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RepR Protein Expression on Plasmid pIP501 Is Controlled by an Antisense RNA-Mediated Transcription Attenuation Mechanism

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Expression of the rate-limiting initiator protein RepR of plasmid pIP501 is controlled by the antisense RNAIII. Mutational alteration of individual G residues within the single-stranded loops of RNAIII led to an increase in copy number. In contrast to the G-rich single-stranded loops, two smaller AT-rich loops of RNAIII were found to be dispensable for its inhibitory function. Reciprocal mutations in the same loop compensated for each other's effect, and a destabilization of the major stem structure of RNAIII also resulted in an increased copy number. These data were consistent with the idea that the interaction of RNAIII with its target starts with the formation of a kissing complex between the single-stranded loops of both molecules. The *repR* mRNA leader sequence, which includes the target of RNAIII, is able to assume two alternative structures due to the presence of two inverted repeats the individual sequences of which are mutually complementary. In the presence of the antisense RNAIII, one of these inverted repeats (IR2) is forced to fold into a transcriptional terminator structure that prevents transcription of the *repR* gene. In the absence of RNAIII, formation of the transcriptional terminator is prevented and expression of the essential *repR* gene can proceed normally. This antisense RNA-driven transcriptional attenuation mechanism was supported by extensive deletional analysis and direct evidence that IR2 functions as a transcriptional terminator.

Plasmid pIP501 (30.2 kb) is a low-copy-number plasmid that was originally isolated from an antibiotic-resistant strain of *Streptococcus agalactiae* (22). Together with plasmids pSM19035 (2, 5) and pAMβ1 (16), it constitutes a group of large incompatible streptococcal plasmids that are able to replicate in a broad range of gram-positive bacteria, including *Bacillus* spp. (14, 16a, 17–19, 30, 37). These plasmids or their derivatives have been found to exhibit a pronounced segregational stability (23), and some of them have been used to stably clone large fragments of foreign DNA (>10 kb) (4, 21, 34). In contrast to the small staphylococcal plasmids most frequently used as cloning vectors in *Bacillus* spp. (20), pIP501 and its related plasmids do not replicate via single-stranded intermediates but rather follow a theta-type mode of replication (13). Surprisingly, the minimal origin of replication of pIP501 has recently been determined to be as small as 52 nucleotides (nt) (7). Its location coincides with the start point of leading-strand synthesis and the termination point of lagging-strand synthesis that have been mapped to the corresponding sequence on plasmid pAMβ1 (13). The replication regions of plasmids pIP501, pSM19035, and pAMβ1 have been sequenced (10, 11, 38, 39), and all three were found to code for two proteins, Cop and Rep. Rep constitutes the specific protein that is essential for initiation of plasmid replication, while Cop is dispensable and involved in copy control. In vitro and in vivo dissection of the transcriptional units together with extensive mutational analysis and gene fusion experiments led to a working model for the regulation of copy number of plasmid pIP501. This model is summarized in Fig. 1. Transcription of the *copR* gene proceeds from promoter pI and is terminated at inverted repeat 1 (IR1), which overlaps promoter pII (12). The latter promoter directs transcription of the *repR* gene. A third promoter, pIII, directs the synthesis of a 136-nt-long antisense RNAIII, which is complementary to part of the *repR* mRNA leader sequence (12). Two levels of copy control have been identified. One level involves the inverted repeat upstream of promoter pII, which is likely to be the target of a negative autoregulation by RepR. There is striking similarity of sequences within IR1 and the origin region (7, 9). The second level includes the CopR protein and the antisense RNAIII. Mutations in either regulatory component cause the same copy effect but are not additive, and mutations within RNAIII or its promoter pIII can be detected only in the presence of CopR (9). The antisense RNAIII can assume a stable secondary structure, which suggested a functional importance of certain single-stranded loops. In addition, the region of complementarity between RNAIII and its target within the *repR* mRNA leader did not include the translational signals of the *repR* gene, thus making the mechanism of its inhibitory action not easily discernible. Furthermore, the functional importance of another inverted repeat (IR2) present within the *repR* mRNA leader remained obscure. In this report, we present data that allow the conclusion that the antisense RNAIII of pIP501 exerts its inhibitory action by causing premature termination of *repR* transcription. This transcriptional attenuation mechanism, which resembles the prototype model described initially for plasmid pT181 (29), requires the structural integrity of the inverted repeat IR2. In addition, mutational analysis of the antisense RNAIII confirmed the importance of its G-rich single-stranded loops for its inhibitory capacity.

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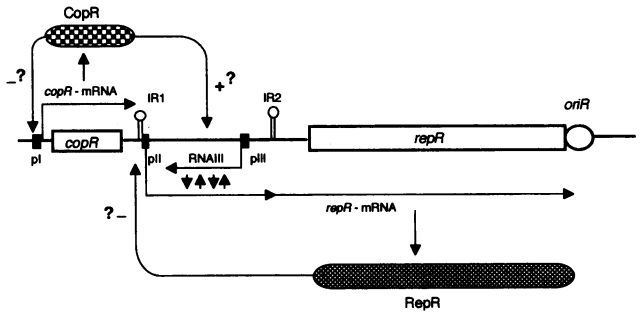

FIG. 1. Working model for the regulatory interactions of components controlling the copy number of plasmid pIP501. *oriR*, origin of replication; ?, hypothetical effects.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The plasmids used in this study to construct new derivatives of pIP501 have been described previously (9, 10). Plasmids were propagated in *Bacillus subtilis* DB104 (24), and *Escherichia coli* TG2 (35) was used for subcloning and mutagenesis experiments. All strains were routinely grown on TY medium.

