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Plasmid pT181 replication is regulated by two countertranscripts

(small RNAs/copy control)

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ABSTRACT **A transcription map of the replication control region of the *Staphylococcus aureus* plasmid pT181 has been constructed. Two major leftward transcripts, RNA III and RNA IV, start at positions 339 and 413, respectively. These two RNAs can serve as mRNAs for a plasmid-specific replication protein RepC. Two short rightward transcripts, RNA I and RNA II, ≈85 and 150 nucleotides long, respectively, start at position 246. These rightward transcripts (referred to as countertranscripts) do not appear to be translated but act directly as negative regulators of plasmid replication, probably by interfering with translation of the RepC mRNAs. There is no significant base sequence homology among the countertranscripts of pT181, ColE1, and R1/NR1/R6-5, suggesting that the structural parallelism has risen by convergent molecular evolution.**

pT181 is a tetracycline-resistance plasmid, 4437 base pairs (bp) long (1); the major elements of its replication control system are shown in Fig. 1. The replication origin is located between nucleotide (nt) 15 and nt 157 (2), within the coding sequence for RepC, a plasmid-specific protein that is required for replication and is presumably involved in initiation (3, 4).

The copy number of pT181 is accurately maintained at 20–25 per cell during steady-state exponential growth (5) by a plasmid-determined control mechanism of the inhibitor–target type (6). The inhibitory component of this system was initially identified as an incompatibility determinant, *inc3A*, that, when cloned to a higher copy-number carrier plasmid, pE194, excluded pT181 unilaterally by inhibiting its replication (6). Analysis of pT181 mutations with elevated copy numbers (7) showed that some of these are insensitive to the inhibition (dominant) and therefore define the genotypic target function. The inhibitory function thus defined, *inc3A/copA*, was shown to control the rate of RepC synthesis and not to affect origin activity directly (6). The regulatory system is therefore of the indirect type (6).

Sequence analysis of copy mutants, some of which are point mutants, others small deletions, has localized the elements of the copy control system to the region just 5′ to the *repC* coding sequence and has shown that the genetic determinants of inhibitor and target overlap (7). This architectural resemblance to other plasmids of the inhibitor–target type, such as the ColE1 (8–10) and IncFII groups (11), is emphasized by our failure to identify any potential polypeptide reading frame in the region specifying the inhibitor, suggesting that the inhibitor may be an RNA molecule.

In this paper, we report that the pT181 RepC mRNA has two tandem starts and a long untranslated 5′ leader and that the known copy control elements are located in this leader region. Two small RNA species are initiated from a single start within the leader and transcribed from the opposite strand, one terminating very close to one mRNA start, the other, ≈60 bp further upstream. We propose that such RNA species be referred to as *countertranscripts*. One or both of these countertranscripts evidently serve(s) as negative control element(s), probably by interfering with translation of the RepC message.

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MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. S1 nuclease and *Escherichia coli* RNA polymerase were from Boehringer Mannheim. Enzymes for RNA sequencing were from Bethesda Research Laboratories. *Bacillus subtilis* RNA polymerase was a gift from R. Losick and was purified by us by the protocol supplied by R. Losick.

Methods. RNA isolation, electrophoresis and hybridization were carried out as described by Thomas (12, 13). *In vitro* transcription reactions were carried out as described by Selzer *et al.* (8).

S1 Nuclease Mapping of RNA. Fifty- or 100-μg amounts of RNA isolated by the method of Chirgwin *et al.* (14) were precipitated along with a molar excess of labeled *Hpa* II–*Rsa* I (nucleotides 78–443) fragment of pT181 and dissolved in 20 μl of hybridization buffer (80% formamide/0.4 M NaCl/40 mM Pipes, pH 6.4/1 mM EDTA). Hybridization was at 39°C for 16 hr. S1 nuclease digestion and electrophoresis were done as described by Weaver and Weissmann (15).

