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Posttranscriptional Control of Plasmid ColIb-P9 *repZ* Gene Expression by a Small RNA

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The replication frequency of plasmid ColIb-P9 depends on the level of *repZ* gene expression, which is negatively regulated by the action of the *inc* gene (C. Hama, T. Takizawa, H. Moriwaki, Y. Urasaki, and K. Mizobuchi, J. Bacteriol. 172:1983–1991, 1990). To further understand the mechanism of this regulation, we analyzed transcripts of the ColIb-P9 replication control region. Four RNA species, designated RNAI to RNAIV, were observed in plasmid pCH11, which contained the whole *inc* gene region and the 5′ portion of the *repZ* gene. RNAII, RNAIII, and RNAIV, with sizes of approximately 200, 500, and 1,500 bases, respectively, were identified as rightward transcripts that shared common transcription initiation sites; RNAIV was determined to be equivalent to a part of *repZ* mRNA, which was observed in pCH10, a plasmid that contained sufficient information for replication and control of ColIb-P9. Conversely, RNAI, with a size of about 70 bases, was transcribed leftward and was identified as the product of the *inc* gene and hence equivalent to *inc* RNA detected by in vitro RNA synthesis. This small RNA was found to be complementary to a part of *repZ* mRNA. These results and quantitative analyses of the transcripts in Inc$^-$ mutants indicate that the *inc* RNA negatively regulates *repZ* expression mainly at the posttranscriptional level through the possible formation of an *inc* RNA-*repZ* mRNA hybrid in the host cells.

In the accompanying paper, we have reported that a 1,845-base-pair (bp) sequence is sufficient for the autonomous replication and control of the 93-kilobase (kb) plasmid ColIb-P9 (6).The sequence has been shown to contain at least two structural genes, designated *repZ* and *inc*. The *repZ* gene encodes a protein with a molecular weight of 39,000, which is essential for ColIb-P9 replication, probably functioning as a replication initiator. The *inc* gene, mapped in the upstream region of *repZ*, phenotypically governs incompatibility and encodes a small RNA (*inc* RNA) with a size of about 70 bases, which acts in *trans* to regulate ColIb-P9 replication by repressing *repZ* gene expression. In addition, characterization of *inc1* and *inc2* mutants suggested that the mutations occurred in a possible promoter region of the *inc* gene.

To further understand the regulatory mechanism(s) of *repZ* gene expression, we have undertaken a study to analyze transcripts of the replication control region of ColIb-P9. In this report, we describe the identification of *inc* and *repZ* RNAs in vivo. *inc* RNA is complementary to a part of *repZ* mRNA. Quantitative analyses of *repZ* mRNA in Inc$^-$ mutants indicate that the negative control of *repZ* gene expression by *inc* RNA appears to proceed mainly at the posttranscriptional level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *Escherichia coli* strains used were W3110 (λ *ind*) (prototroph) and MC1061 [*araD139* Δ(*ara-leu*) *7697 lacX74 galU galK hsdR rpsL*] (3). Seven plasmids, pCH10, pCH11, pCH11-I1 (*inc1*), pCH11-I2 (*inc2*), pCH140, pCH140-I1 (*inc1*), and pCH140-I2 (*inc2*), are described in the accompanying paper (6). These plasmids carried the 1,120-bp *Eco*RI-*Sal*I or 1,118-bp *Eco*RI-*Hinc*II fragment of ColIb-P9 including the intact *inc* gene region and only the 5′ portion of *repZ*. Plasmids pCH140 and its Inc$^-$ derivatives, which contained the translational *repZ-lacZ* fusions, were used for quantitative analysis of *repZ* mRNA synthesis. Plasmids pDX13 and pDX14 were constructed by cloning the 1,120-bp *Eco*RI-*Sal*I fragment of pCH11 into the *Eco*RI-*Sal*I sites of vectors pTZ18R and pTZ19R (13), respectively, and used to prepare strand-specific single-stranded DNA by infecting helper bacteriophage M13KO7 as described previously (19). LB broth (10) and M9 minimal medium (14) containing 1 μg of thiamine per ml and 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.) were used for the growth of bacteria. Ampicillin (100 μg/ml) was added to maintain plasmids.