DNA preparation and manipulation. Plasmid DNA was isolated from *B. subtilis* as reported previously (10). DNA manipulations (restriction enzyme cleavage, ligation, and fill-in reactions with the Klenow fragment of DNA polymerase I) were carried out at the conditions specified by the manufacturer or according to standard protocols (35). A GenAmp polymerase chain reaction (PCR) kit from Perkin Elmer/Cetus was used for PCR. DNA sequencing of all mutations or fragments generated by PCR was performed according to the dideoxy-chain termination method (36) with a Sequenase kit from U.S. Biochemical.

Construction of mutations within RNAIII. All pIP501 derivatives carrying point mutations or deletions within the *repR* mRNA leader-RNAIII region were generated by two-step PCRs with plasmid pUC118-F or plasmid pUC119-F as the template. During the first step, the fragments located proximal and distal to the deletion or point mutation were separately amplified by using the universal or the reverse sequencing primer and one of the following mutagenic oligonucleotides: 5′-AATTACTTTATTAATCCCCATTAT ATCGGGT-3′ and 5′-ACCCGATATAATGGGGATTAATA AAGTAATT-3′ (pPR25); 5′-GACCCAAAATTTGGTAAT CAGATACTTAAAT-3′ and 5′-ATTTAAGTATCTGATTA CCAAATTTTGGGTC-3′ (pPR26); 5′-CGACCAGTTAAAG TCTGAGAAATTTTA-3′ and 5′-TAAAATTTCTCAGACT TTAACTGGTCG-3′ (pPR347); 5′-CCAGTTAAAGCCTGG AAATTTTAACTGC-3′ and 5′-GCAGTTAAAATTTCCAG GCTTTAACTGG-3′ (pPR351); 5′-CCTTAATTGATTATC ACCAATCAATTAA-3′ and 5′-TTAATTGATTGGTGATA ATCAATTAAGG-3′ (pPR382); 5′-CCTTAATTGATTAC TACCAATCAATTAA-3′ and 5′-TTAATTGATTGGTAGT AATCAATTAAGG-3′ (pPR383); 5′-CTTAATTGATTAC CGCCAATCAATTAAA-3′ and 5′-TTTAATTGATTGGCG GTAATCAATTAAG-3′ (pPR384); 5′-CTTAATTGATTAC TGCCAATCAATTAA-3′ and 5′-TTAATTGATTGGCAG TAATCAATTAAG-3′ (pPR383-384); 5′-TTAATTGATTAC CATCAATCAATTAAAGA-3′ and 5′-TCTTTAATTGATT GATGGTAATCAATTAA-3′ (pPR385); and 5′-AAGAAGT CGAGATCCAAAATTTGG-3′ and 5′-CCAAATTTTGGAT CTCGACTTCTT-3′ (pPR408).

In the second step, the PCR products of the first reactions were joined and amplified as a single fragment by using the universal and reverse sequencing primer only. These fragments were finally digested with *Bam*HI and *Eco*RI and inserted into plasmid pBT48 (10) cleaved with the same pair of enzymes. The pPR series plasmids thus obtained did not yet carry intact *copR* genes. Reconstitution of functional *copR* genes was accomplished by transferring an *Xba*I-*Kpn*I cassette from plasmid pCOP2 (9) onto each of the pPR series plasmids, giving rise to the corresponding pCOP series derivatives. All pPR and pCOP derivatives were initially established in *E. coli* and subsequently transformed into *B. subtilis*. Plasmids pPR347-383 and pPR347-408, which carried double-point mutations, were constructed with pPR347 as the template for the initial PCR and the same mutagenic oligonucleotides that were used to generate the single-point mutations on pPR383 and pPR408, respectively. All deletions and point mutations were confirmed by DNA sequencing. During these sequencing controls, we identified a triple-mutant plasmid, pPR347-383-397, that resulted from a spontaneous mutation (A-397→T) during the construction of pPR347-383. Furthermore, the sequencing controls of all mutant plasmids revealed another point mutation (position 356) that requires correction of the previously published sequence of the pIP501 replication region (see Results).

Construction of mutations within the attenuator structure. The point mutation present on plasmid pCOP517 (G-517→A) and the deletions on plasmids pCOP22 and pCOP23 were constructed by two-step PCRs exactly as outlined above. The following mutagenic oligonucleotides were used: 5′-TA AGAAGATACCAAGCAATCAATTAAGA-3′ and 5′-TCTT AATTGATTGCTTGGTATCTTCTTA-3′ (pPR517); 5′-GAG AAATTTTAACTGCGAGCACCAATCAATTAAAGA AG-3′ and 5′-CTTCTTTAATTGATTGGTGCTCGCAGTT AAAATTTCTC-3′ (pPR22); and 5′-AACTTAGTTGATTGC CG-TTGTGATTCAACTTTG-3′ and 5′-CAAAGTTGAATC ACAACGGCAATCAACTAAGTT-3′ (pPR23).

To construct plasmids pCOP19, pCOP20, and pCOP21, the fragments located 5′ and 3′ of the deletions were initially amplified by PCR with pUC118-F as the template, the universal or the reverse sequencing primers, and one of the following oligonucleotides: 5′-TCTAGAGTCGACTTGAAA TCCCCTCA-3′ and 5′-TCTAGAGTCGACGTTGTGATTC AACT-3′ (pPR19); 5′-TCTAGAGTCGACTGGTCGTGGCT CTT-3′ and 5′-TCTAGAGTCGACGTTGTGATTCAACT-3′ (pPR20); and 5′-TCTAGAGTCGACTTGAAATCCCCTCA-3′ and 5′-TCTAGAGTCGACTTAGTTGATTGCCTTTTTT G-3′ (pPR21). The amplified 5′ fragments were subsequently cleaved with *Bam*HI and *Sal*I, and the 3′ fragments were digested with *Sal*I and *Eco*RI. Both fragments were then jointly inserted into plasmid pBT48 (10) and linearized with *Bam*HI and *Eco*RI, and the *copR* gene was eventually reconstituted as described above to yield plasmids pCOP19, pCOP20, and pCOP21.

