid R1, although substantially different in amino acid sequence from the *repA2* protein of NR1 (27), appears to play a similar role in the regulation of R1 transcription (11, 22). However, the RNA-A transcription promoters of NR1 and R1 also are nonhomologous (27), and the transcription repressors do not cross-react with the nonhomologous operators (12). A third open reading frame in the DNA sequence, *repA3*, lies between the *repA2* and *repA1* genes (24) and is transcribed by RNA-CX and RNA-A (Fig. 1). However, no function has yet been found for the *repA3* gene.

The translation of *repA1* mRNA (RNA-CX and RNA-A) is regulated by the product of the *inc* gene, RNA-E, which is transcribed from the opposite DNA strand (Fig. 1) and is not translated. RNA-E inhibits translation of *repA1* mRNA by direct interaction between the complementary transcripts (13, 34). The combined regulation of transcription and translation of the gene for the *repA1* initiation protein results in a regulated plasmid copy number. The effects of each of the components of the replication control system on plasmid copy number, incompatibility, and expression of the *repA1* gene have been examined (12, 13, 20, 33, 34). In this communication we present a detailed analysis of the in vivo regulation of *repA1* mRNA transcription as a function of the gene dosage of the *repA2* transcription repressor protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *E. coli* K-12 KP245 (20) and NK5031 (8) were used for plasmid construction and lysogen construction, respectively. The plasmids and phages used in this study are described in the first table of reference 33. The construction of lysogens with recombinant λRS205 phage derivatives (2) has been described previously (5, 6). The region of miniplasmid DNA between the *Eco*RI site in the *cat* gene and the first counterclockwise *Sal*I site (Fig. 1) was inserted into λRS205 to place β-galactosidase synthesis under the control of the rightward NR1 replication transcripts (Fig. 2).

Culture media. L broth (10) was used for outgrowth after transformation. 1xA medium (21) at 30°C was used for the labeling of RNA and β-galactosidase measurements. Antibiotics were included, when appropriate, to select for cells carrying plasmids: tetracycline hydrochloride, 5 μg/ml; sodium ampicillin, 25 μg/ml; chloramphenicol, 20 μg/ml. Growth was monitored by turbidity at 600 nm with a Gilford model 260 spectrophotometer.

Isolation and in vitro manipulation of plasmid and phage DNA. 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 (19, 20). Restriction fragments from NR1 or its derivatives (Fig. 1) were purified by agarose gel electrophoresis (16) and then ligated to plasmid pBR322 (3) or plasmid pUC8 (31) which had been digested with an appropriate restriction enzyme. The orientations of the inserted DNA were determined by restriction analysis (data

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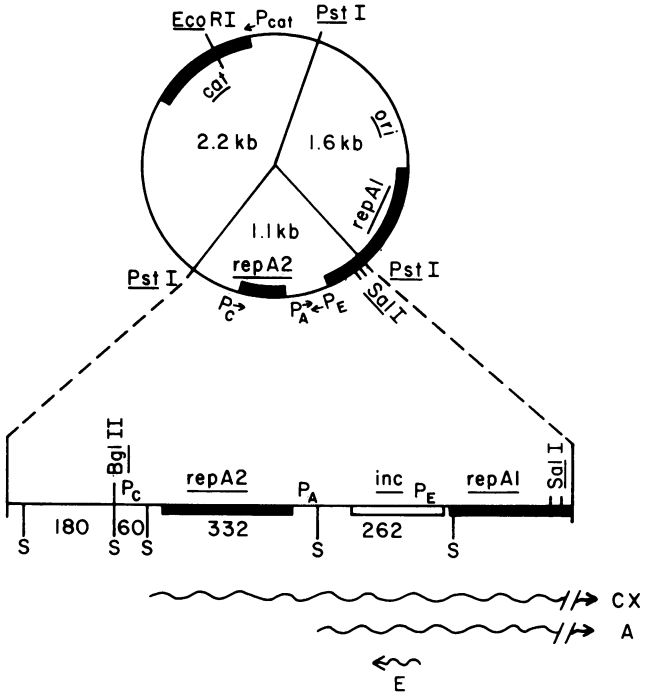