Enzymes, radioactive materials, and oligodeoxyribonucleotides. All enzymes used were obtained from Takara Shuzo (Kyoto, Japan). Reactions for restriction enzymes were carried out as specified by the manufacturer. [α-^{32}P]dCTP (400 Ci/mmol) and [γ-^{32}P]ATP (6,000 Ci/mmol) were supplied by Amersham Corp. (Arlington Heights, Ill.) and Dupont, NEN Research Products (Boston, Mass.), respectively. Four 17-base oligodeoxyribonucleotides (oligo-1, 5′ GAACTTGGCGGAAAGAC3′; oligo-2, 5′CTTCAGTCTTC TGACTT5′; oligo-3, 5′GACTCCAGTGTACTGCG3′; and oligo-4, 5′TGCGCAGGCTCTGCTCG3′) were chemically synthesized and used after purification by Sephadex G-25 DNA-grade columns (Pharmacia, Uppsala, Sweden) or OPC cartridges (Applied Biosystems, Inc., Foster City, Calif.). These oligodeoxyribonucleotides corresponded to nucleotides 321 to 337, 275 to 259, 500 to 484, and 746 to 730 for oligo-1, -2, -3, and -4, respectively (6).

Isolation of cellular RNA. Total cellular RNA was isolated by the method of Aiba et al. (1), with a slight modification. Cells harboring plasmids were grown either in LB [for W3110 (λ *ind*)(pCH10) or pCH11 derivatives] or M9 minimal medium [for MC1061 (pCH140) derivatives] at 37°C to an optical density at 650 nm of 0.35 to 0.45, harvested by centrifugation, and suspended in a 1/20 volume of lysis buffer (0.5% sodium dodecyl sulfate [SDS], 1 mM disodium EDTA,

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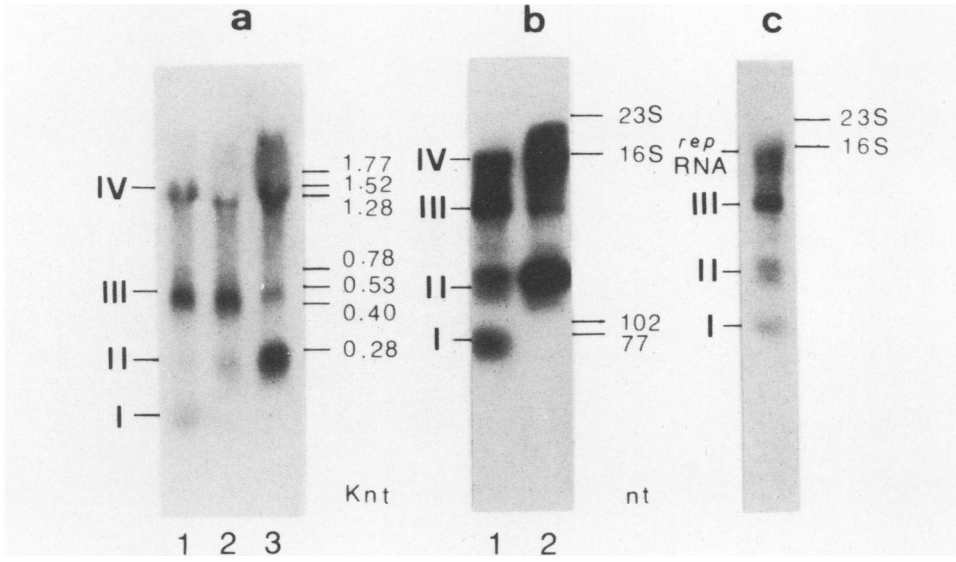