RESULTS

Identification of Transcripts Involved in Copy Control. Total RNA extracted from strains carrying pT181 and pT181 *cop-608* was analyzed by blot hybridization using as probes the pT181 restriction fragments, *Taq* I C and *Mbo* I D (Fig. 1), labeled by nick-translation (13). pT181 *cop-608* has a spontaneous deletion of nt 183–362 that eliminates both inhibitor and target and increases the copy number 50-fold (4–6). The *Taq* I C fragment (nt 3942–158) contains two-thirds of the *repC* coding sequence. The *Mbo* I D fragment (nt 31–376) contains the 5′ end of the *repC* coding sequence and most of the untranslated leader region. As shown in Fig. 2*a*, the *Taq* I C fragment is homologous to an RNA species of ≈1 kilobase (kb) in extracts from both pT181- and *cop-608*-containing strains. The amount of 1-kb RNA in the *cop-608* strain is much greater than that in the pT181 strain, consistent with the much higher copy number of the former. This band is presumed to represent the RepC message (see below). With the *Mbo* I D probe, in addition to the 1-kb species, two low-molecular-weight RNA species appeared in the pT181 preparation but not in the *cop-608* preparation. The smallest RNA (RNA I) has a mobility corresponding to that of a tRNA; hence, it is calculated to be ≈80 nt long. The second RNA (RNA II) has a mobility corresponding to a length of about 150 nt. The fact that these small RNAs are not seen in extracts from *cop-608*-containing strains indicates that the 180-

Abbreviations: bp, base pair(s); nt, nucleotide(s).

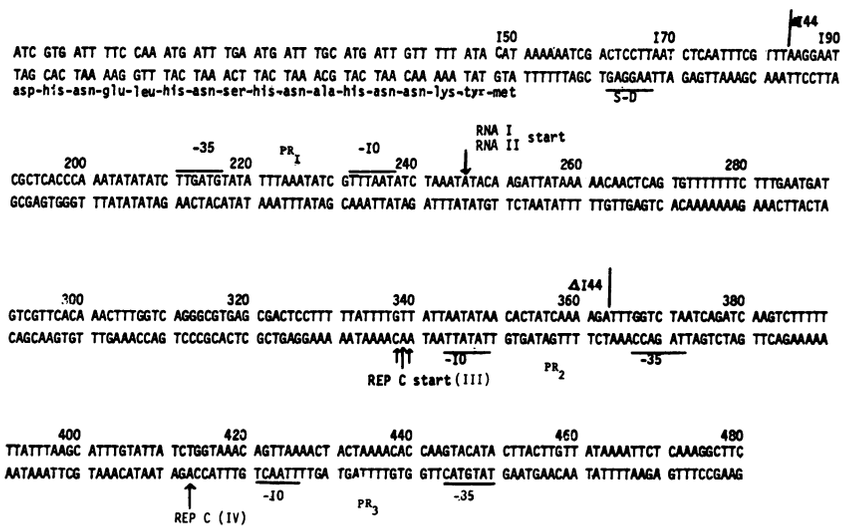

FIG. 1. Plasmid pT181. The nucleotide sequence from position 100 to position 500 is shown indicating the start of the *repC* coding region. Promoter sequences for small RNAs and RepC mRNAs are underlined, with arrows showing the respective initiation sites. Deletion Δ144 in *cop-608* is indicated by two vertical lines. The putative Shine–Dalgarno sequence (S-D) is also underlined.

bp deletion, Δ144, contains sequences required for their synthesis.

***inc3A/copA* Activity Is Correlated with the Ability to Synthesize RNA I and RNA II.** The *inc3A/copA* function of pT181 is ordinarily scored as exclusion of autonomous pT181 by a cloned copy of the plasmid or of a fragment containing the inhibitor gene. This exclusion activity has been mapped on an *Xba* I clone of pT181::pE194 by enzymatic generation of deletions starting from the unique *Pvu* I site at pT181 position zero. Deletions ending before the *Taq* I site at position 158 (deletions 141, 142, and 242) retained the activity whereas deletions extending beyond that site (deletions 143, 240, and 243) lacked it (6). RNA blot analysis of these strains (Fig. 2*c*) indicated that the three CopA$^+$ derivatives synthesize RNA I and RNA II whereas the CopA$^-$ derivatives do not. Further, both small RNAs were found in elevated quantities in extracts from each of several dominant copy mutants but were either reduced in quantity or absent in extracts from recessive (inc3A-sensitive) copy mutants (data not shown). It is concluded, therefore, that one or both of these RNAs is the negative control element(s) for pT181 replication.