FIG. 1. Structure of NR1 minireplicator plasmids composed of three *Pst*I restriction fragments: the 2.2-kb fragment encoding chloramphenicol acetyltransferase (*cat*), the 1.1-kb fragment encoding incompatibility (*inc*), and the 1.6-kb fragment containing the origin of replication (*ori*). The protein coding regions for *cat*, *repA2*, and *repA1* are indicated by the heavy lines. Sites for cleavage by *Sau*3A restriction endonuclease in the 1.1-kb *PstI inc* fragment are indicated by S, and the sizes of the *Sau*3A restriction fragments are shown below the line in bp. The promoter for RNA-CX transcription (p_C) is in the 60-bp *Sau*3A fragment, the promoter for RNA-A transcription (p_A) is at the right end of the 332-bp *Sau*3A fragment, and the promoter for RNA-E transcription (p_E) is in the 262-bp *Sau*3A fragment. The transcripts are indicated by the wavy arrows below the map. The protein coding sequence of *repA2* is within the 332-bp *Sau*3A fragment, whereas the protein coding sequence for *repA1* begins in the 262-bp *Sau*3A fragment and extends into the 1.6-kb *Pst*I fragment, ending near the origin of replication. A third open reading frame, *repA3*, is indicated by the open box in the 262-bp *Sau*3A fragment.

not shown). Deletion mutant pDXRR14 was produced by *Bgl*II digestion of pDXRR12 (Table 1; 33), followed by treatment with BAL 31 exonuclease. Blunt-end ligation of this DNA, followed by transformation and selection for ampicillin resistance, resulted in a series of plasmids with different-size deletions. pDXRR14 had lost ca. 200 base pairs (bp) of DNA (data not shown), including the RNA-CX promoter and the amino-terminal coding region of *repA2* (Fig. 2).

β-Galactosidase enzyme assay. The β-galactosidase activity of exponential phase cultures of the λRS205 lysogens was assayed by a modification of the method of Miller (21) as described previously (5). A lysogen with λRS205 produced 39 ± 7 U of β-galactosidase activity, and this was substracted as background from the activities of the other lysogens. Steady-state induction of *lac* transcription by different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) was performed as described by Miller (21).

Plasmid copy number measurements. Relative plasmid copy numbers were estimated by ultracentrifugation of bacterial DNA from exponential-phase cultures in ethidium bromide-cesium chloride density gradients (33) or from gene dosage effects by measuring the chloramphenicol acetyltransferase enzyme specific activity in cell extracts prepared from exponential-phase cultures of cells harboring *cat*$^+$ plasmids (5, 28).

RNA labeling and hybridization. Exponential-phase cultures were pulse-labeled with 5-[^{3}H]uridine, and RNA was extracted as previously described (34). The RNA was quantitatively hybridized to single-stranded DNA fixed to nitrocellulose filters as previously described (34). Single-stranded DNA probes contained the *Sau*3A fragments from the NR1 replication control region (Fig. 1) cloned into phage M13mp10 (18) in either orientation relative to the viral strand. This allowed strand-specific hybridization of the RNA transcripts, as described in detail elsewhere (D. D. Womble, P. Sampathkumar, A. M. Easton, V. A. Luckow, and R. H. Rownd, J. Mol. Biol., in press). The filters were treated with ribonuclease before measuring the bound counts per minute, and the amount bound was normalized to the extent of homology to the restriction fragment in the probe (either 332 or 262 bases). The transcription rates were then normalized to the rate of 5′ RNA-CX transcription in the λ1910 lysogen.

RESULTS

Repression of RNA-A transcription by the *repA2* protein. To examine transcription from the *repA1* promoters, p_C and p_A, *lac* fusions were constructed with the cloning vector λRS205 and various portions of NR1 plasmid DNA (Fig. 2). Rightward transcription initiated at p_C or p_A, or both, in the NR1 portion continues through the *lacZ* region of the λRS205 portion. The amount of rightward transcription can be estimated as β-galactosidase enzyme activity in lysogens which contain single copies of these recombinant prophages (2, 5). λ1910 contains the wild-type NR1 regulatory components (Table 1, Fig. 2), whereas λ1909 has deleted the 60-bp *Sau*3A fragment which contains the RNA-CX promoter and λ1911 has deleted both the 60- and 332-bp *Sau*3A fragments which contain the RNA-CX and RNA-A promoters, respectively. λDXRR12 contains a 100-fold down mutation in p_E. The only active NR1 transcription promoter in λDXRR14 is p_A, owing to both the down mutation in p_E and the deletion of p_C (Fig. 2).

To vary the amount of *repA2* repressor protein in *trans*, the 332-bp *Sau*3A fragment from NR1 (Fig. 1) was cloned into the high-copy-number vector pUC8, a derivative of pBR322 (31). Transcription of the cloned *repA2* gene in the resulting plasmid, pVLRR7, is under the control of the *lac* promoter-operator of the vector.

The vector plasmid pBR322, which by itself has no significant effect on transcription from the NR1 promoters (5, 34), was introduced into each of the lysogens as a control. The λ1910 lysogen produced 163 U of β-galactosidase as a result of transcription from p_C and p_A (Table 1). The β-galactosidase activity in the λ1909 lysogen was twofold higher, presumably owing to fully derepressed transcription from the RNA-A promoter in the absence of *repA2* repressor protein. Only a background level of β-galactosidase activity was produced by the λ1911 lysogen. In the λDXRR12 lysogen, which has no RNA-E synthesis, the β-galactosidase activity also was stimulated compared with the λ1910 lysogen. The β-galactosidase activity in the λDXRR14 lysogen, which has neither *repA2* nor RNA-E, was stimulated even further (Table 1).

