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Replication control in plasmid R1: duplex formation between the antisense RNA, CopA, and its target, CopT, is not required for inhibition of RepA synthesis

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The replication frequency of plasmid R1 is regulated by an antisense RNA, CopA, which inhibits the synthesis of the rate-limiting initiator protein RepA. The inhibition requires an interaction between the antisense RNA and its target, CopT, in the leader of the RepA mRNA. This binding reaction has previously been studied *in vitro*, and the formation of a complete RNA duplex between the two RNAs has been demonstrated *in vitro* and *in vivo*. Here we investigate whether complete duplex formation is required for CopA-mediated inhibition *in vivo*. A mutated *copA* gene was constructed, encoding a truncated CopA which is impaired in its ability to form a complete CopA/CopT duplex, but which forms a primary binding intermediate (the 'kissing complex'). The mutated CopA species (S-CopA) mediated incompatibility against wild-type R1 plasmids and inhibited RepA-LacZ fusion protein synthesis. Northern blot, primer extension and S1 analyses indicated that S-CopA did not form a complete duplex with CopT *in vivo* since bands corresponding to RNase III cleavage products were missing. An *in vitro* analysis supported the same conclusion. These data suggest that formation of the 'kissing complex' suffices to inhibit RepA synthesis, and that complete CopA/CopT duplex formation is not required. The implications of these findings are discussed.

Key words: antisense RNA/plasmid R1/RNase III/replication control/RNA duplex

Introduction

Antisense RNAs act as inhibitors of gene expression in many prokaryotic systems. These systems are found almost exclusively in accessory elements such as plasmids, phages and transposons (for a recent review, see Simons and Kleckner, 1988). The inhibitory function of an antisense RNA can involve the down-regulation of synthesis of a gene product, or be manifest as an inhibition of primer formation, as observed in replication control of the ColE1-related plasmids (Polisky, 1988).

In the antisense RNA systems that have been studied in some detail *in vitro*, complete RNA duplex formation between inhibitor and target RNAs has been demonstrated (Tomizawa, 1984; Persson *et al.*, 1988; Kittle *et al.*, 1989). Most antisense/target RNA pairs show rapid duplex formation with an apparent second order rate constant in the order of 10^6 M^{-1} s^{-1}. Tomizawa (1990a,b) and Persson *et al.* (1990a,b) have dissected the kinetic pathways, from the free RNA species to the complete RNA duplex for RNAI/RNAII of plasmid ColE1 and CopA/CopT of plasmid R1, respectively. Their results show that the rate-limiting step in the binding pathway is the formation of an early intermediate (a 'kissing complex') which involves primarily loop sequences in the interacting RNA species.

Duplexes between antisense RNAs and their target RNAs have also been shown to be formed *in vivo*, as demonstrated by the observation that the host enzyme RNase III cleaves the RNA duplex specifically (Blomberg *et al.*, 1990; Case *et al.*, 1990; Krinke and Wulff, 1990a,b; K.Gerdes, personal communication).

Assuming that most, if not all, antisense RNAs can form duplexes with their target counterparts *in vivo*, the question arises whether the inhibitory function of an antisense RNA requires full duplex formation, or whether the primary interaction may be sufficient for inhibition. From the proposed mechanisms by which various antisense RNAs affect the activities of their target RNAs, we can identify some cases where a requirement for complete duplex formation is expected, whereas in other cases such a requirement *a priori* does not seem obvious. The first class of systems can be regarded as 'direct' ones, implying that the sites of antisense RNA binding and inhibition are the same (or overlapping). The second class of systems are the 'indirect' ones, where the site of antisense/target RNA binding is separate from the presumed site at which the effect is accomplished. Inhibition of transposase translation in IS*10* has been shown to involve sequestering of the ribosome binding site (RBS) in an RNA duplex with the antisense RNA, RNA-OUT (Ma and Simons, 1990). The blocking of ribosome access to the mRNA suggests a requirement for duplex formation. Likewise, the OOP-RNA of phage λ appears to require complete duplex formation with the 3′-region of the cII mRNA, since the inhibition is dependent on an RNase III-mediated cleavage of the RNA duplex and subsequent destabilization of the mRNA (Krinke and Wulff, 1990a). In several plasmids, the antisense RNAs regulate by an indirect mechanism, and in at least two of them, pT181 (Novick *et al.*, 1989) and ColE1 (Masukata and Tomizawa, 1986), the regulation involves altered folding pathways for the target RNAs. Here, it is conceivable that the effect on the folding of the nascent target RNAs does not require complete duplex formation, since the trapping of RNA folding intermediates may be accomplished by loop-loop contacts.

The fact that, in plasmid R1, the site of CopA binding in the leader of the RepA mRNA (CopT) is situated ~80 nucleotides upstream of the presumed site of inhibition (the translation initiation region of the *repA* reading frame) also suggests an indirect mode of inhibition. The mechanism by which RepA synthesis is down-regulated is not understood, but the effects of CopA both on folding of the RepA RBS region (Dong *et al.*, 1987) and on duplex-dependent enhanced mRNA decay (see Blomberg *et al.*,

1990) may represent possible pathways. We have previously investigated the *in vivo* transcription patterns in strains harbouring various R1 plasmids and subclones thereof. Blomberg *et al.* (1990) have shown that RNase III processes the duplex between CopA and CopT and appears to be involved in the inhibitory event. If RNase III-catalysed processing is a key event in the inhibition of RepA synthesis, then formation of a complete CopA/CopT duplex should be required for inhibition.