Initiation Sites of RNA I and RNA II. Preparations of supercoiled pT181 and *cop-608* DNAs were transcribed with purified RNA polymerases from *B. subtilis* or *E. coli*, because the homologous enzyme from *Staphylococcus aureus* has not been well characterized. RNA molecules synthesized in the presence of [α-^{32}P]UTP *in vitro* were electrophoresed on long thin 6% polyacrylamide 8 M urea gels. As shown in Fig. 3, both polymerases synthesized RNA species indistinguishable in size from RNA I and RNA II from T181 DNA but not from *cop-608* DNA. When the pT181 *Mbo* I D fragment was used as the template for *in vitro* transcription, the two small RNAs were still synthesized but the size of the RNA II was decreased (Fig. 3*b*), suggesting that the *Mbo* I site at position 376 is within the RNA II gene but not within the RNA I gene. This was confirmed by determining the sequences of the 5′ ends of both RNAs. Incubation of pT181 DNA with *E. coli* RNA polymerase in the presence of [γ-^{32}P]ATP gave labeled RNA I and RNA II transcripts, indicating that both are initiated with ATP. [γ-^{32}P]RNA I and [γ-^{32}P]RNA II were isolated from the acrylamide gels and analyzed by the enzymatic sequencing method of Donis-Keller *et al.* (16). The results indicated that RNA I and RNA II have the same 5′-terminal sequence starting at position 246, 95 bases 5′ to the *repC* protein start, and are read from left to right (Fig. 4). Upstream from the transcriptional start site, sequences 5′ G-T-T-T-A-A-T 3′ and 5′ T-T-G-A-T-G 3′ show extensive homology with the consensus −10 and −35 regions of prokaryotic promoters (18). The identification of this promoter sequence is supported by the observation that, in the recessive copy mutant, *cop-619* (7), a single base change from thymine to cytosine at position 233, within the putative −10 sequence (Fig. 1), results in very low levels of both RNA species (data not shown). The termination site of RNA I would then be at about nt 330, before *Mbo* I (376), whereas that of RNA II would be at about nt 400, past *Mbo* I (376), which would explain the foreshortening of RNA II but not of RNA I with the *Mbo* I D template.

Localization of the 5′ Terminus of RepC mRNA. Two approaches were used to map the 5′ end of the putative RepC mRNA identified in Fig. 2—analysis of run-off transcripts generated with *E. coli* RNA polymerase and S1 nuclease mapping of *in vivo* RNA. When *Hpa* II-digested pT181 DNA

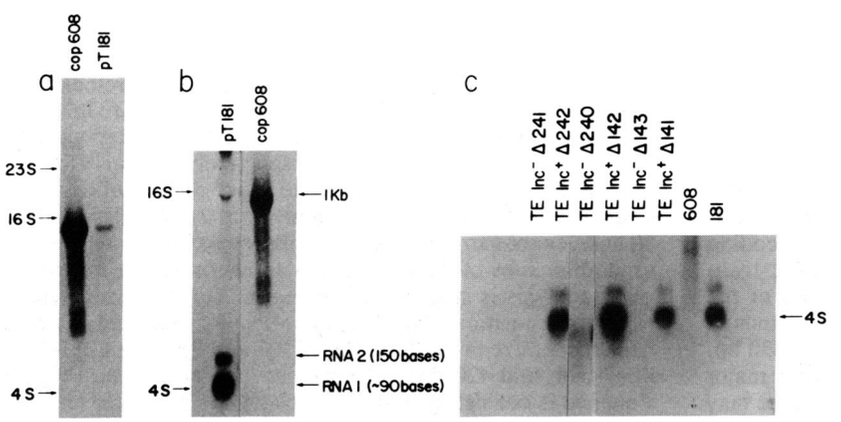

FIG. 2. Blot analyses of RNAs isolated from *S. aureus* strains carrying different plasmids. (*a*) Total RNA isolated from strains carrying pT181 or *cop-608* was fractionated by electrophoresis on 1.5% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose filters, and hybridized to the nick-translated *Taq* I C fragment from pT181. (*b*) As in *a* except that the *Mbo* I D fragment was used as the hybridization probe. (*c*) RNA derived from strains carrying various deletion derivatives of the pT181::pE194 cointegrate was analyzed using the *Mbo* I D fragment as probe.