The effects of excess *repA2* protein were examined by introduction of plasmid pVLRR7 into each of the lysogens

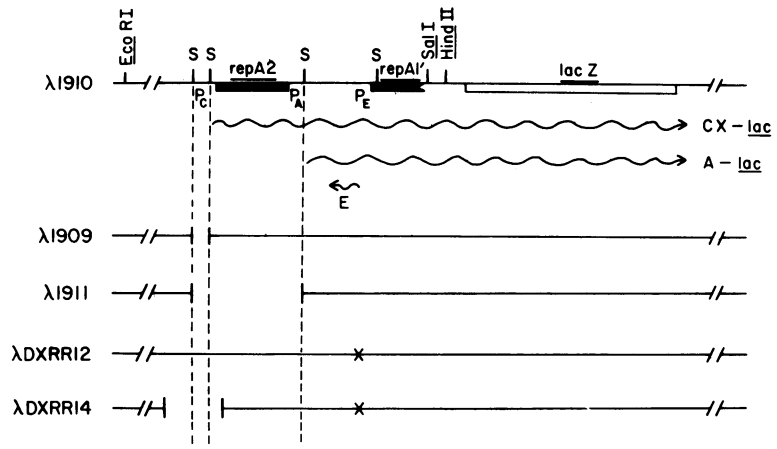

FIG. 2. Structures of the recombinant λRS205 prophage derivatives. The genes, sites, and transcripts are as described in the legend to Fig. 1. The prophages are named after the source of the inserted NR1 DNA, such as λ1910 from pRR1910 (Table 1 in reference 33). The gaps in the lower structures represent deletions, whereas the X's in the λDXRR12 and λDXRR14 structures represent the down mutation in p_E.

(Table 1). The excess *repA2* protein from the cloned *repA2* gene in pVLRR7 reduced the β-galactosidase activity in all of the lysogens which transcribed *lacZ* from p_A. β-Galactosidase was not reduced in the lysogen with λ *lacP*$^+$, in which the wild-type *lac* promoter was inserted into λRS205 (2, 5). In the lysogens whose only source of *lac* mRNA was transcription from the RNA-A promoter (λ1909 and λDXRR14), the excess *repA2* protein reduced the β-galactosidase activity more than 20-fold, essentially to the background level (Table 1). In the lysogens with both the RNA-CX and RNA-A promoters (λ1910 and λDXRR12), the excess *repA2* protein reduced the β-galactosidase activities only two- to threefold, and the remaining activity was significantly higher than background (Table 1). Three conclusions about the regulation of transcription of *repA1* mRNA can be drawn from these experiments: (i) excess *repA2* protein can repress nearly all transcription from p_A; (ii) when transcription from p_A is repressed, constitutive RNA-CX synthesis can still provide a basal level of rightward mRNA; and (iii) the indigenous amount of *repA2* protein synthesized in the λ1910 and λDXRR12 lysogens is insufficient to repress completely the RNA-A promoter. Because each λ-*lac* prophage was integrated as a single copy into the chromosome, these lysogens could be considered to have a gene dosage of *repA2* of approximately one.

Effects of varying the *trans repA2* protein concentration. Transcription of the *repA2* gene in pVLRR7 is under the control of the *lac* promoter-operator of the pUC8 vector. The transcription from the *lac* promoter-operator can be repressed by the *lac* repressor, provided by the *lacI*^{Q} gene on plasmid pVLRR10 (Fig. 3). When pVLRR7 and pVLRR10 are present in the same cell, the amount of *repA2* protein synthesized can be varied by the addition of different concentrations of the inducer IPTG. This system can be used to vary the amount of *trans repA2* protein in the lysogens with the recombinant λ-*lac* prophages (Fig. 3A).

The scheme illustrated in Fig. 3B is similar, except that the λ *lacP*$^+$ prophage contains the wild-type *lac* promoter-operator. There are many copies of the *lac* promoter-operator in the lysogen with λ *lacP*$^+$ and pVLRR7, but only the one in the prophage provides mRNA for β-galactosidase. The amount of β-galactosidase synthesized in this lysogen reflects the amount of transcription from a *lac* promoter in the cell. Therefore, it can be considered to reflect the average amount of *repA2* mRNA synthesized from transcription initiated at the majority of the *lac* promoters in the cell, which are located on the pVLRR7 plasmid. This can be used to calibrate the relative amount of *repA2* protein synthesized in these lysogens at the various levels of IPTG inducer.