To test the requirement for complete duplex formation between antisense RNA and target RNA *in vivo*, we made use of some experimental results obtained recently. Persson *et al.* (1990a,b) demonstrated that a truncated CopA species, denoted CopI, was very ineffective in forming a persistent duplex with CopT *in vitro*, whereas it formed the kissing complex as efficiently as wild-type CopA. This truncated CopA, consisting only of stem−loop II (see Figure 1 for an example of such a CopA species), acted as a competitive inhibitor of CopA binding to the target RNA. We therefore reasoned that a mutated *copA* gene which produces a similarly truncated CopA *in vivo* could be used to assess the biological function of a CopA species unable to form a complete duplex with its target RNA.

Here we report such an analysis and show that formation of a kissing complex appears to be sufficient for most, if not all, of the observed CopA-dependent inhibition of RepA synthesis.

Results

A 5′-truncated CopA species (S-CopA) expresses incompatibility towards a wild-type R1 replicon

To obtain a gene producing a CopA RNA species analogous to the one previously shown to be essentially inactive in duplex formation *in vitro*, but capable of forming the primary 'kissing intermediate' (Persson *et al.*, 1990a,b), we introduced a deletion of 30 bp into the *copA* gene by oligo-directed mutagenesis (see Materials and methods). Plasmid pGW58-SCA has a deletion of the sequences encoding the middle region and the 5′-proximal stem−loop I and was expected to produce a CopA species of the sequences and structures shown in Figure 1. This CopA RNA, denoted S-CopA (short CopA), carries one additional nucleotide at its 5′-end compared with the comparable CopA species studied by Persson *et al.* (1990b), since we wished to preserve the authentic transcription initiation nucleotide.

CopA RNA is an incompatibility element, and, hence, the presence of a wild-type *copA* gene *in trans* inhibits replication of a co-existing wild-type R1 plasmid, resulting in loss of the latter plasmid. We therefore tested whether the truncated *copA* gene mediated incompatibility against a wild-type R1 plasmid. Table I shows the result of such an experiment (see Materials and methods for details). Plasmid pKN1562 (a mini-R1 derivative) was displaced by introducing plasmids carrying either the wild-type (pGW58) or truncated *copA* gene (pGW58-SCA), but not by the vector itself (pMa5.8). The pGW58 constructs, in addition to the *copA* gene, also contain sequences of R1 encoding the *repA* promoter, so that CopA activity is lower due to convergent transcription (Stougaard *et al.*, 1982). In agreement with this, plasmids carrying only the *copA* genes and lacking a *repA* promoter showed stronger incompatibility against pKN1562 (Table I: pGW643, pGW643-SCA). The data indicate that the deletion in the *copA* gene did not impair its function as an incompatibility element, and that the inhibitory activity of S-CopA is similar to that of wild-type CopA.

S-CopA is almost as efficient as wild-type CopA in inhibition of RepA−LacZ activity

To obtain more quantitative measurements of the inhibitory activities of the mutant and wild-type *copA* genes, we measured their effect *in trans* on RepA−LacZ protein synthesis, with the fusion protein encoded on a co-resident plasmid. Plasmids pJL99 and pJL133 harbour translational *repA−lacZ* fusions on p15 or pMB1 replicons, respectively. Plasmid pJL133 has an ~5-fold higher copy number than pJL99 (data not shown). Due to convergent transcription, both plasmids synthesize only small amounts of endogenous CopA RNA (Stougaard *et al.*, 1982; P.Blomberg, unpublished). External CopA was provided by co-existing, compatible replicons; for pJL99, the second replicon was a derivative of pSP64 (Melton *et al.*, 1984), and derivatives