Plasmid pCOP24, carrying a combination of the two deletions present on pCOP21 and pCOP22, was constructed by PCR using plasmid pCOP22 as a template and the same pair of primers as for amplification of the 5′ fragment of plasmid pCOP21. The resulting fragment contained the deletion present on pCOP22 and extended to the left border of the deletion present on pCOP21. After this amplified fragment was cleaved with *Bam*HI and *Sal*I, it was used to replace the corresponding fragment on plasmid pCOP21.

Determination of plasmid copy number. The copy number of all pIP501 derivatives in *B. subtilis* DB104 was determined as described before (9).

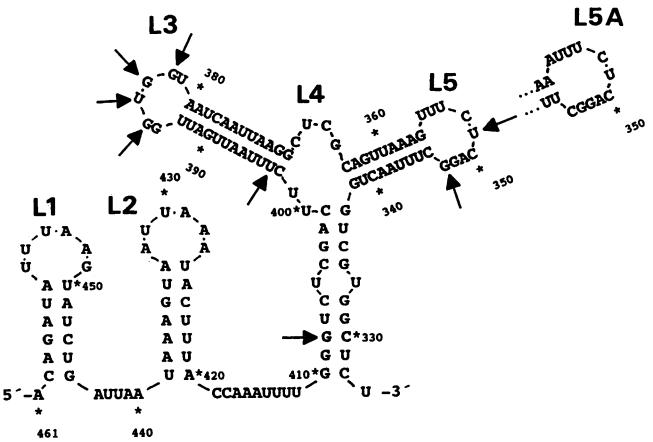

FIG. 2. Secondary structure of the antisense RNAIII. The change in the secondary structure of loop L5 caused by the mutation in position 356 is indicated as loop L5A. Nucleotide numbering refers to the original sequence data for the pIP501 replication region (10). L1 to L5 designate the individual single-stranded loops. The point mutations introduced are indicated by arrows.

Nuclease S1 mapping of the transcriptional terminator IR2. For nuclease S1 mapping, a 215-bp *Taq*I-*Pvu*II fragment (nt 399 to 613 [10]) covering the putative transcriptional terminator IR2 was isolated and 3′ labeled with [^{32}P]dCTP and the Klenow fragment of DNA polymerase I. Total RNA from pCOP4 (expressing *copR*) as well as pGB354 and pPR12 (both Δ*copR* [3, 9]) was used for hybridization. Strand separation, hybridization, and digestion with nuclease S1 were done exactly as outlined by Barthelemy et al. (1). Protected fragments were electrophoresed on 6% denaturing polyacrylamide gels with both a Sanger sequencing reaction and a Maxam-Gilbert purine reaction of the same fragment as size standards.

RESULTS

Correction of the DNA sequence coding for RNAIII. During the mutagenesis experiments presented in this report, we noticed in position 356 a single nucleotide difference (C instead of T) from the previously published DNA sequence of RNAIII (10, 12). The published DNA sequence of the pIP501 replication region was derived from a DNA fragment that was cloned into pUC118 (plasmid pUC118-F [10]) for sequencing purposes. During this cloning step, a spontaneous mutation which changed the C in position 356 (10) to a T had obviously occurred. This difference was initially noted after sequencing of the corresponding region on pUC119-F, and the C-356 residue was subsequently confirmed to be the actual wild-type nucleotide by determining the respective region directly on the pIP501 derivative pGB3631. This single nucleotide difference resulted in a change of the secondary structure of the antisense RNAIII (reduction in size of loop L5; Fig. 2). However, copy control was not affected by this mutation, as the copy numbers of the wild-type and mutant plasmids were identical in *B. subtilis*. Both pUC118-F and pUC199-F were used as templates for PCR to construct the point or deletion mutants presented below. Thus, mutants pCOP385 and pCOP383-384 (Table 1) carry the wild-type sequence in position 356 while all others carry the additional mutation in this position.

TABLE 1. Relative copy numbers of pIP501 derivatives carrying point mutations or deletions within RNAIII

Plasmid[a]	Mutation[b]	Copy no.	Relative copy no.
pCOP356	None (wild type)	5	1
pCOP4*	G-356→A	5	1
pCOP25*	Δnt 446–466	5	1
pCOP26*	Δnt 420–440	5–10	1–2
pCOP347*	G-347→A	25–50	5–10
pCOP351*	Δnt 351 (U)	5	1
pCOP382*	G-382→A	50–100	10–20
pCOP383*	G-383→A	25–50	5–10
pCOP385	G-385→A	50–100	10–20
pCOP408*	G-408→A	50–100	10–20
pCOP347-408*	G-347→A		
	G-408→A	50–100	10–20
pCOP347-383*	G-347→A		
	G-383→A	25–50	5–10
pCOP383-384	G-383→A		
	U-384→C	5	1
pCOP347-383-397*	G-347→A		
	G-383→A		
	U-397→A	5	1

[a] Plasmids marked with an asterisk carry the mutant A residue in position 356; all others carry the wild-type sequence. The mutation is indicated only for pCOP4.
[b] As manifested in the RNAIII sequence.