The results of varying the IPTG concentration on β-galactosidase activity in the λDXRR14 and the λ *lacP*$^+$ lysogens are shown in Fig. 4. As the concentration of IPTG increased, increasing the amount of *repA2* protein in the cell, the amount of β-galactosidase resulting from RNA-A transcription in the λDXRR14 lysogen decreased (Fig. 4A). In contrast, the amount of β-galactosidase in the λ *lacP*$^+$ lysogen increased with an increasing concentration of IPTG (Fig. 4B), reflecting the average gene expression from the *lac* promoters in these lysogens at each level of IPTG. Results essentially identical to those in Fig. 4A were obtained when IPTG was varied in the λ1909 lysogen harboring pVLRR7 and pVLRR10 (data not shown), in which *lacZ* transcription also is initiated solely from p_A (Fig. 2). Similar results also were obtained with the λ1910 and λDXRR12 lysogens, except that the minimum β-galactosidase activity at the highest IPTG concentrations was significantly higher than background, owing to the basal level of RNA-CX transcription (data not shown).

The relationship between the *repA2* protein concentration in the cell and the amount of transcription from the RNA-A promoter can be estimated from the data in Fig. 4 with two assumptions. The first assumption is that the amount of *repA2* protein synthesized at any given concentration of

TABLE 1. Effects of additional *repA2* protein on β-galactosidase synthesis in strains lysogenic for λ-*lac* fusion phages

Prophage	Control elements coded by prophage				U of β-galactosidase in lysogens harboring the indicated plasmid	
	repA2	RNA-CX	RNA-A	RNA-E	pBR322	pVLRR7 (*repA2*$^+$)
λ1910	+	+	+	+	163 ± 8	58 ± 12
λ1909	–	–	+	+	377 ± 106	0 ± 3
λ1911	–	–	–	+	0 ± 7	0 ± 13
λDXRR12	+	+	+	–	276 ± 29	141 ± 35
λDXRR14	–	–	+	–	593 ± 130	29 ± 29
λ *lacP*$^+$	–	–	–	–	662	647