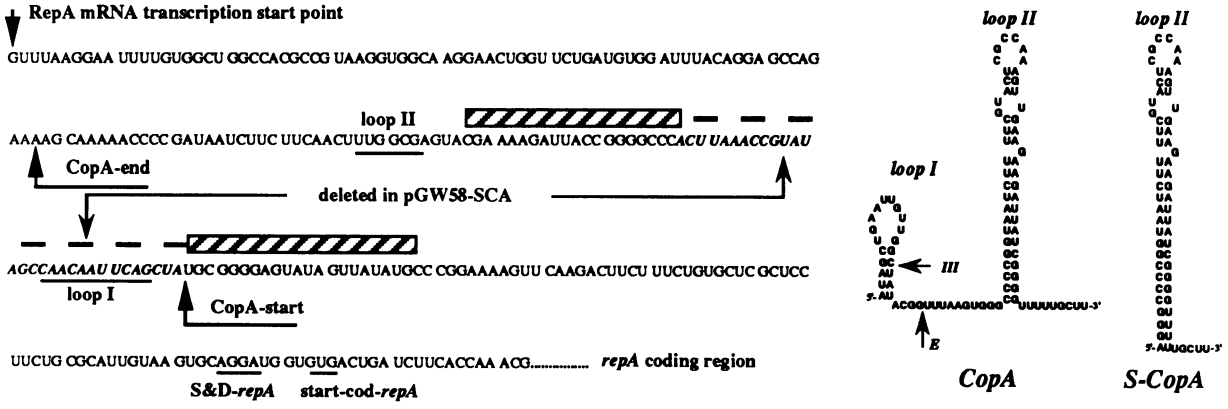

Fig. 1. Nucleotide sequence of mutant and wild-type CopT RNA, and the secondary structures of mutant and wild-type CopA RNAs. The left-hand panel shows the sequence of the leader region of the RepA mRNA (CopT) with several relevant landmarks. Letters in italics correspond to nucleotides deleted in plasmid pGW58-SCA. The position of the oligodeoxyribonucleotide used for the creation of the mutation is indicated by the striped bar (interrupted by the deleted nucleotides). The right-hand panel shows the secondary structures of wild-type CopA and mutant CopA (S-CopA) (Wagner and Nordström, 1986; E.G.H.Wagner, unpublished). The arrows labelled III and E indicate the identified cleavage positions of RNase III and E, respectively (Blomberg *et al.*, 1990; P.Blomberg, unpublished). Note that the cleavage by RNase III occurs only when CopA is present in a duplex with CopT (Blomberg *et al.*, 1990, and see Results).

of pSU2718 (Martinez *et al.*, 1988) were used in conjunction with pJL133. This experimental design also permits an assessment of the effect of CopA activity at different CopA/CopT ratios, since the copy numbers of the CopA donor plasmids are reversed relative to those of the test plasmids. Table II shows that with both test plasmids (pJL99 and pJL133) the *in vivo* β-galactosidase activity was severely inhibited by both wild-type CopA and S-CopA. In both cases, CopA appeared to be approximately three times as effective as S-CopA.

S-CopA is severely impaired in its ability to form a duplex with CopT in vitro

The *in vitro* binding properties of a short CopA species, obtained by *in vitro* manipulations (called CopI, Persson *et al.*, 1990a,b), have been studied previously. The mutated *copA* gene in pGW58-SCA should yield a product which contains only one additional 5′-nucleotide compared with CopI, and presumably folds into an identical structure (see Figure 1). We investigated whether *in vitro* transcription of the mutant *copA* gene yielded the expected product. S-CopA was shown to be one nucleotide longer than the previously described CopI species (data not shown). The rate constant for duplex formation with CopT was found to be the same for S-CopA and CopI. Both mutant RNA pairs showed a decrease in the apparent second order binding rate constant of ~130-fold, compared with a wild-type CopA/CopT pair, at a CopT concentration of 4×10^{-9} M (data not shown). In conclusion, the *in vitro* properties of the S-CopA species were indistinguishable from those of the previously characterized CopI species, implying that it is severely impaired in its ability to form a complete duplex with CopT, but is capable of forming the primary binding intermediate.

Table I. Both *S-copA* and *copA* (wt) genes express incompatibility against plasmid R1

Plasmids	Colonies on Km plates (mini-R1)	Colonies on Ap plates (CopA-donor plasmid)
pKN1562 + pGW58-SCA	~500	~3000
pKN1562 + pGW58	50–100	~3000
pKN1562 + pMa5.8	~3000	~3000
pKN1562 + pGW643-SCA	0	~3000
pKN1562 + pGW643	0	~3000
pKN1562 + pSP64	~3000	~3000

Table II. S-CopA and CopA (wt) inhibit the synthesis of translational RepA–LacZ fusion protein *in vivo*

Plasmid with *repA–lacZ* fusion	CopA-donor plasmid	Relative β-Gal activities[a]	Degree of repression[b]
pJL99	pSP64	100.0	
pJL99	pGW643	0.8	125×
pJL99	pGW643-SCA	1.6	62×
–	pSP64	<0.1	
pJL133	pSU2718	100.0	
pJL133	pGW15	4.4	23×
pJL133	pGW15-SCA	12.2	8×

[a] The β-galactosidase activities are expressed as relative values, and the activity of cells carrying the fusion construct in the presence of insertless vector was set to 100%.
[b] The repression effect is the ratio between activities from cells harbouring the fusion plasmid in the presence of the insertless control plasmid and those in the presence of a *copA* gene donor plasmid. In the absence of external CopA activity, cells carrying pJL99 had a 2-fold higher activity than those harbouring pJL133.