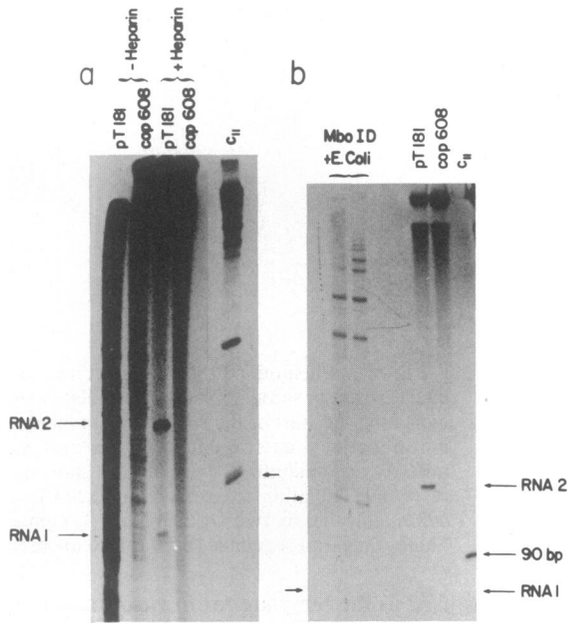

FIG. 3. Transcription of plasmid DNAs *in vitro* with purified RNA polymerases. (*a*) *E. coli* RNA polymerase was used for transcription of supercoiled pT181 and *cop-608* DNAs. C_{11} (provided by R. Losick) is a pBR322 derivative containing a chromosomal *B. subtilis* insert that, when cleaved with *Bam*HI, generates a 90-base run-off transcript. This run-off transcript was used as a length marker (shown with an arrow). The other bands in the marker lane correspond presumably to pBR322 transcripts. End-labeled fragments of pT181 DNA double digested with *Mbo* I and *Dde* I were also used as molecular weight standards (not shown). (*b*) As in *a* except that *B. subtilis* RNA polymerase (provided by R. Losick) was used instead of the *E. coli* enzyme. C_{11} template gives rise to only the 90-nt run-off transcript with *B. subtilis* RNA polymerase. This autoradiogram also shows the transcripts generated by using the *Mbo* I D fragment of pT181 as a template for *E. coli* RNA polymerase.

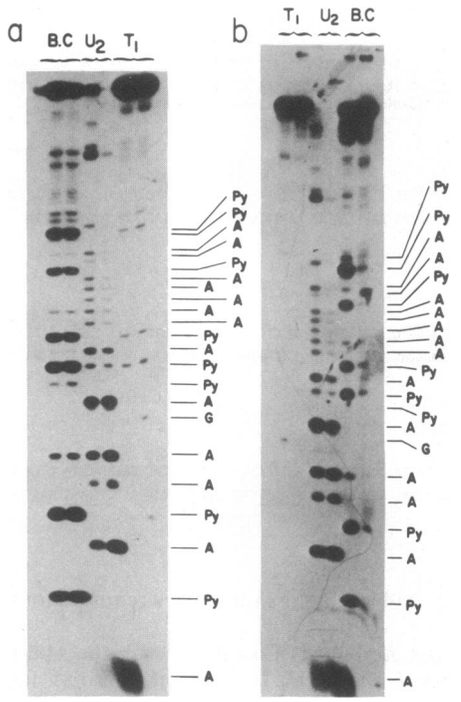

FIG. 4. Nucleotide sequence analysis of *in vitro*-synthesized RNA I and RNA II. 5′-End-labeled RNA I and RNA II were synthesized in the presence of 40 μM [γ-^{32}P]ATP (100 Ci/mmol; 1 Ci = 37 GBq) under *in vitro* conditions with *E. coli* RNA polymerase, using pT181 DNA as template. The two RNAs were separated by electrophoresis on 6% acrylamide/8 M urea gels (17) and RNA sequencing reactions were carried out as described by Donis-Keller *et al.* (16).