FIG. 1. Northern blot analysis of transcripts of the ColIb-P9 replication control region. (a) Autoradiograms of a 1% agarose gel of RNAs. Total cellular RNA (20 μg) isolated from cells harboring plasmids pCH11 (*inc*$^+$) (lane 1), pCH11-I2 (*inc2*) (lane 2), and pCH11-I1 (*inc1*) (lane 3) was electrophoresed on a 1% agarose gel, transferred onto a nylon membrane, and hybridized with a [α-^{32}P]dCTP-labeled 1,120-base *Eco*RI-*Sal*I fragment from pCH11. Positions of four major transcripts are indicated by I (RNAI), II (RNAII), III (RNAIII), and IV (RNAIV). As size markers, a 0.16- to 1.77-kb RNA ladder (Bethesda Research Laboratories) was used. (b) Separation of ColIb-P9 RNAs on a 3% agarose gel. Cellular RNA (10 μg) from cells harboring pCH11 (lane 1) and pCH11-I1 (lane 2) was fractionated on a 3% agarose gel and was hybridized to estimate the size of RNAI with the same probe used for panel a. As molecular markers, denatured 102-base *Hin*fI and 77-base *Hap*II fragments of pCH11 and *E. coli* 16S and 23S rRNAs were used. (c) Gel electrophoretic pattern of RNAs of pCH10. Cellular RNA (20 μg) isolated from cells carrying pCH10 was analyzed by the Northern blot method, using the same probe as used for panel a. The band located in the gel top has a size of about 1,400 bases and is designated *rep* RNA.

20 mM sodium acetate [pH 4.8]). Total cellular RNA was extracted twice with phenol at 60°C for 5 min, precipitated three times with ethanol, and finally suspended in a small volume of H_2O treated with 0.1% diethylpyrocarbonate.

Northern (RNA) blot analysis of RNA. After denaturation in 50% deionized formamide–2.2 M formaldehyde–0.5× MOPS (9) at 65°C for 5 min, the cellular RNA was fractionated by electrophoresis on either a 1 or 3% agarose gel containing 2.2 formamide in 1× MOPS and then transferred onto a nylon membrane (GeneScreen Plus; Dupont NEN) with 20× SSPE (1× SSPE is 0.18 M sodium chloride, 10 mM sodium phosphate [pH 7.4], and 1 mM disodium EDTA). The blotted membrane was baked in vacuo at 80°C for 2 h. In some experiments, the gel was soaked with 50 mM NaOH for 15 min to ensure the transfer of large RNA molecules. RNA was detected by RNA-DNA hybridization, using either a [α-^{32}P]dCTP-labeled 1,120-bp *Eco*RI-*Sal*I fragment or [γ-^{32}P]ATP-end-labeled oligodeoxyribonucleotides as probes. The [α-^{32}P]dCTP-labeled DNA probe was prepared by the random priming method (5), using a hexadeoxynucleotide mixture (Takara Shuzo) and the purified 1,120-bp *Eco*RI-*Sal*I fragment as the template. The 5′ ends of oligodeoxyribonucleotides were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase.

When RNA was analyzed by the [α-^{32}P]dCTP-labeled DNA, the blotted membrane was preincubated at 42°C for 4 h in 50% deionized formamide–10% sodium dextran sulfate–1% SDS, 1 M sodium chloride–100 μg of denatured salmon testis DNA per ml. A sample of the DNA probe, denatured before use by treatment at 95°C for 10 min, was added to the preincubated membrane and incubated at 42°C for 12 h. The membrane was then successively washed twice with 2× SSPE at room temperature for 5 min each, twice with 2× SSPE containing 1% SDS at 65°C for 30 min each, and twice with 0.1× SSPE at room temperature for 30 min each. The washed membrane was dried and subjected to autoradiography.

When 5′-end-labeled oligodeoxyribonucleotides were used as probes, the blotted membrane was preincubated at 42°C for 4 h in 1% SDS–1 M sodium chloride–10× Denhardt solution (12)–50 mM Tris hydrochloride (pH 7.5)–0.1% sodium pyrophosphate. A sample of the probe was added to the membrane and incubated for another 12 h at 2 to 3°C below the dissociation temperature (T_d) of oligodeoxyribonucleotide-RNA duplex, which was calculated by using the empirical formula $T_d = 2(A + T) + 4(G + C)$ (20). The membrane was then washed by the procedure described above except that washing with 1% SDS–2× SSPE was performed at the hybridization temperature used.