S-CopA does not form a persistent duplex with CopT in vivo

Duplex formation between CopA and CopT can be assayed *in vivo*, since Blomberg *et al.* (1990) demonstrated that the duplex is specifically processed by the host enzyme RNase III. We therefore employed Northern analyses to test for duplex-specific cleavages under conditions where S-CopA represses RepA–LacZ activity (see above). The analyses were done with RNA from both series of cell cultures (the pJL99 and the pJL133 series; Table II). Total RNA was extracted from the same cultures and at the same time points as used for the β-galactosidase activity determinations. Figure 2 shows an analysis of CopA RNA species from the pJL99 series as well as from cells carrying only the CopA donor plasmids. RNA from strains with plasmid pGW643, encoding a wild-type *copA* gene, displays the characteristic two bands corresponding to full-length CopA and a processed form, respectively. The lower band is generated independently of CopT transcription (Blomberg *et al.*, 1990) and is dependent on the presence of RNase E (P.Blomberg, unpublished). The lane designated pGW643-SCA shows a

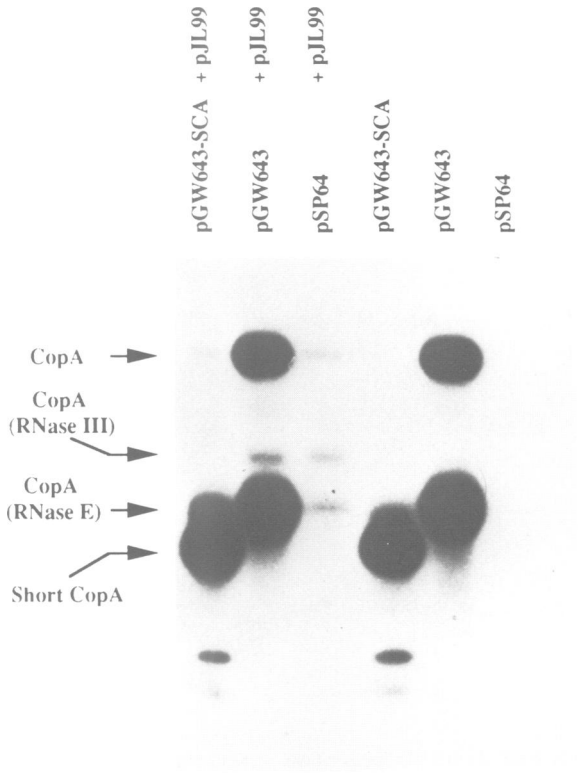

Fig. 2. Northern analysis of CopA RNA species in the presence and absence of a co-resident *repA–lacZ* fusion plasmid. Total RNA was separated on an 8% sequencing gel, and Northern analysis was performed as described in Materials and methods. Plasmids contained within the cells used for RNA extraction are indicated. Positions of bands corresponding to relevant RNAs (discussed in the text) are shown.

major band whose migration corresponds to the expected size of the mutant CopA of 61 nucleotides. Two minor bands that probably represent breakdown products can be seen below. Their origin was not investigated further. The CopA RNA pattern from a strain harbouring pJL99 and the insertless vector plasmid pSP64 showed three bands, where the upper and lower bands again represent full-length CopA and the RNase E-processed form, respectively. The middle band represents the duplex-dependent CopA species generated by RNase III cleavage (Blomberg *et al.*, 1990). Introduction of a wild-type *copA* gene (lane pJL99 + pGW643) resulted in a drastic increase in the intensity of the upper and lower bands, indicating a large increase in *copA* gene dosage, and, hence, in intracellular concentration of CopA RNA. Only a slight enhancement of intensity of the middle band was observed, indicating that, as expected, the duplex-dependent cleavage was limited by the low abundance of CopT RNA. The presence of the mutant *copA* gene on the donor plasmid