was used as a template in the *in vitro* transcription reaction, a 260-base transcript and a minor 330-base transcript were seen along with the two small RNAs. *Pvu* I-digested DNA generated a major transcript about 330 nt long and a longer minor transcript (data not shown). These results place the start site of the major transcript at about nt 330 (Fig. 1), establish its directionality, and confirm its identity as the RepC message. To locate the 5′ terminus more precisely, we isolated a run-off transcript using the *Dde* I C restriction fragment (position 265–769) as the template. This run-off transcript was electrophoresed on a DNA sequencing gel along with samples of the *Dde* I–*Alu* I fragment (position 265–600) that had been 5′-end labeled at the *Dde* I site and fragmented by the standard Maxam–Gilbert sequencing reactions (17).

As shown in Fig. 5*a* and indicated in Fig. 1, the major RepC mRNA species is initiated at about position 339. This result was confirmed by S1 nuclease mapping analysis (15) using total RNA isolated from strains carrying pT181 or *cop-615*, a copy mutant with a single base change at position 310 (7). pT181 DNA was cleaved with *Hpa* II, 5′-end labeled, and then cleaved with *Rsa* I. The 365-bp *Hpa* II–*Rsa* I fragment from position 78 to position 443 was hybridized to total RNA under appropriate conditions and then digested with S1 nuclease. The remaining RNA·DNA hybrids were denatured and electrophoresed on a denaturing polyacrylamide gel. Since the *Hpa* II site at position 78 is within the *repC* coding sequence, this analysis detects only *repC*-specific transcripts. As shown in Fig. 5*b*, S1 nuclease digestion of the hybrids generated a major band of ≈260 bp and two bands of lighter intensity corresponding to RNA species of ≈330 bp. This analysis is consistent with the 5′ terminus of a major *repC* transcript at position 339 and suggests that there may be one or two minor starts at about position 410. Since deletion Δ144 in *cop-608* DNA (Fig. 1) must remove part of the promoter for the position 339 start, there must be an upstream RepC mRNA start for this plasmid. Accordingly, the 5′ terminus of RepC mRNA in *cop-608* DNA was determined. *Hpa* II-cleaved *cop-608* DNA was used to generate a run-off transcript that was electrophoresed along with the chemical sequencing fragments of the *Hpa* I–*Dde* I (position 78–769) fragment from *cop-608* DNA, which had been end labeled at the *Hpa* II site. As shown in Fig. 5*c*, the *cop-608* RepC mRNA starts at position 413. The presence in wild-type extracts of a transcript that protects a 330-nt fragment suggests that this promoter functions in wild type as well as in *cop-608*. Furthermore, transcription *in vitro* of the *Dde* I C fragment from pT181 also results in two run-off transcripts, a major one starting at about position 339 and a minor one starting at about position 413 (results not shown).

DISCUSSION

We have analyzed the transcription pattern of the control region of the *S. aureus* plasmid pT181. We have identified two short clockwise (rightward) transcripts, RNA I and RNA II, ≈80 and 150 nt long, respectively, that are initiated from a single promoter plus two longer counterclockwise (leftward) transcripts, RNA II and RNA IV, that are initiated from tandem promoters ≈75 bp apart and have long untranslated leaders of 190 and 260 nt, respectively. Because RNA I and RNA II are complementary to the 5′ regions of RNA III and RNA IV, we refer to them as 5′ *countertranscripts*. These countertranscripts terminate close to the respective initiation sites of the two mRNA transcripts. The transcription analysis is summarized in Fig. 6. Three transcriptional starts, at positions 246, 339, and 413, are indicated, placing the respective promoters, PR_1, PR_2, and PR_3, at nt 211–238, 347–374, and 420–447, respectively. As noted, the PR_1 sequence is consistent with the prokaryotic promoter consen-