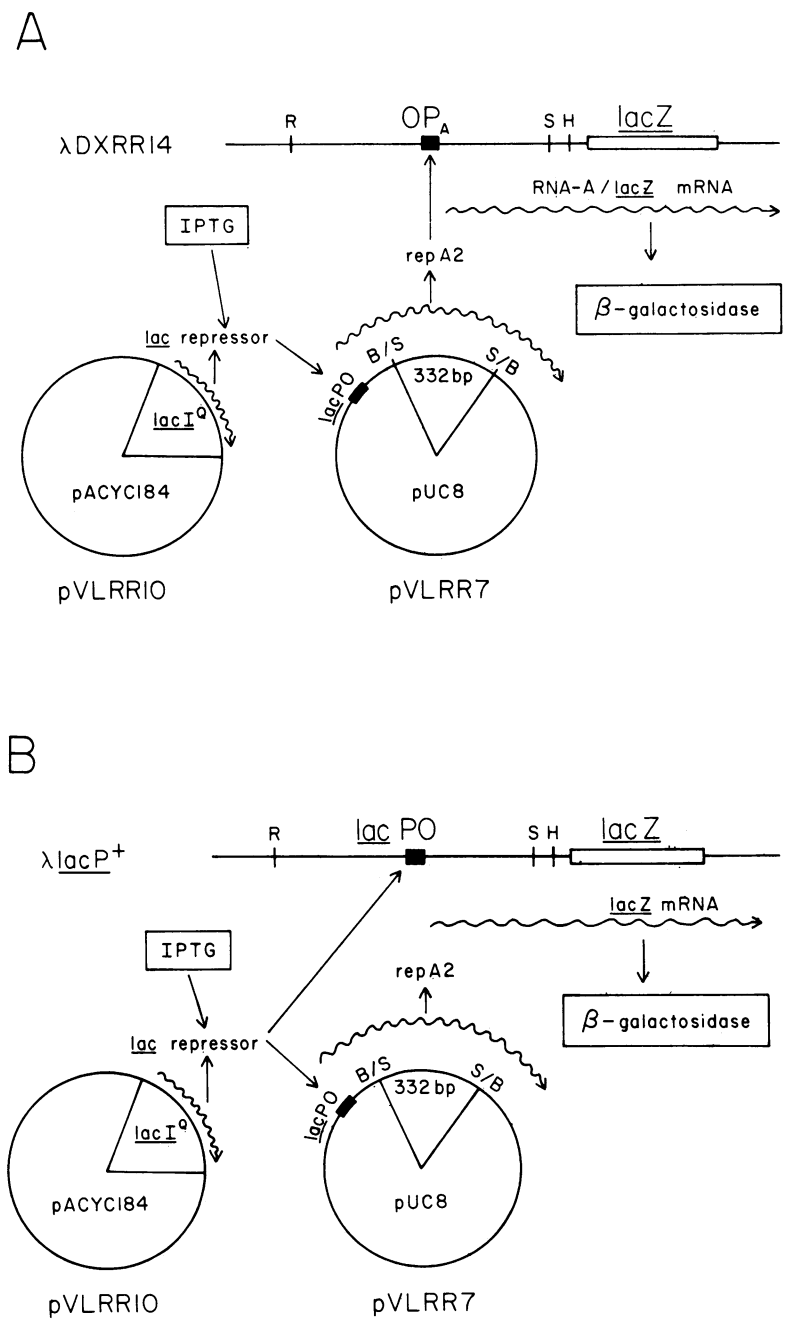

FIG. 3. Scheme for varying the level of *repA2* protein in the lysogens. (A) The only active NR1 promoter in the λDXRR14 prophage is p_A. Transcription from p_A proceeds through the *lacZ* coding region of the prophage, resulting in synthesis of β-galactosidase. Transcription from the *lac* promoter-operator of the vector plasmid pUC8 proceeds through the cloned 332-bp *Sau*3A fragment in pVLRR7, resulting in synthesis of the *repA2* repressor protein. The *repA2* protein represses transcription from p_A by binding to the operator, O, resulting in less synthesis of β-galactosidase. The amount of *repA2* protein synthesized is regulated by the *lac* repressor protein, encoded by plasmid pVLRR10, and the chemical inducer IPTG. Variation of the concentration of IPTG in the medium results in different levels of *repA2* protein synthesis, the effects of which are monitored as β-galactosidase activity. (B) Scheme for estimating the level of *repA2* protein synthesis as a function of IPTG concentration. β-Galactosidase is synthesized as a result of transcription from the *lac* promoter-operator in the prophage. Transcription initiated at the majority of *lac* promoter-operators in the cell, on plasmid pVLRR7, results in synthesis of *repA2* protein, as in A. The restriction sites indicated are for *Eco*RI (R), *Sal*I (S), *Hin*dIII (H), and *Bam*HI-*Sau*3A fusions (B/S, S/B).

IPTG is directly proportional to the β-galactosidase activity in the λ *lacP*$^+$ lysogen (Fig. 4B), since both are transcribed from the *lac* promoter. The second assumption is that the maximum level of β-galactosidase activity in the λDXRR14 lysogen, without the addition of IPTG, reflects the fully derepressed level of transcription from the RNA-A promoter in the absence of *repA2* repressor protein. From this maximum, the extent of repression of the RNA-A promoters at any given concentration of IPTG can be calculated. For example, the extent of repression of the RNA-A promoter of

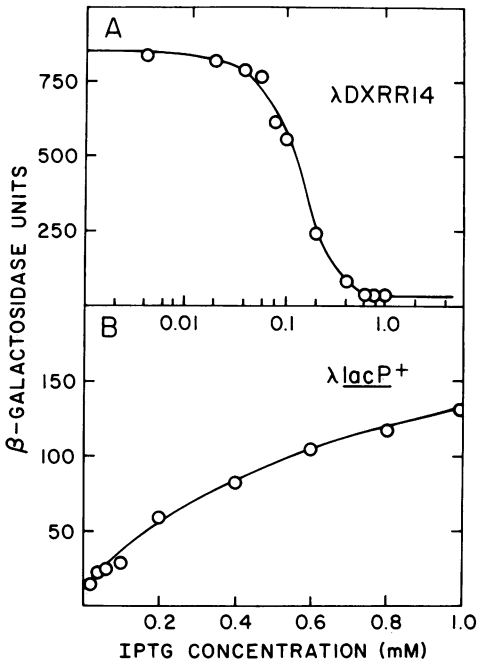

FIG. 4. Variation of β-galactosidase activity as a function of IPTG inducer concentration. The schemes for these experiments are shown in Fig. 3.

λDXRR14 at 0.1 mM IPTG was 0.38. At 0.1 mM IPTG, the β-galactosidase activity of the λ *lacP*$^+$ lysogen was 29 U (Fig. 4B), corresponding to 29 U of *repA2* protein from pVLRR7. The data from the λDXRR14 lysogen (Fig. 4A) and also from the λ1909 lysogen (data not shown) were converted to the fraction repressed and plotted in Fig. 5 against the *repA2* repressor concentration given in units of β-galactosidase, which were estimated from the λ *lacP*$^+$ lysogen at each level of IPTG (Fig. 4B). Normalized to the maximum level of expression in each lysogen, the results for λDXRR14 and λ1909 both fell approximately on the same repression curve (Fig. 5). The sigmoidal shape of this curve suggested cooperativity in the repression of the RNA-A promoter by the *repA2* repressor. Hill plot analysis (30) of these data resulted in a slope of ca. 3 (Fig. 6), which suggests that there may be at least three subunits involved.