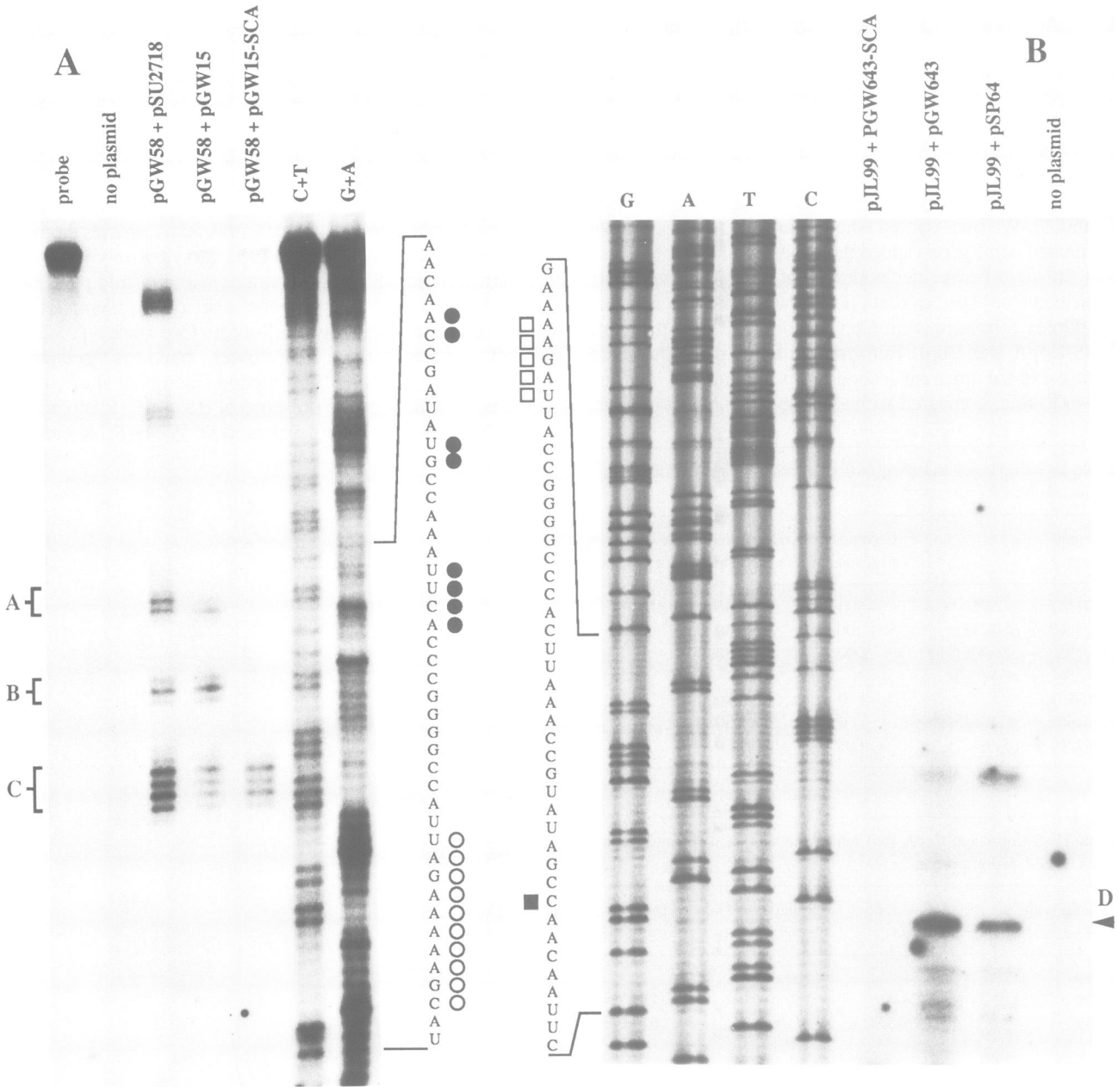

Fig. 3. Analysis of cleavage positions in RepA mRNA. (**A**) S1 analysis was performed on total RNA extracted from cells harbouring the plasmids indicated above the lanes. A Maxam–Gilbert sequencing reaction performed on the S1 probe (see Materials and methods) was used as a marker. A relevant segment of the sequence is shown alongside the autoradiogram. Filled circles correspond to the sets of bands denoted A, B and C. Open circles indicate the approximate positions of S1-protected fragments that should appear if RNase III cleavage of an S-CopA/CopT RNA duplex had occurred. Band C represents a duplex-independent 3′-endpoint in the RepA mRNA, resulting from transcriptional pausing or from a block in exonucleolytic degradation (Blomberg *et al.*, 1990). (**B**) Primer extension analysis was performed on total RNA extracted from cells harbouring the plasmids indicated above the lanes (see Materials and methods). Dideoxy sequencing reactions, performed on R1-DNA using the same primer, were used as a reference. The filled square corresponding to band D indicates the major RNase III-generated cleavage position in CopT. The approximate positions of expected, RNase III-generated, RepA mRNA 5′-endpoints (if an S-CopA/CopT RNA duplex had formed) are indicated by open squares. Note that the RNA sequence shown alongside the autoradiogram is complementary to the DNA sequence seen on the film.

yielded two results. The middle band corresponding to the RNase III-processed CopA was practically absent, and no new bands appeared below the band representing the S-CopA signal (cf. lane pJL99 + pGW643-SCA with lane pGW643-SCA). This result suggests that (i) due to its much higher intracellular concentration, S-CopA outcompetes the endogenous wild-type CopA in binding to CopT, and (ii) the interaction between S-CopA and CopT does not result in an RNA duplex, as indicated by the absence of RNase III cleavage. If duplex-dependent RNase III cleavage had occurred, we should have detected an RNA species ~13–15 nucleotides shorter than S-CopA, since the enzyme introduces a primary cut at this distance from the 5′-end of the shorter RNA species in an RNA duplex (Blomberg *et al.*, 1990; Krinke and Wulff, 1990a,b; E.G.H.Wagner, unpublished). Northern analyses of CopT RNAs supported these conclusions (data not shown).

To look directly for the appearance of RNase III cleavages in CopT, dependent on the formation of an S-CopA/CopT duplex, we performed primer extension and S1 analyses. Figure 3A shows an autoradiogram of an S1 analysis, and Figure 3B that of a primer extension experiment. The pattern of bands corresponding to RepA mRNA 3′- and 5′-ends was essentially as shown previously (Blomberg *et al.*, 1990). The positions of RNA endpoints dependent on duplex formation between wild-type CopA and CopT are denoted A, B and D, and are indicated by filled symbols. The expected approximate positions of RNase III cleavages due to the formation of a S-CopA/CopT duplex are indicated by open symbols. No bands consistent with such cleavages were apparent. In conclusion, S-CopA appears to interact with CopT without forming a complete duplex under conditions where RepA–LacZ activity is severely repressed.