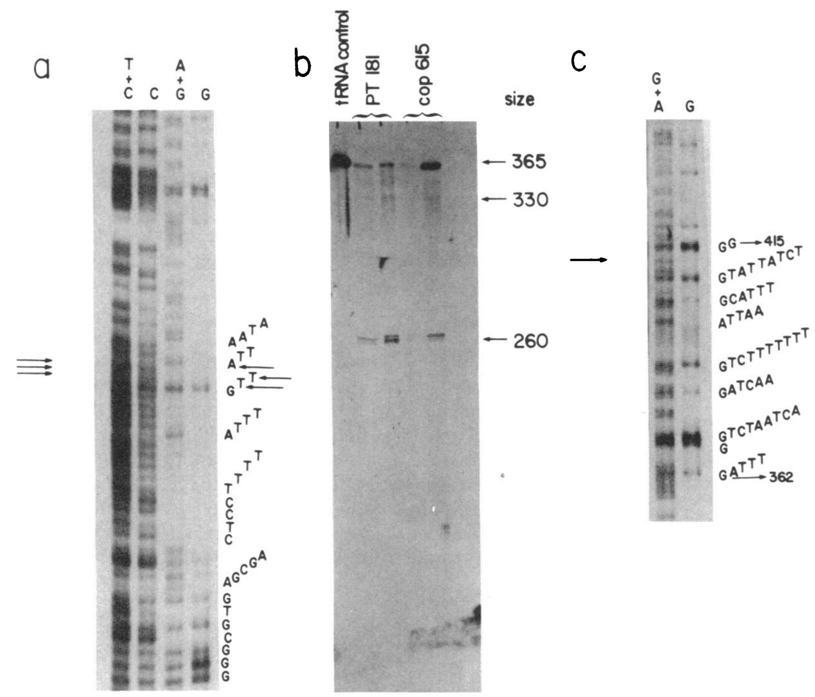

FIG. 5. Localization of transcription initiation site for RepC mRNA. (*a*) A 74-base run-off transcript was generated from purified *Dde* I C fragment by *E. coli* RNA polymerase as described in the legend to Fig. 3 and electrophoresed through an 8% DNA sequencing gel together with base-specific fragments of a *Dde* I–*Alu* I (position 265–600) fragment that had been end labeled at the *Dde* I site. (*b*) S1 nuclease mapping analysis of the 5′ terminus of *in vivo*-synthesized RepC mRNA. Total RNA from cells carrying pT181 or *cop-615* DNA was incubated under hybridization conditions with denatured *Hpa* II–*Rsa* I (position 78–443) DNA that had been end labeled with ^{32}P phosphate at the *Hpa* II site. After hybridization and digestion with S1 nuclease, the remaining DNA·RNA hybrids were electrophoresed through a 6% acrylamide/8 M urea gel. End-labeled fragments of pT181 DNA obtained by double digestion with *Mbo* I and *Dde* I were used as molecular weight standards. The extra bands above the 330-bp one are believed to be the result of breathing during S1 digestion. (*c*) A run-off transcript generated from *Hpa* II-cleaved *cop-608* DNA by *E. coli* RNA polymerase was electrophoresed through a 6% DNA sequencing gel together with G- and (G+A)-specific fragments of an *Hpa* II–*Dde* I (position 78–769 of *cop-608*) fragment that had been end labeled at the *Hpa* II site.

sus sequence. The −10 region for PR_2 contains the sequence T-A-T-A-T-T, which is a good fit to the −10 consensus sequence. In addition, the −35 region, T-A-G-A-C-C, is a recognizable variant of the −35 consensus. The −10 and −35 regions of PR_3, corresponding to the 413 start, T-T-T-A-A-C and A-T-G-T-A-C, respectively, also constitute a recognizable promoter by *E. coli* standards. These two promoters are both functionally weak, as inferred from the RNA blot analysis shown in Fig. 2. The transcriptional data presented here coupled with genetic data presented elsewhere (7) suggest that both leftward transcripts can serve as mRNAs for the replication protein RepC. The relative weakness of their promoters is consistent with an initiator protein that is rate-limiting and is synthesized in very small quantities.