The relationship of the repression curve in Fig. 5 to the wild-type levels of *repA2* protein produced from RNA-CX transcription could be determined by a comparison with the data in Table 1. Because the lysogens contain only a single copy of the λ-*lac* prophages, the effects of one dose of *repA2* protein could be estimated by comparing the β-galactosidase activity in the λ1910 lysogen with that in the λ1909 lysogen or by comparing the β-galactosidase activity in the λDXRR12 lysogen with that in the λDXRR14 lysogen. The contribution to the total β-galactosidase activity from RNA-CX transcription in the λ1910 and λDXRR12 lysogens was estimated from the β-galactosidase levels in the presence of excess *repA2* protein provided by pVLRR7 (Table 1). The remainder of the activity without excess *repA2* repressor reflects the contribution from RNA-A transcription. For example, the total β-galactosidase activity in the λDXRR12 lysogen was 276 U (Table 1). The contribution from RNA-CX was 141 U (Table 1), giving a value of 135 U of β-galactosidase from RNA-A, at one dose of *repA2* protein in the λDXRR12 lysogen. The maximum value for β-galactosidase from fully

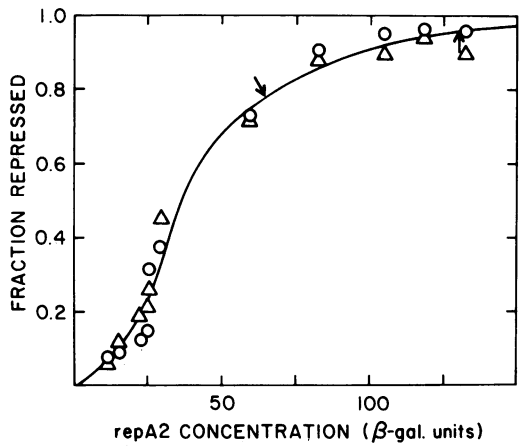

FIG. 5. Repression curve of the RNA-A promoter versus the level of *repA2* repressor protein. The data for the λDXRR14 lysogen (○) are from Fig. 4, whereas the data from the λ1909 lysogen (△) are from a similar experiment. The *repA2* repressor concentration at each level of IPTG inducer was calculated from Fig. 4B. The fraction repressed was calculated from the maximum level and the observed levels of β-galactosidase activity at each level of IPTG inducer. The fraction repressed equals (maximum − observed)/maximum. The arrows indicate the positions equivalent to one dose and two doses of *repA2* repressor.

derepressed RNA-A transcription in the λDXRR14 lysogen under the same conditions was 593 U (Table 1). The extent of repression of RNA-A promoters by one dose of *repA2* protein in the λDXRR12 lysogen was therefore 0.77.

In Fig. 5, the fraction of RNA-A promoters repressed equals 0.77 when the *repA2* repressor level is 64.5 U of β-galactosidase, and therefore this level is equivalent to one dose of *repA2* protein. The wild-type copy number of NR1 is ca. two per chromosome (25, 35). Therefore, at the normal copy number of NR1 there will be two doses of *repA2* protein. This corresponds to 129 U of β-galactosidase in Fig.

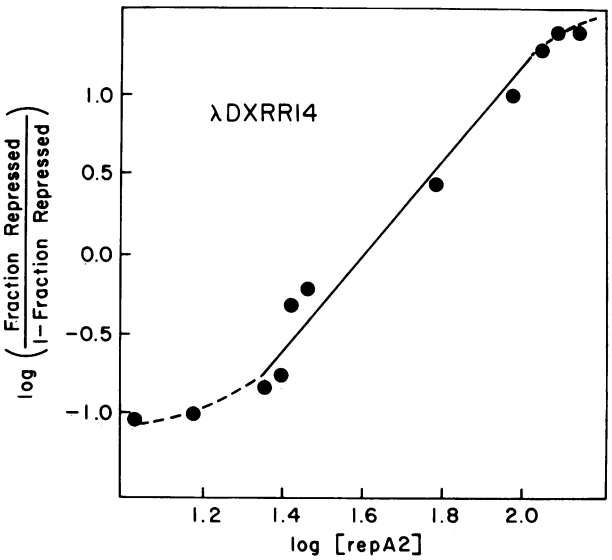

FIG. 6. Hill plot analysis of the repressor-operator binding data. The λDXRR14 case shown here was obtained by replotting the points from Fig. 5. The slope in the central portion of the graph is ca. 3. A similar plot was obtained for the case with λ1909 (not shown).