S-CopA does not form a double-stranded RNA substrate for RNase III in vitro

In the analyses presented in Figures 2 and 3, we could not detect any duplex-dependent cleavage of either S-CopA or CopT RNAs when present in the same cell. Although we infer that this observation is indicative of an interaction leading only to the primary kissing intermediate, and a blockage of the pathway to the full duplex due to the absence of the necessary structure/sequence determinants identified previously (Persson *et al.*, 1990a), we wanted to test whether an artificially made S-CopA/CopT duplex could serve as a substrate for RNase III *in vitro*. Figure 4A shows such an analysis, and a simplified interpretation of the results is represented in the drawing in Figure 4B. ^{32}P-labelled CopT was synthesized and purified (Materials and methods). Binding of ^{3}H-labelled CopA or S-CopA was permitted to occur, and subsequently RNase III was added. As can be seen (Figure 4A, lane native; CopT/CopA), CopA RNA formed a duplex with CopT which was cleaved by RNase III to yield the expected pattern. Under the same conditions, S-CopA did not induce cleavage of CopT, suggesting the absence of a duplex structure (Figure 4A, lane native; CopT/S-CopA). When CopA or S-CopA were heated and slowly cooled in the presence of CopT to permit the

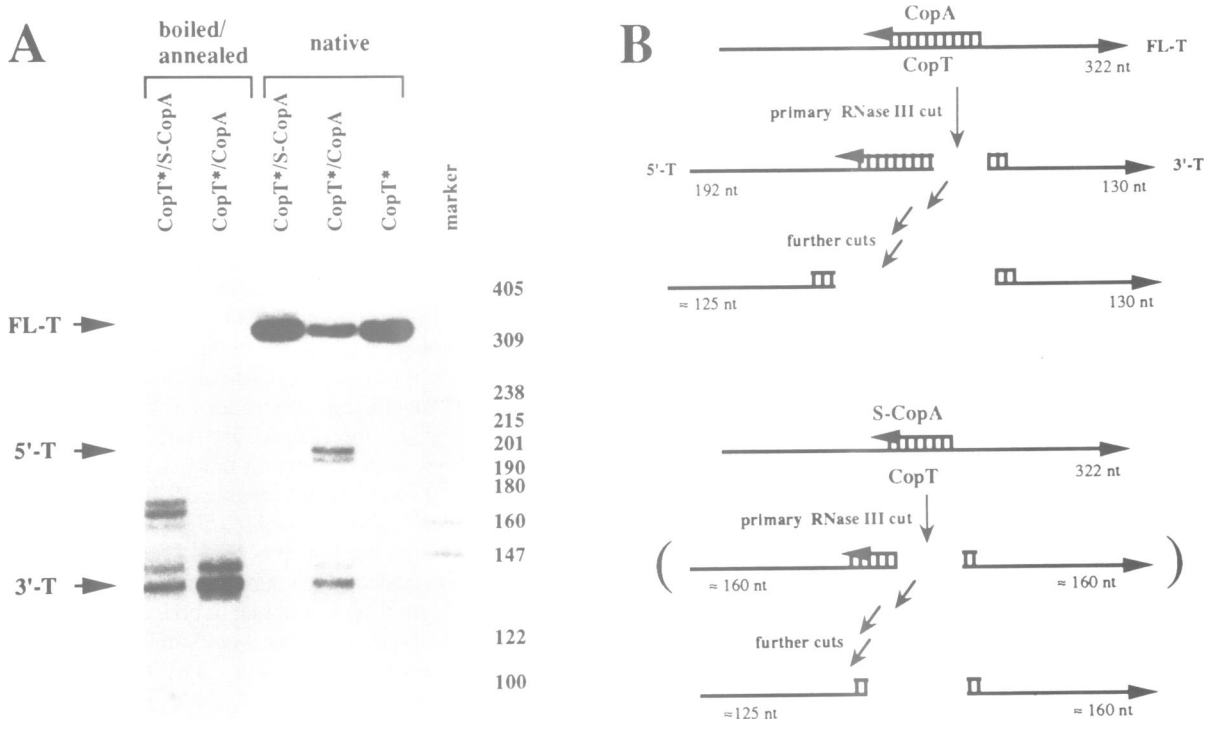

Fig. 4. RNase III cleavage of CopA/CopT duplexes formed *in vitro*. (**A**) Native and artificial duplexes between CopA (or S-CopA) and CopT were cleaved with RNase III as described in Materials and methods. The asterisks indicate that CopT is the only ^{32}P-labelled RNA species. Arrows indicate the bands corresponding to full-length CopT (FL-T) and the fragments resulting from the primary RNase III-cut in the CopA/CopT duplex (3′-T, 5′-T). See (B) for a simplified interpretation. (**B**) Schematic representation of the RNase III cleavages occurring in a CopA/CopT duplex. The primary cuts in CopA/CopT are the ones identified both *in vitro* and *in vivo* by Blomberg *et al.* (1990). Parentheses in the case of S-CopA/CopT indicate the absence of this cleavage pattern due to the difficulty of forming this duplex species from native RNAs (see text). The approximate lengths of the RNA fragments resulting from RNase III cleavages are indicated.