Dot blot analysis of RNA. The denatured cellular RNA samples were directly dotted onto a nylon membrane using a Milliblot-D blotting apparatus (Millipore Corp., Bedford, Mass.) and treated in vacuo at 80°C for 2 h. Conditions for RNA-DNA hybridization were exactly as described for Northern blot analysis.

Primer extension mapping. The method used to determine the 5′ ends of *inc* and *repZ* RNAs was essentially that described previously (21). Total cellular RNA (8 μg) was annealed to 5′-end-labeled oligo-1 or oligo-2 at 65°C for 20 min in a solution containing 50 mM Tris hydrochloride (pH 8.2), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Then 0.5 mM each of four deoxynucleoside triphosphates and 10 U of reverse transcriptase of Rous-associated virus-2 were added to the solution, and the primer extension reaction was carried out at 42°C for 1 h. Primer-extended products were extracted with phenol-chloroform, precipitated twice with ethanol, dissolved in the TE buffer (12), and electrophoresed on a 8% polyacrylamide gel containing 8 M

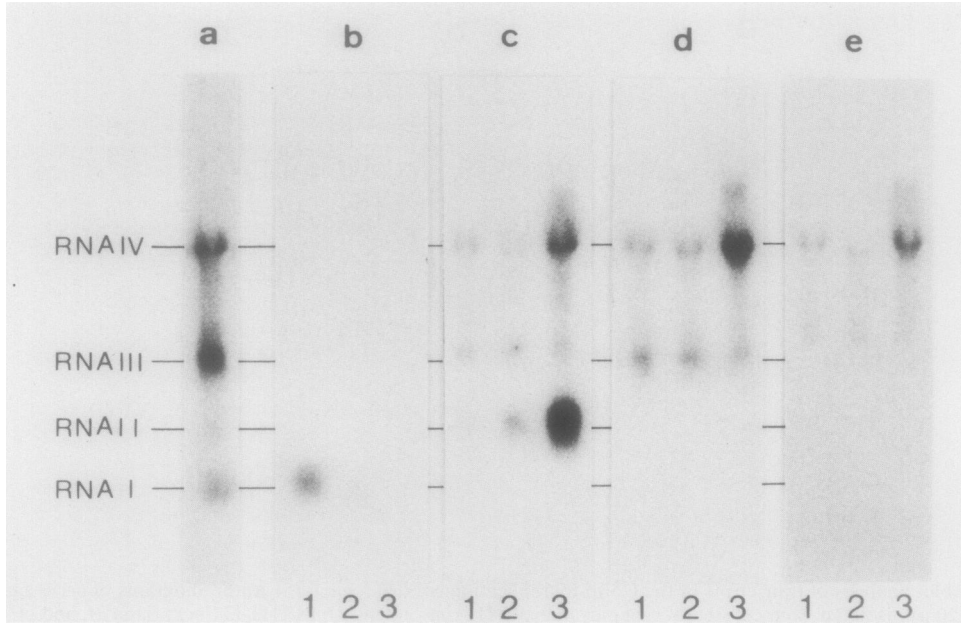

FIG. 2. Northern blot analyses of RNAs of ColIb-P9 by synthetic oligodeoxyribonucleotide probes. Total cellular RNA (20 μg) from cells harboring plasmids was fractionated by electrophoresis on a 1% agarose gel and transferred to nylon membranes. Each RNA species was detected by hybridization with ^{32}P-5′-end-labeled synthetic 17-base oligodeoxyribonucleotides as probes (oligo-1, oligo-2, oligo-3, and oligo-4 in panels b, c, d, and e, respectively). RNAs detected by a [α-^{32}]dCTP-labeled 1,120-base *Eco*RI-*Sal*I fragment are shown in panel a as a reference. Lanes: 1, pCH11 (*inc*$^+$); 2, pCH11-I2 (*inc2*); 3, pCH11-I1 (*inc1*).

urea with a sequencing ladder, using the same DNA primers. The products were visualized by autoradiography.