Mutations within RNAIII affect the copy number. The interaction between sense and antisense RNAs has been shown in several systems to start with the formation of an initial, transient complex. This kissing complex is formed between the single-stranded loops of both RNAs and subsequently converted into a completely paired region by a zipper-like reaction. The strength of the initial complex formation is thus influenced by the nucleotides present in the single-stranded loops of the sense and antisense RNAs. To determine which of the single-stranded loops of RNAIII are critical for its regulatory role, a number of point mutations and deletions were constructed. The copy numbers of these mutant plasmids were compared with that of pCOP4, carrying all components known to be involved in copy control of pIP501. The two small stem-loop structures L1 and L2 (Fig. 2) were obviously not important for the regulatory function of RNAIII. Deletion of loop L1 (plasmid pCOP25; Table 1) did not affect the copy number at all, and deletion of loop L2 (plasmid pCOP26; Table 1) resulted in only a slight, if any, increase in copy number. In contrast, point mutations within loops L3 and L5 of the large branched structure of RNAIII had a strong effect on the copy number of the respective mutant plasmids. Initially the G's in positions 382, 383, and 385 of loop L3 and position 347 of loop L5 were converted into A residues. These changes were thought to reduce the strength of the initial interaction between target and antisense RNA, and thus an increase in copy number was expected. As shown in Table 1, all of these point mutations resulted in copy numbers at least 5- to 10-fold higher than that of pCOP4. It was interesting to note that the increase in copy number was not uniform. Plasmids pCOP382 and pCOP385 showed copy numbers that were increased 10- to 20-fold. A reversion of the copy number up-mutations was attempted by introducing a compensating secondary mutation. For this purpose, the U in position 384 of loop L3 was changed to a C residue. Introduction of this secondary mutation into plasmid pCOP383 resulted in the expected

reversion of the copy number to the wild-type level of pCOP4.

A double mutation of single G's in loops L3 (position 383) and L5 (position 347) had no additive effect and did not lead to a further increase in copy number (Table 1). During the construction of this double mutant, we found a triple mutant which carried an additional and spontaneous mutation in position 397 (pCOP347-383-397; Table 1). Surprisingly, this mutant plasmid exhibited a reduced copy number, which was again identical with that of pCOP4. The mutation in position 397 obviously increased the size of loop L4 and extended the single-stranded region by two additional G residues.

Three further mutations were constructed to study the effect of loop size reduction and the importance of the stability of the stem of the large branched structure of RNAIII (Fig. 2). Deletion of nt 351, which reduced the size of loop L5, was without effect on the plasmid copy number (Table 1). In contrast, the change of the G in position 408 into an A residue caused a 10- to 20-fold increase in copy number. Thus, destabilization of the stem of the large branched structure of RNAIII resulted in a severe reduction of its regulatory function. A combination of the point mutations in positions 408 and 347 (loop L5; Fig. 2) did not give rise to a further increase in copy number.

The leader region of *repR* mRNA may fold into structures resembling a transcriptional attenuator. On the basis of the algorithm of Zuker and Stiegler (44; RNAfold in the PC/GENE software package), an extensive computer-based analysis of structural features within the *repR* mRNA leader region was carried out to develop a hypothesis for how the antisense RNAIII may regulate the expression of *repR*. The target region of RNAIII within the *repR* mRNA leader region did not include the translational initiation region of the *repR* gene, nor did it code for any short leader peptide (9, 10) (Fig. 1). Regulation at the translational level therefore appeared to be unlikely. Depending on the presence or absence of RNAIII, the leader region of *repR* mRNA was, however, found to assume two possible structures (Fig. 3). We previously noted the presence of an inverted repeat (IR2; nt 517 to 530 and 536 to 549 [9, 12]) upstream of promoter pIII that resembled a typical Rho-independent transcriptional terminator (Fig. 1 and 3A). Folding analysis of the *repR* mRNA leader region now revealed that the left branch of IR2 (nt 517 to 530; designated a in Fig. 3A and B) was complementary also to a second region within the *repR* mRNA leader (nt 370 to 383; designated A in Fig. 3A and B). This additional complementary region was, however, part of one of the inverted repeat structures present within the target sequence of RNAIII. During transcription of the *repR* gene, the leader region is likely to initially fold into a structure that is a mirror image of the structure of antisense RNAIII at least with respect to the functionally important stem-loop structures (Fig. 3A). As transcription proceeds in the absence of the antisense RNAIII, this structure becomes unravelled again and is rearranged into a more stable one (shown in Fig. 3B) that is likely to emerge for energy-related reasons. Annealing of A and a, however, prevents the formation of IR2 and thus leads to a silencing of the putative transcriptional terminator. On the other hand, in the presence of RNAIII and CopR, the antisense RNA would hybridize to its target region and thereby render this part of the *repR* mRNA leader region unavailable for intrastrand pairing. As a consequence, intrastrand annealing of A and a is abolished, the transcriptional terminator structure IR2 can be formed as shown in Fig. 3A, and expression of *repR* will be prevented by premature termination of transcription. The folding alternatives of the *repR* mRNA leader region in the presence or absence of the antisense RNAIII were thus reminiscent of a transcriptional attenuator which is controlled by a regulatory antisense RNA.

Deletional analysis supports the transcriptional attenuation hypothesis. The transcriptional attenuation hypothesis also accommodated our previous observation that plasmid pCOP10 (9) failed to replicate in *B. subtilis*. This plasmid lacked the RNAIII coding region and thus the antisense target but still retained an intact IR2 (Fig. 4). Constitutive formation of the transcriptional terminator IR2 on pCOP10 was therefore likely to prevent expression of *repR*, which is essential for plasmid replication. In fact, plasmid pCOP10 could be successfully transformed into a *B. subtilis* strain that supplied RepR in *trans* from a *repR* gene inserted into the chromosomal α-amylase locus. Furthermore, removal of IR2 from the *repR* mRNA leader region by extending the deletion on pCOP10 (plasmid pCOP20; Fig. 4) reconstituted the ability of pCOP20 to replicate in *B. subtilis*. This plasmid had a copy number that was increased approximately 10-fold. Both the restoration of plasmid replication and the increase in copy number agreed well with the proposed hypothesis.