TABLE 2. Transcription rates versus copy number: gene dosage effects

Template[a]	Relative copy no.	Rightward promoters		Relative transcription rate[b]		(RNA-CX + RNA-A)/ RNA-CX
		p_C	p_A	RNA-CX	RNA-CX + RNA-A	
λ1910	1	+	+	1.0	2.6	2.6
pWFRR2	1.5	+	+	1.6	2.7	1.7
pRR933	2	+	+	1.9	1.4	0.76
pRR942	8	+	+	6.6	4.2	0.64
pRR935	40	+	+	33	20	0.60
λ1909	1	−	+	0.0	3.5	
pRR5406	4	−	+	0.0	17	

[a] λ1910 and λ1909 are λRS205 prophage derivatives; pWFRR2, pRR933, pRR942, and pRR5406 are autonomous minireplicator plasmids; and pRR935 is a pBR322 clone containing the NR1 replication control region.

[b] The transcription rates were determined by quantitative hybridization of pulse-labeled RNA to single-stranded DNA probes. The rate of RNA-CX transcription was determined using the plus strand of 332-bp *Sau*3A fragment, whereas the combined total of RNA-CX plus RNA-A was determined by using the plus strand of the 262-bp *Sau*3A fragment.

5, which gives a fraction repressed of 0.96. Similarly, it can be calculated that at one-half dose of *repA2* protein, the fraction repressed equals 0.40, and so on.

Transcription rates as a function of plasmid copy number. Derivatives of NR1 with a variety of different copy numbers were used to measure the rates of in vivo transcription from the NR1 replication control region (Table 2). The plasmid and prophage derivatives are described in detail in reference 33. Plasmid pRR933 is a minireplicator plasmid derived from wild-type NR1 and has a wild-type copy number. In the cells with λ1909 or pRR5406, owing to the lack of an active p_C, there is no RNA-CX transcription, and therefore no *repA2* repressor is synthesized. The transcription of RNA-A from λ1909 and pRR5406 is therefore fully derepressed. The other templates in Table 2 all have intact RNA-CX promoters, and they synthesized both RNA-CX and *repA2* repressor.

The in vivo transcription rates for RNA-CX and RNA-A were measured by quantitative hybridization methods (Table 2). Hybridization to the plus strand of the 332-bp *Sau*3A fragment (Fig. 1) was used to measure synthesis of the 5′ end of RNA-CX alone, whereas hybridization to the plus strand of the 262-bp *Sau*3A fragment (Fig. 1) measured synthesis of the combined total of RNA-CX plus RNA-A. A greater amount of hybridization to the 262-bp plus probe reflected RNA-A synthesis. RNA-CX transcription, measured by the 332-bp plus hybridization probe, was constitutive and approximately proportional to gene dosage from templates with an intact RNA-CX promoter (Table 2). For λ1909 and pRR5406, derepressed RNA-A transcription also was proportional to gene dosage (Table 2). RNA-A transcription was repressed at the higher copy numbers from the templates which synthesized *repA2* protein, resulting in a reduction in the ratio of hybridization to the two probes (Table 2). This ratio was ca. 0.6 at the highest copy numbers. The dose of *repA2* at the highest copy numbers is sufficient to fully repress the RNA-A promoter (Table 1, Fig. 4), and therefore the ratio 0.6 should reflect the basal level of RNA-CX transcription through the 262-bp *Sau*3A fragment when the RNA-A promoter is repressed. With this ratio, the amount of transcription resulting from RNA-A synthesis can be calculated at any copy number by subtracting the value of RNA-CX from the total. For example, at the wild-type copy number of pRR933, the rate of RNA-CX transcription through the 262-bp fragment is estimated as: $1.9 \times 0.6 = 1.1$. The rate of RNA-A transcription is then given by the difference: $1.4 - 1.1 = 0.3$, or 0.15 per plasmid copy. This represents only ca. 4% of the maximum rate per copy estimated from the fully derepressed value for λ1909 (Table 2). This suggests that the extent of repression of RNA-A promoters at the normal NR1 copy number is ca. 0.96, in good agreement with the value calculated from the β-galactosidase data (Fig. 5).

DISCUSSION

Replication of IncFII plasmids such as NR1 is controlled by regulating the synthesis of the *repA1* initiation protein (5, 13, 26). This is regulated by limiting both the transcription and translation of *repA1* mRNA (5, 34). Transcription is regulated by the *repA2* repressor protein (12, 15), whereas translation is regulated by the incompatibility inhibitor, RNA-E (13, 34).