RESULTS AND DISCUSSION

Transcripts of the replication control region. We first performed Northern blot RNA-DNA hybridization analyses to identify and characterize transcripts in the replication control region. Total cellular RNA was isolated from cells harboring pCH11 (*inc*$^+$) or its Inc$^-$ derivatives, all of which carried the 1,120-bp *Eco*RI-*Sal*I fragment containing the whole *inc* gene region but three-fifths of the 5′ portion of the *repZ* gene (6). Figure 1 shows the results of experiments in which the uniformly [α-^{32}P]dCTP-labeled *Eco*RI-*Sal*I DNA was used as a probe. Plasmid pCH11 produced four major transcripts, designated RNAI, RNAII, RNAIII, and RNAIV (Fig. 1a, lane 1). Similarly, pCH11-I1 (*inc1*) and pCH11-I2 (*inc2*) showed essentially the same patterns as did pCH11 except that the level of RNAI was substantially reduced in *inc1* and *inc2* mutants and there was a corresponding increase in the levels of RNAII. The sizes of the RNA species were determined to be approximately 70, 200, 500, and 1,500 bases for RNAI, RNAII, RNAIII, and RNAIV, respectively, by electrophoresis on either a 1 or 3% agarose gel, using appropriate size markers (Fig. 1a and b).

Since the ColIb region of pCH11 and its derivatives carried only the 5′ portion of the *repZ* reading frame, we also analyzed the transcripts by using plasmid pCH10, which contains sufficient information for replication and control of the ColIb-P9 replicon. As in the case of pCH11, four RNA species were observed (Fig. 1c). One of them corresponded to about 1,400 bases in size, and the others were identical to RNAI, RNAII, and RNAIII. Disappearance of RNAIV with concomitant appearance of the 1,400-base RNA species indicate that these two RNAs are related to each other.

Next, the direction and region of each transcript were investigated by using specific oligodeoxyribonucleotides as probes. When oligo-1 (corresponding to positions 321 to 337) was used, only RNAI was detected (Fig. 2b), indicating that this RNA was transcribed leftward. The amounts of RNAI observed by this probe varied, depending on the *inc* mutations, as has been observed with the *Eco*RI-*Sal*I probe. Conversely, RNAII, RNAIII, and RNAIV were detected by the probes complementary to rightward transcripts; that is, RNAII was detected by the oligo-2 probe (corresponding to positions 275 to 259); RNAIII was detected by the oligo-2 and oligo-3 probes (corresponding to positions 500 to 484); and RNAIV was detected by the oligo-2, oligo-3, and oligo-4 probes (corresponding to positions 746 to 730) (Fig. 2c to e).

Primer extension mapping. The primer extension method was used to determine the 5′ end of each RNA species. For RNAI, the 5′-end-labeled oligo-1 probe was annealed to cellular RNA isolated from cells harboring either pCH11, pCH11-I1, or pCH11-I2. DNA was synthesized from this primer by reverse transcriptase. Figure 3a shows autoradiograms of the primer-extended products. Under the experimental conditions used, one major and several minor DNA bands were synthesized in pCH11 and pCH11-I2. The intensity of DNA bands in pCH11 was higher than that in pCH11-I2. Conversely, we could not detect any DNA band in pCH11-I1 (Fig. 3a, lane 2). These results are consistent with the results shown in Fig. 1 and 2.

Comparison of the DNA bands with a DNA sequencing ladder as a standard showed that the 5′ end of the major band corresponded to position A-363. Within this region, possible *E. coli* promoter sequences (TATACT [positions 377 to 372] for the -10 region and TTGAAT [positions 400 to 395] for the -35 region in the opposite strand) could be seen. Moreover, *inc2* (pCH11-I2) and *inc1* (pCH11-I1) mutations occurred at positions T-374 to C-374 and A-400 to C-400, corresponding to the possible -10 and -35 regions, respectively (6). Accord-