To further substantiate the transcriptional attenuation hypothesis, a number of additional deletion mutants were constructed (summarized in Fig. 4). Complete removal of the IR2 structure (plasmid pCOP19) from the *repR* mRNA leader resulted in a 10-fold-increased copy number although neither the RNAIII coding region nor promoter pIII was affected. Deletion of either one of the inverted repeat branches A and a also gave rise to the expected pattern. Deletion of a (plasmid pCOP21; Fig. 4) again resulted in a 10-fold-increased copy number. In contrast, deletion of A caused a failure of plasmid pCOP22 to replicate in *B. subtilis* as the consequence of constitutive formation of IR2. Like plasmid pCOP10, pCOP22 could, however, be introduced into a *B. subtilis* strain providing RepR in *trans* from the chromosome. Deletion of both A and a (plasmid pCOP24; Fig. 4) did not have an additive effect, and the increase in copy number was identical with that of pCOP19 or pCOP21. However, this observation ruled out an extreme instability of *repR* mRNA as the cause for the failure of pCOP22 to replicate. Deletion of the poly(U) stretch on plasmid pCOP23 (Fig. 4) also caused a 10-fold increase in copy number. This poly(U) stretch is likely essential for transcription to be terminated at IR2. Finally, the importance of correct positioning of the poly(U) stretch was further supported by analysis of a point mutation that changed the G at position 517 into an A residue. This mutation destabilized the base of the terminator stem and moved the poly(U) stretch away from the IR2 structure. As a result, a fivefold increase in copy number was observed for the respective mutant plasmid pCOP517 (Fig. 4). In summary, all deletions introduced into the *repR* mRNA leader region strongly supported the transcriptional attenuation hypothesis.

Direct demonstration of transcriptional termination at IR2. Since plasmid pIP501 (as well as its derivative pCOP4) is a low-copy-number plasmid, it proved difficult to directly demonstrate transcriptional termination at IR2 by nuclease S1 mapping. Results from previous in vitro runoff transcription experiments with *B. subtilis* σ^{43} RNA polymerase (12), however, showed that transcriptional termination in fact occurred at IR2. Transcription starting from promoter pII always generated RNA molecules of distinct length that were considerably shorter (270 nt) than the runoff transcripts

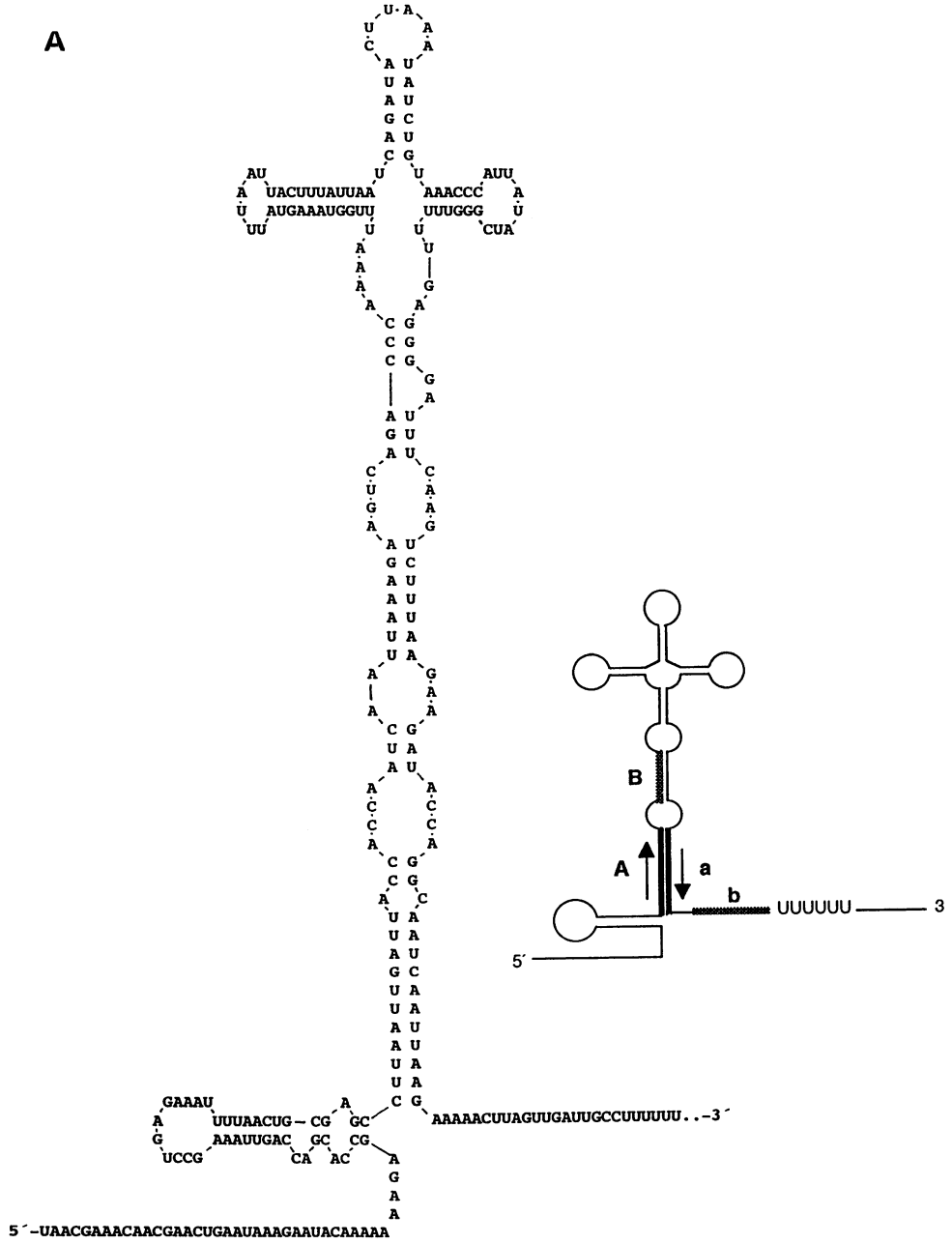

FIG. 3. Alternative folding pattern of the leader sequence of the *repR* mRNA. Both the RNA sequences and a schematic representation of the folding patterns are indicated. (A) Folding of the leader sequence in the absence of RNAIII. The repeats A and a anneal, and formation of the transcriptional terminator IR2 is prevented. (B) Folding of the leader sequence in the presence of RNAIII. The sequences included in the boxed area represent the region of hybridization between RNAIII and its target. Occupation of A by interaction with RNAIII renders a available for annealing with b, which results in the terminator structure IR2.