formation of RNA duplexes in both combinations, RNase III was able to digest both substrates. The size difference between the resulting 3′-CopT fragments corresponds to the difference in 5′-start points of the two CopA species (Figure 4A, lanes boiled/annealed; CopT/CopA and CopT/S-CopA). CopA, S-CopA and CopT alone were not cleaved by RNase III under these conditions (lane CopT, and data not shown). Thus, both the *in vivo* results shown in Figures 2, 3 and the *in vitro* results in Figure 4 suggest that the absence of duplex-dependent RNase III cleavage reflects an inability of S-CopA to proceed from the primary intermediate state to the full RNA duplex.

Discussion

The results presented in this communication indicate that inhibition of RepA synthesis by CopA RNA can be accomplished without complete pairing between the antisense RNA and its target. This conclusion is based on the results obtained with a CopA species that can form a kissing intermediate, but is severely impaired in its ability to form a complete RNA duplex. A truncated *copA* gene was constructed, and the effect of the mutant CopA on plasmid replication and RepA−LacZ protein synthesis was determined. In both kinds of experiments, S-CopA appeared to function as an efficient inhibitor, comparable with intact CopA. The most critical point in the present analysis is the demonstration that, to the best of our knowledge, S-CopA achieves its effect without forming a complete duplex with CopT. We do not have methods at our disposal that enable us to demonstrate CopA/CopT duplexes (or the absence of them) directly *in vivo*. The absence of duplex formation between S-CopA and CopT *in vitro* cannot be taken as a safe indication that the binding process occurs in the same fashion *in vivo*. Although no involvement of proteins, plasmid-encoded or host chromosome-encoded, has been demonstrated in the binding process between the antisense and target RNAs of plasmid R1, this possibility cannot be ruled out. It is thus conceivable that RNA binding proteins could affect a melting of RNA secondary structure so that, in contrast to the *in vitro* situation, the kissing intermediate could be converted into a full duplex *in vivo*. A Rom/Rop-like protein like the one encoded by ColE1 would not be a candidate for such a function, since it stabilizes the initial complex rather than affecting the hybridization rate constant (Tomizawa, 1985). Recently, Andersen and Delihas (1990) have demonstrated that MicF RNA binds to a protein present in *E.coli* cell extracts. It is not known whether binding of the protein affects formation of the MicF RNA/OmpF mRNA duplex *in vivo* or *in vitro*. We have therefore probed for the presence of CopA/CopT or S-CopA/CopT RNA duplexes by making use of the observation that antisense/target RNA duplexes are efficiently cleaved by the host enzyme RNase III (Blomberg *et al.*, 1990; Krinke and Wulff, 1990a,b; Ma and Simons, 1990). Using Northern analysis, primer extension and S1 nuclease protection assays, we show here that the expected duplex-specific, processed, RNA species are present in cells containing a wild-type *copA* gene in addition to a wild-type *repA−lacZ* fusion plasmid construct, but are absent in cells where S-CopA is supplied *in trans* (Figure 3). We estimate that if inhibition of RepA−LacZ fusion protein synthesis had been dependent on S-CopA/CopT duplex formation, the analysis shown above would have detected the processed RNAs. We also show that a complete duplex between S-CopA and CopT, formed artificially by heating and cooling the two RNAs, is a substrate for RNase III *in vitro*. This experiment rules out that this particular RNA duplex is refractory to RNase III processing.

S-CopA was shown to be efficient in inhibiting RepA−LacZ synthesis, and showed a degree of inhibition only ~3-fold lower than that obtained with intact CopA. The small difference in effectiveness between S-CopA and CopA could either reflect the ~2-fold difference in intracellular steady-state concentration of the antisense RNA between cells containing either the mutant or the wild-type gene (data not shown) or be indicative of a higher specific activity of the wild-type gene product. In either case, it is striking that the first 30 nucleotides of CopA contribute very little to the inhibitory activity of the antisense RNA.

Does antisense RNA-promoted inhibition in other, indirect, control systems function in the absence of complete duplex formation? To our knowledge, this problem has not previously been addressed, and no *in vivo* results clearly answer this question. However, Lin-Chao and Cohen (1991) have demonstrated that a mutated RNAI is functional in replication control of plasmid ColE1. This mutant RNA, called pppRNAI$_{-5}$, lacks five nucleotides at the 5′-end of RNAI. A requirement for these nucleotides for RNAI/RNAII duplex formation has been demonstrated previously *in vitro* (Tomizawa, 1984; Tamm and Polisky, 1985). We suggest, therefore, that duplex formation might not be required for the inhibitory activity of the antisense RNAs in the replication control of the ColE1-like plasmids.