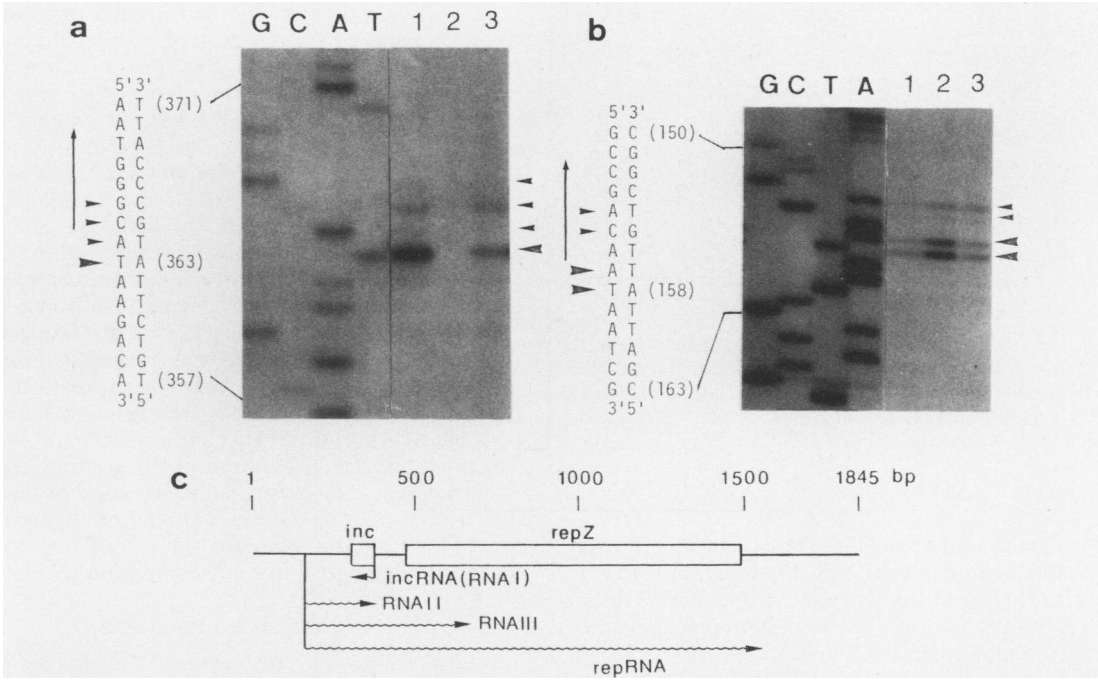

FIG. 3. Primer extension mapping of the 5′ ends of leftward and rightward transcripts. (a) Primer extension mapping of RNAI. Cellular RNA (8 μg) isolated from cells harboring plasmids was hybridized with oligo-1, whose 5′ end was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. DNA was synthesized from this primer by the reaction of reverse transcriptase, electrophoresed on an 8% polyacrylamide gel with 8 M urea, and subjected to autoradiography. Lanes: 1, pCH11 (*inc*$^+$); 2, pCH11-I1 (*inc1*); 3, pCH11-I2 (*inc2*); G, C, A, and T, a standard sequence ladder using the same synthetic primer and its complementary single-stranded DNA from pDX13. (b) Primer extension mapping of the rightward transcripts, RNAII, RNAIII, and RNAIV. The RNA samples used were the same as those in panel a. ^{32}P-5′-end-labeled oligo-2, which hybridizes with all rightward transcripts, was used. Lanes: 1, pCH11 (*inc*$^+$); 2, pCH11-I1 (*inc1*); 3, pCH11-I2 (*inc2*); G, C, T, and A, a standard sequence ladder using the oligo-2 primer and its complementary single-stranded DNA prepared from pDX14. Large and small arrowheads indicate the major and minor 5′ ends of primer-extended products, respectively. Arrows denote the transcription directions determined. Numbers in parentheses indicate nucleotide sequence numbers of ColIb-P9 (6). (c) Transcription mapping of the ColIb-P9 replication control region. The results of the Northern blot and primer extension experiments with pCH11 and pCH10 are summarized. RNAI is equivalent to *inc* RNA detected by in vitro RNA synthesis. RNAIV observed in pCH11 is a part of *rep* RNA detected in pCH10. Wavy lines with arrowheads indicate the directions and positions of the transcripts.

ingly, the substantial reduction of RNAI synthesis in these mutants can be explained as indicating that these two mutations were of the promoter-down type.