(580 or 330 nt). Reexamination of these results confirmed that the endpoint of the shorter transcripts coincided exactly with the position of IR2 (12). In vivo evidence for transcriptional termination at IR2 was obtained by nuclease S1 mapping with mRNA isolated from a *B. subtilis* strain carrying the copy mutant plasmid pPR12 (3, 9). As shown in Fig. 5, a very faint transcriptional termination signal was detectable at the poly(U) stretch following the IR2 structure. In contrast, no signal was detectable when mRNA of pCOP4-containing cells was hybridized with the same probe, ruling out that the faint bands observed with pPR12 mRNA were the result of a probe artifact. The strength of the signal was expected to be low, if at all detectable, since the high copy number of plasmid pPR12 was due to a lack of CopR. The amount of pPR12 *repR* mRNA transcripts terminated at IR2 under these conditions was in fact very low, since a strong signal for the start point of promoter pII (12) was

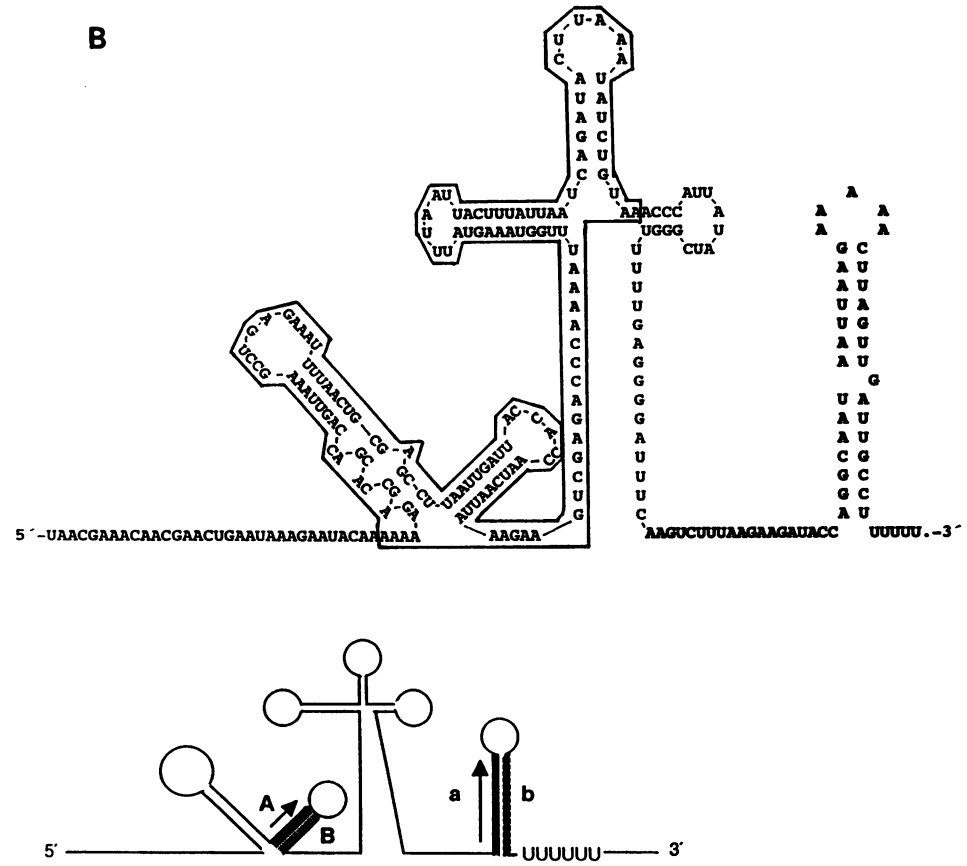

FIG. 3—*Continued*

obtained with the same mRNA preparation under comparable experimental conditions.

DISCUSSION

The replication frequency of plasmid molecules during the cell cycle is assumed to follow a Poisson distribution rather than to occur at a constant rate (27). As a consequence, individual cells inherit differently sized plasmid pools and regulatory mechanisms are required that respond rapidly in particular to down fluctuations of the copy number in order to ensure segregational stability of the plasmid. From the data presented here, we conclude that plasmid pIP501 has evolved antisense RNA-driven transcriptional attenuation as a mechanism that allows a rapid upward correction of its copy number. Transcription from promoter pIII gives rise to the 136-nt-long antisense RNAIII which is complementary to a noncoding region within the *repR* mRNA leader sequence (12). Our data support the conclusion that the primary result of the interaction of antisense RNAIII with its target inside the *repR* mRNA leader appears to be the formation of a transcriptional terminator (IR2) that aborts the majority of transcripts initiated at the *repR* promoter pII. Any drop in the concentration of the antisense RNAIII caused by an uneven distribution of plasmid molecules during the cell cycle or by other physiological effects would, thus, result in a reduced frequency of interaction of RNAIII with its target sequence. The failure to form the transcriptional terminator IR2 then leads to increased read-through transcription and eventually to a rise in the concentration of RepR that is the rate-limiting factor for pIP501 replication in *B. subtilis*. Copy numbers of pIP501 derivatives have previously been shown to vary colinearily with the amount of RepR protein expressed (8). Antisense RNA-driven transcriptional attenuation, thus, is an efficient control mechanism to ensure that the copy number of pIP501 does not drop below a critical level. An efficient mechanism like this is particularly important for pIP501, since it is a low-copy-number plasmid and thus especially prone to loss by imbalances in plasmid distribution or replication. Transcriptional attenuation is a control mechanism that is also particularly efficient from an economic point of view. Full transcription of the *repR* gene is initiated only when expression of the gene is actually required for plasmid maintenance.