pT181 replication is regulated by an inhibitor–target system in which the countertranscripts serve as the inhibitory

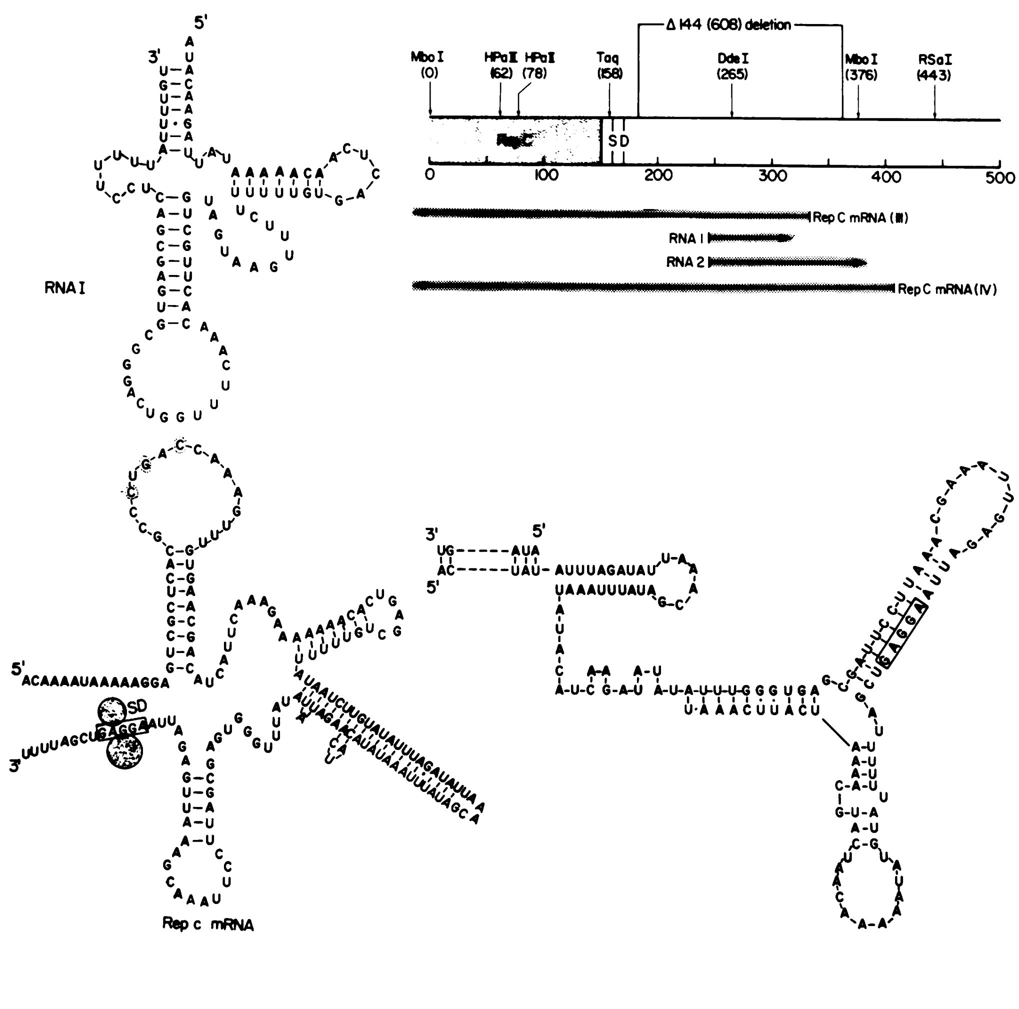

FIG. 6. Transcription map of the copy control region of plasmid pT181 and computer-generated secondary structures of RNA I, the RepC mRNA (RNA III) leader region, and the complex between RNA I and the RNA III leader. The latter structure was obtained by omitting from the computer run the first 90 nt of RepC mRNA. The Shine–Dalgarno sequence A-G-G-A-G is shown boxed.