To determine the 5′ ends of RNAII, RNAIII, and RNAIV, the 5′- end-labeled oligo-2 probe, which could hybridize with all rightward transcripts, was used. The patterns of DNA synthesis were identical in three strains, pCH11, pCH11-I1, and pCH11-I2 (Fig. 3b). Two major bands (corresponding to positions T-157 and A-158) and two minor bands (corresponding to positions T-154 and G-155) were detected. These results indicate that RNAII, RNAIII, and RNAIV are transcribed from the same initiation site. In addition, possible promoter sequences, TATATT (positions 144 to 148) for the -10 region and TTTCAA (positions 121 to 126) for the -35 region, were found in the upstream region of the initiation sites. Therefore, we provisionally determined A-158 or T-157 to be the transcription initiation site of the rightward transcripts.

The Northern blot and primer extension analyses indicated three points about the nature of transcripts of the ColIb-P9 replication control region. First, RNAI, a leftward transcript with the size of about 70 bases, is transcribed mainly from position A-363, and its production is reduced by *inc1* and *inc2* mutations. These data indicate that RNAI is equivalent to *inc* RNA, which has been detected by in vitro RNA synthesis (6). Thus, we designate RNAI as *inc* RNA.

Nikoletti et al. (15) reported the nucleotide sequences and products of *inc* genes from two IncIα (=IncI$_1$)-group plasmids, R64-11 and R144-3. Comparison of our data with theirs shows that the transcription initiation sites of *inc* genes are essentially identical in all plasmids. However, the size of *inc* RNA differs: approximately 70 bases for ColIb-P9 and 112 bases for R64-11 and R144-3. In this regard, our preliminary result of S1 nuclease mapping indicates that the 3′ end of ColIb-P9 *inc* RNA is A-294. If that is so, *inc* RNA consists of exactly 70 bases.

Second, RNAII, RNAIII, and RNAIV are transcribed rightward from the same transcription initiation site, corresponding to position A-158 or T-157, an indication that these species are related to each other. It has been shown that the reading frame (including the termination codon) of *repZ* is located at positions 455 to 1,486 (6). This means that the size of *repZ* mRNA is at least 1,032 bases. Indeed, a 1,400-base RNA species was observed in pCH10. These results imply that this RNA can satisfy the criterion of *repZ* mRNA. Thus, we conclude that the 1,400-base RNA is *repZ* mRNA and that RNAIV is a part of *repZ* mRNA. The fact that RNAIV is larger than *repZ* mRNA is probably due to the readthrough transcription of *repZ* mRNA in pCH11.

Determination of the size and initiation site of each transcript permits us to illustrate a transcription map of the replication control region of ColIb-P9 (Fig. 3c). This map indicates that *inc* RNA is complementary to a 5′ portion of the rightward transcripts including *repZ* mRNA and presum-

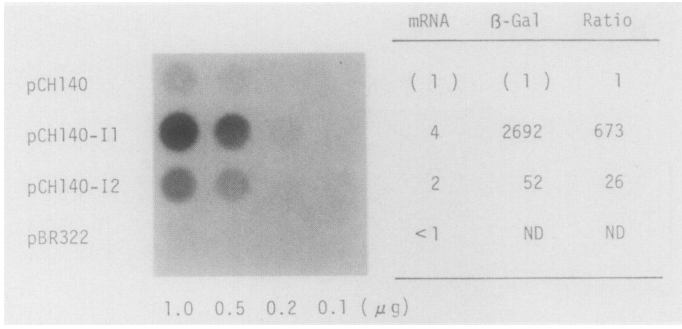

FIG. 4. Dot blot analyses of *repZ* mRNA synthesis. Cellular RNA isolated from MC1061 cells harboring translational *repZ-lacZ* fusions of pCH140 (*inc*$^+$), pCH140 (*inc1*), and pCH140-I2 (*inc2*) was dot blotted on a nylon membrane. *repZ* mRNA was detected by ^{32}P-end-labeled oligo-4 specific to this RNA or RNAIV. β-Gal, β-Galactosidase.

ably functions as an antisense RNA, as has been shown in several plasmids, including ColE1 (8, 17, 18), IncFII (2, 11, 16, 22), and pT181 (7). Indeed, when incubated at 37°C for 30 min, *inc* RNA forms a hybrid with *repZ* mRNA at the entire complementary region (data not shown).