Antisense RNA-driven transcriptional attenuation has also been found to be the mechanism that regulates synthesis of the rate-limiting replication initiator protein of another group of plasmids from gram-positive bacteria (29). The prototype plasmid of this group, pT181, also encodes an antisense RNA (25) which acts by inducing premature termination of *repC* transcription (29). However, in contrast to pIP501, pT181 directs the synthesis of two antisense RNAs (RNAI and RNAII) which differ in length and are obviously both required for efficient transcriptional attenuation to occur (15, 25, 28, 29). Plasmid pT181 belongs to a group of plasmids that has been thoroughly studied. Comparison of the DNA sequences of six plasmids of the pT181 group revealed a strong conservation of the structural requirements needed for the attenuation mechanism, i.e., conservation of the two pairs of mutually complementary inverted

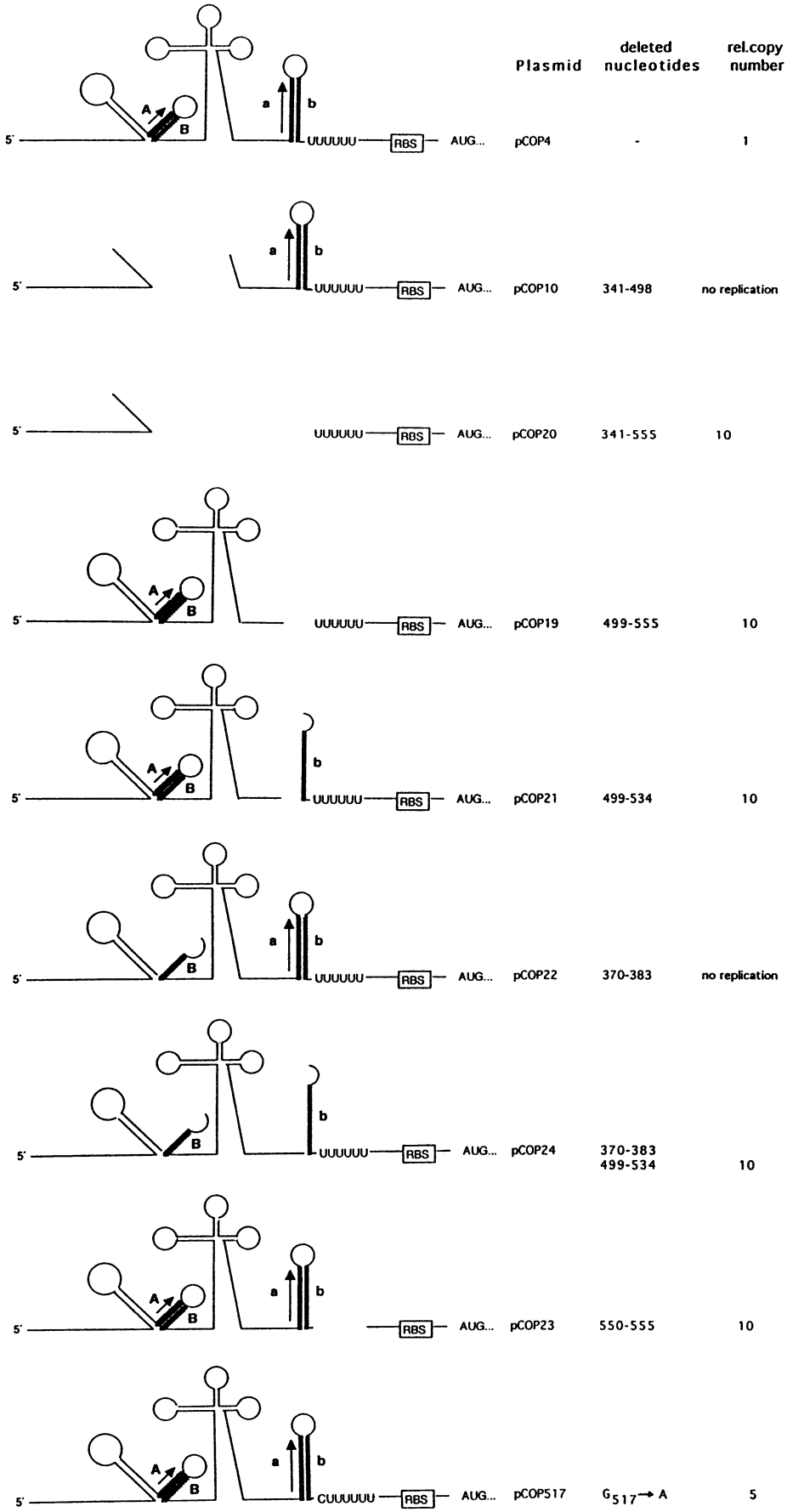

FIG. 4. Compilation and schematic representation of the deletions and point mutations introduced into the *repR* mRNA leader sequence and their effects on the copy number of the respective pIP501 derivatives. Nucleotide positions refer to the original sequence data for the pIP501 replication region (10). RBS, ribosome binding site.