component. We have found that mutations or deletions of the countertranscript promoter cause an increase in copy number, as do mutations affecting the terminator of either species (J. Kornblum and S. Projan, personal communication). The target component is defined by copy mutations with decreased sensitivity to the wild-type inhibitor; these overproduce both countertranscripts (unpublished data). All of these mutations and deletions map in a short segment of the RNA III/RNA IV leader region, indicating that the determinants of inhibitor and target overlap (7). Because we can identify no significant open reading frame in the RNA III/RNA IV leader region, we conclude that the inhibitory activity is due to the countertranscripts themselves.

It is possible that either of the leftward transcripts could serve as a primer and that regulation of replication could be at the level of primer formation as with ColE1 (10). However, the indirect nature of the regulation system suggests that RepC synthesis rather than primer formation is the primary regulatory target and evidence presented elsewhere (6) supports this view. A consideration of predicted secondary structures for the leader transcripts and countertranscripts suggests a possible mechanism for this regulation. Secondary structures of RNA I and an RNA I–RNA III duplex were predicted by computer analysis according to the program of Zucker and Stiegler (19). As shown in Fig. 6, RNA I can form a folded structure with a large central loop. Most of the copy mutants that have been analyzed by DNA sequence analysis (7) have base changes clustered in this loop area (Fig. 6). Thus, it seems likely that the primary interaction between RNA I and RepC mRNA involves base pairing between the corresponding single-stranded loops of the two complementary transcripts in much the same way as has been found for the ColE1 (9, 20) and IncFII plasmids (11, 21). Changes in the loop structure may result in decreased affinity between the inhibitor and its target as a consequence either of less effective base pairing (A·U replacing G·C) or of alterations in secondary structure. We note that the putative ribosome binding site for RepC translation is located in the distal stem of a potential stem and loop structure (from nt 158–192) whose formation would be expected to interfere with translation of the message. As shown in Fig. 6, the secondary structure analysis is consistent with the hypothesis that RNA I facilitates formation of the above-mentioned stem and loop structure; we propose, therefore, that regulation of pT181 replication is likely to be at the level of RepC translation, which would be inhibited by the formation of a countertranscript–mRNA complex. The computer-predicted optimal folding for RNA II and the RNA IV leader display the same structures in the target region as do RNA I and the RNA III leader. These results therefore place pT181 within the group of regulatory systems that involve complementary base pairing between a functional transcript and a shorter 5′ countertranscript, such that function of the major transcript is inhibited. These systems include two unrelated groups of plasmids, the ColE1 group (10) and the IncFII group (22), and transposon Tn*10* (23), and it seems safe to say that countertranscript regulation is now established and doubtless other examples will soon be identified.

These systems are distinctly different from those of plasmids such as R6K, RK2, F, and P1, which also encode diffusible initiator proteins. For R6K, it has been shown that the negative regulator is the initiator protein itself, which is autoregulated, and its negative function involves DNA binding at directly repeated oligonucleotides near the replication origin (24).

The architectural parallel among the countertranscript regulons is particularly striking because the base sequences involved are entirely unrelated and there are two different control mechanisms: inhibition of preprimer processing for ColE1-like plasmids (10) and inhibition of mRNA translation for the IncFII plasmids (22), Tn*10* (23), and probably also pT181. Given unrelated sequences and differing functions using the same functional organization in unrelated species, one is led to conclude that these regulatory systems have evolved independently and therefore constitute convergent evolution at the molecular level.

We are grateful to R. C. Crouch for carrying out the computer analysis and to R. Rownd for the idea of eliminating the region of mRNA leader complementary to the countertranscript in the computer analysis of secondary structure. We thank our laboratory colleagues, Stephen Carleton, Dr. Steve Projan, Sarah Highlander, and Dr. Alexandra Gruss, for both intellectual and experimental contributions to this work. This work was supported by Grant MV-8E from the American Cancer Society (to R.P.N.). C.C.K. holds a postdoctoral fellowship from the Leukemia Society of America, Inc.

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