There are two transcription promoters for synthesis of *repA1* mRNA, producing RNA-CX and RNA-A (Fig. 1) (6, 26). The 5′ end of RNA-CX also is the mRNA for *repA2*. RNA-CX transcription is constitutive at a low level (Table 2), and therefore synthesis of *repA2* protein is gene dosage dependent (11). An excess of *repA2* protein can completely repress transcription from the RNA-A promoter (Table 1). The data from Fig. 4 and 5 and Table 2 suggest that at the wild-type level of *repA2* protein in the cell, the RNA-A promoter is ca. 96% repressed. When RNA-A synthesis is repressed, RNA-CX provides the basal level of *repA1* mRNA (Table 1). At or above the normal copy number of NR1, RNA-CX is the primary source of *repA1* mRNA. Under these conditions, the inhibition of *repA1* mRNA translation by the interaction with RNA-E is the primary means of regulating NR1 replication (26). An excess of *repA2* protein has very little effect on copy number or incompatibility of the minireplicator plasmids which synthesize RNA-CX, such as pRR933 or pRR942 (26, 33). This is consistent with the idea that RNA-A transcription is already repressed by the dose of *repA2* protein provided by the normal plasmid copy number. In contrast, minireplicator plasmid pRR5406 is incompatible with any plasmid which synthesizes *repA2* protein (33). The only source of *repA1* mRNA for pRR5406 is RNA-A (Table 2), and therefore its replication is prevented when RNA-A synthesis is repressed by *repA2* protein.

When the copy number of NR1 falls below the wild-type level, transcription of RNA-A is derepressed (Fig. 5, Table 2). The RNA-A promoter is about sixfold stronger than the RNA-CX promoter, so that derepression of RNA-A transcription dramatically increases the total amount of *repA1* mRNA synthesis (Tables 1 and 2). The repression curve in Fig. 5 is sigmoidal, which suggests that there is a cooperativity factor in the interaction between *repA2* protein and the operator at the RNA-A promoter (p_A). Unlike the case for the cooperative binding of lambda phage repressor and operator (1, 9), there are no obvious multiple binding sites in the nucleotide sequence of p_A (24). This suggests that the cooperativity could result from protein-protein interactions, as in the assembly of protein subunits into a multimer. Hill plot analysis of these data results in a slope of ca. 3 (Fig. 6). Because the slope obtained in a Hill plot frequently underestimates the number of subunits (30), we suggest that the active form of the *repA2* repressor may be a tetramer.

The closely related IncFII plasmid R1 has an analogous transcription repressor protein called *copB* (12, 22). Although the carboxy-terminal portion of the *copB* protein differs from that of the *repA2* repressor (27), the transcrip-

tion promoter (p_C) and amino-terminal portion of the *copB* and *repA2* genes are identical at the nucleotide level (27). It therefore seems reasonable to assume that the two proteins will be expressed at similar levels in the cell. Light and Molin (11) have estimated that there are ca. 2,000 *copB* proteins synthesized per cell from an R1 plasmid with a wild-type copy number.

At 2,000 monomers per cell, the total concentration of NR1 *repA2* protein in the cell would be ca. 10-fold higher than for lambda repressor in a lysogen (200 monomers per cell) (9) and 50-fold higher than for *lac* repressor in *lac*$^+$ *E. coli* (40 monomers per cell) (7, 9). The volume within an average *E. coli* cell is 1.6×10^{-15} liter (32), and therefore the wild-type *repA2* protein concentration will be ca. 2.1 μM. For *repA2* protein to be in equilibrium between monomers and active multimers at that concentration, the protein-protein interaction would have to be much weaker than that for *lac* repressor protein, all of which is found as active tetramers in vivo (7). However, under in vivo conditions, the lambda repressor protein is in equilibrium between monomers and active dimers (1, 9). At the wild-type NR1 copy number, the concentration of RNA-A operators in the cell will be about twice that of the *lac* operator or the lambda operator in a lysogen (1, 9). If the RNA-A promoter is 96% repressed under in vivo conditions, then the interaction of *repA2* repressor and operator must be fairly weak in comparison to the *lac* system (14). The best fit to the points in Fig. 5 is given by an active tetramer in equilibrium with inactive monomers and with a weak repressor-operator dissociation constant similar to that for the lambda repressor-operator binding (1, 9).

The combination of a high total concentration of monomers in the cell, a cooperative equilibrium between monomers and active multimers, and a weak binding of active repressor to the operator would result in the dramatic derepression of the RNA-A promoter when the plasmid copy number (and *repA2* repressor gene dosage) is decreased by only a factor of two below the wild-type level (Fig. 5, Table 2). The resulting increase in the synthesis of *repA1* mRNA would increase the probability of plasmid DNA replication under conditions where, by random fluctuation, the copy number had drifted to a below-normal level. This mechanism probably sets the minimum plasmid copy number in the cell, which is very important for a low-copy-number plasmid such as NR1. Low-copy-number IncFII plasmids are inherited very stably in a cell population even in the absence of selection (20, 23), so that there are never fewer than two plasmids present at the time of cell division. It seems likely that the derepression of RNA-A transcription at a below-normal copy number may help to ensure that that is the case.

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