Third, although the functions of RNAII and RNAIII are not known at present, it is interesting that the reduction in transcription initiation from the *inc* RNA promoter increases the synthesis or accumulation of RNAII (Fig. 1 and 2c). In addition, it is noteworthy that, on the basis of their sizes, the 3′ ends of RNAII and RNAIII appear to coincide with the positions of G+C-rich palindromic sequences, namely, CCCCCACTATCTTTC-(21 bp)-GAAAGATAGTGGGGG (positions 302 to 352) and AGCTGCT-(4 bp)-AGCGCT (positions 667 to 672) for RNAII and RNAIII, respectively. In particular, the former corresponds to the G+C-rich region of the *inc* gene. Two possibilities, among several, could be considered regarding the synthesis or accumulation of RNAII and RNAIII. One possibility is that these RNA species are derived from RNAIV during the process of its degradation. This possibility is based on the hypothesis that the sequences corresponding to the 3′ portions of RNAII and RNAIII have potentials to form stable stem-loop structures of RNA but do not correspond to *rho*-independent terminators. In this context, the lower level of synthesis or accumulation of RNAII in the wild-type strain than in *inc* mutants can be interpreted as suggesting that hybridization of *repZ* mRNA with *inc* RNA accelerates the degradation of RNAII. The other possibility is that *repZ* transcription pauses in some manner at the specific secondary-structure regions, thereby regulating the synthesis of mature *repZ* mRNA, as has been reported for the regulation of RepA1 synthesis in the IncFII NR1 plasmid (4). These possibilities remain to be verified.

Regulation of *repZ* gene expression by *inc* RNA. We have shown that the *inc* gene negatively regulates ColIb-P9 replication through repressing *repZ* gene expression (6). To elucidate at which level *repZ* gene expression is controlled, transcriptional, posttranscriptional, or both, synthesis of *repZ* mRNA was analyzed by the RNA dot blot hybridization method. For this purpose, total cellular RNA was prepared from MC1061 cells harboring translational *repZ-lacZ* fusion plasmid, pCH140 (*inc*$^+$), pCH140-I1 (*inc1*), and pCH140-I2 (*inc2*), transferred onto a nylon membrane, and then hybridized with the ^{32}P-end-labeled oligo-4 probe, which could detect only *repZ* mRNA. Relative amounts of *repZ* mRNA, estimated by the intensity of autoradiograms, were 1, 2, and 4 for the wild type, the *inc2* mutant, and the *inc1* mutant, respectively (Fig. 4), an indication that the production of *repZ* mRNA increased at some extent in Inc$^-$ mutants. On the other hand, when the extent of *repZ* gene expression was measured by the β-galactosidase activity of the translational *repZ-lacZ* fusions, *inc2* and *inc1* mutations increased the activity 52 (67/1.3)- and 2,692 (3,500/1.3)-fold, respectively, compared with the wild-type level. Comparison of the ratio of translation to transcription between wild type and Inc$^-$ mutants clearly demonstrates that translation is effected to a much higher degree than transcription.

The contrast between transcription and translation and the data showing that *inc* RNA can form a hybrid with *repZ* mRNA strongly indicate that the expression of *repZ* is regulated mainly at the posttranscriptional level through possible hybridization between *inc* RNA and *repZ* mRNA. The precise mechanism of this posttranscriptional regulation is not known. However, characterization of the interaction of *inc* RNA and *repZ* mRNA in relation to possible alternation of higher-order structure of *repZ* mRNA would provide insight to the control of *repZ* expression by *inc* RNA.

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