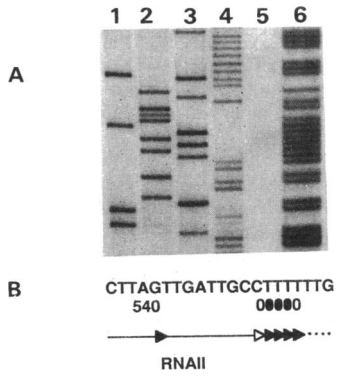

FIG. 5. S1 nuclease mapping of the transcriptional terminator IR2. (A) Lanes: 1 to 4, GATC Sanger sequencing reactions of a size standard; 5, nuclease S1 mapping of the terminator IR2 with mRNA isolated from a *B. subtilis* strain carrying the high-copy-number derivative pPR12 (9) of pIP501; 6, Maxam-Gilbert purine reaction of the same single strand that was used for the nuclease S1 mapping. (B) Schematic representation of the mapping data. Nucleotide numbering refers to the original sequence data for the pIP501 replication region (10).

repeats, although there were clearly differences in the respective DNA sequences of the individual plasmids (29). A similar situation is true for plasmid pIP501 and its related plasmids pSM19035 and pAMβ1. The DNA sequences of the replication regions of the latter two plasmids have also been determined (11, 38, 39), and a comparison revealed a similar organization of transcriptional units (12). All three plasmids carry sequences A/B and a/b as a structural basis for the attenuation mechanism (a compilation of the respective sequences is shown in Fig. 6). While the respective regions on plasmids pIP501 and pSM19035 are identical, there is considerable difference (37% of the nucleotides) from the corresponding DNA sequence on pAMβ1. Accordingly, the proposed secondary structures of the antisense RNAs coded for by pIP501 and pSM19035 on the one hand and pAMβ1 on the other are quite different. Nevertheless, despite these differences, complementarity between the distantly located repeats of pAMβ1 is maintained. This structural conservation points to strong selective pressure to maintain this control mechanism of *rep* expression. Although the pT181 and pIP501 plasmid families have evolved similar mechanisms to control expression of their rate-limiting initiator proteins, these two plasmids are clearly of different evolutionary origins. While pIP501-related plasmids use a theta-type mode of replication, replication of pT181 plasmids occurs via single-stranded intermediates by a rolling-circle type of mechanism (20).

The topological organization of the replication region of pIP501 exhibits similarity to that of the gram-negative plasmid R1 (6, 26). Synthesis of the RepA initiator protein of R1 is also controlled by an antisense RNA complementary to the leader region of the *repA* mRNA (26, 32, 33). It has, however, recently been found that the CopA antisense RNA of R1 acts at the translational level rather than on transcription. Interaction between CopA and its target CopT prevents translation of a short leader peptide and thereby interrupts translational coupling between the leader peptide and RepA (6).

A characteristic property of regulatory antisense RNA molecules is their ability to assume distinct secondary structures. This is also true for RNAIII of pIP501. Interaction of such highly structured antisense RNA molecules with their targets is likely to involve several steps and starts with the formation of so-called kissing complexes between the single-stranded loops of antisense and target RNA. This concept was initially proposed by Tomizawa (40–42) for the interaction of RNAI and RNAII of ColE1 and was later supported by both in vivo and in vitro studies of the interaction of the CopA antisense RNA of plasmid R1 with its target CopT (31–33). In vitro experiments with the R1 system even suggested that formation of the kissing complex is sufficient for the inhibitory action of CopA to occur (43). These results stress the critical role of single-stranded loops within antisense RNA molecules for their regulatory function. The mutational analysis of RNAIII presented here supports this concept. We have previously pointed out (12) that two of the single-stranded loops of RNAIII (L3 and L5) are particularly G rich. A change of these G residues to A residues resulted in an upregulation of the copy number of the pIP501 derivatives, most probably as a result of a reduced ability to form the initial kissing complex with the *repR* mRNA leader. In contrast, deletion of the other two (AT-rich) loops L1 and L2 was virtually without effect on the regulatory function of RNAIII in vivo. Similar differences in the functional importance of individual antisense RNA loops have also been noted for CopA of R1 (33). As expected, reciprocal double mutations within the same loop (pCOP383-384) compensated for each other. It was, however, interesting to note that mutations in different loops had also a compensating effect (pCOP347-383-397). The increase in the size of loop L4 of RNAIII obviously reverted the effect of the copy number up-mutations in L3 and L5. The mutation in position 397

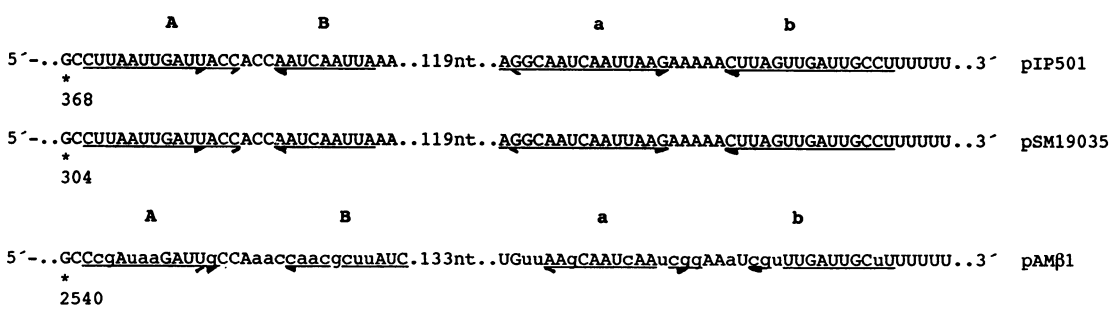

FIG. 6. Comparison of the *repR* mRNA leader sequences of plasmids pIP501, pSM19035, and pAMβ1. The sequences were taken from Brantl et al. (10, 11), Sorokin and Khazak (38), and Swinfield et al. (39). **A**, **B**, **a**, and **b** refer to the inverted repeat sequences that are involved in the antisense RNA-driven transcriptional attenuation mechanism.

resulted in an increased size of loop L4 with the exposure of additional G residues in the single-stranded region. Furthermore, the importance of stable secondary structure for the function of the antisense RNAIII was revealed by the copy-up effect of mutant pCOP408. In summary, the results of the mutational analysis of RNAIII are compatible with the concept that the initial kissing complexes between RNAIII and its target are critical for its inhibitory function. Future in vitro analysis is needed to resolve whether full duplex formation between RNAIII and its target is essential for its function. Although the inhibitory mechanism of RNAIII is indirect, full duplex formation may nevertheless be required to efficiently prevent the folding of IR2.

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