Does the repression level obtained in the presence of mutated versus wild-type CopA make sense when we consider the kinetics of RNA−RNA interaction? Persson *et al.* (1990a,b) demonstrated that the binding between CopA and CopT is kinetically irreversible, with the formation of the kissing complex being the rate-limiting step. S-CopA (there called CopI) was shown to form the intermediate with CopT with the same kinetics as CopA, and was only impaired in a subsequent step. In addition, the dissociation constant of the kissing intermediate determined *in vitro* was $\sim 10^{-12}$ M (Persson *et al.*, 1990b). This implies that under *in vivo* conditions, the half-life of the kissing complex would by far exceed the time intervals relevant for its regulatory function. In conclusion, differences in kinetics between S-CopA and wild-type CopA are not likely to lead to inconsistencies with respect to the results obtained.

If we accept that kissing complex formation between S-CopA and CopT suffices to inhibit RepA synthesis, we have to consider which mechanisms could account for the results obtained. One possibility is that the mechanism of inhibition is the same as for CopA, and that in both cases, RNA duplex formation is not required. This would imply that CopA/CopT duplex formation is fortuitous, and may simply be a consequence of the ability of these RNAs to undergo complete hybridization. This might also suggest that the most likely effect of both mutant and wild-type CopAs is an effect on RepA mRNA folding pathways, and would rule out duplex-dependent processing by RNase III as the major factor in the observed inhibition. This is apparently contradictory to results reported by us recently (Blomberg *et al.*, 1990), where we presented data that suggested the involvement of RNase III in the regulation of RepA synthesis. One

suggestion was that RNase III-mediated cleavage could lead to a facilitated decay of the RepA mRNA. We have tested the effect of duplex-dependent cleavage on RepA mRNA stability and found that it decreases its half-life by a factor of two to three (P.Blomberg, unpublished). The magnitude of the change in mRNA half-life is too small to account for the effect of CopA on RepA expression, therefore, duplex-dependent cleavage by RNase III cannot be the major factor in the inhibition pathway. The effect of RNase III on the replication properties of R1 plasmids is complex and will be analysed further. A second possibility is that several pathways for repression are possible, and that in the absence of RNA duplex formation, S-CopA inhibits RepA synthesis via a pathway different from that used in the presence of CopA. At this point, we cannot distinguish between these possibilities.

Recent data obtained in our laboratory could explain the reason for the difficulty in obtaining evidence for transcription termination, RNA folding changes near the RepA RBS, and facilitated mRNA decay. We can show now that a leader peptide is encoded in the intergenic region between *copA* and the *repA* reading frame (P.Blomberg and E.G.H.Wagner, in preparation). The gene encoding this reading frame is highly expressed, regulated by CopA, and we can demonstrate translational coupling to RepA. The Shine–Dalgarno sequence of the leader peptide is located only two nucleotides downstream of the 5′-end of CopA RNA when present in a duplex with CopT, which might suggest steric interference with ribosome binding. Alternatively, secondary structure changes affecting the ribosome binding site of the leader peptide reading frame (rather than the RepA RBS) could still represent a mechanism accounting for both the CopA and the S-CopA-mediated inhibition. A further possibility relevant to the inhibition by S-CopA is that this RNA inhibits the formation of an activator structure, for example a pseudoknot formed by a downstream segment of the RepA mRNA with the target loop II. A precedence for this possibility has been found recently by Asano *et al.* (1991) in plasmid ColIb-P9. So far, our results do not provide a test for the validity of such a mechanism for inhibition of *repA* expression by S-CopA (or even for CopA).

In conclusion, the data presented in this communication appear to rule out that RNase III-dependent processing of the RepA mRNA/CopA duplex is an obligatory event in replication control of plasmid R1. They also suggest that alternative pathways for inhibition of RepA synthesis are possible, and that, at least for one such pathway, RNA duplex formation is not required.

Table III. Plasmids used

Plasmid	Parent plasmid(s)	Relevant characteristics[a]	Source or reference
pGW15	pSU2718 + pGW643	p15A vector with *copA* gene	This work
pGW15-SCA	pSU2718 + pGW643-SCA	p15 vector with Δ*copA* gene	This work
pGW58		cloned R1 control region, pMB1 replicon	Blomberg *et al.* (1990)
pGW58-SCA	pGW58	Δ in *copA* gene	This work
pGW640		SP6 promoter in front of CopT sequence	Persson *et al.* (1988)
pGW643		pMB1 vector with *copA* gene	Wagner *et al.* (1987)
pGW643-SCA	pSP64 + pGW58-SCA	pMB1 vector with Δ*copA* gene	This work
pJL99		Short transl. *repA–lacZ* fusion, p15A replicon	Light and Molin (1981)
pJL133		Long transl. *repA–lacZ* fusion, pMB1 replicon	Light and Molin (1981)
pKN1562		Mini-R1, Km^r, $copA^+$, $copB^+$	Molin *et al.* (1979)
pSP64		pMB1 vector	Melton *et al.* (1984)
pSU2718		p15A cloning vector	Martinez *et al.* (1988)

[a] Only characteristics relevant to the present study are specified.

Materials and methods

Enzymes

Restriction enzymes and other enzymes used in the cloning steps were purchased from IBI or Pharmacia.

Cell growth and media

Cells were grown in LB medium supplemented with 0.2% glucose or on LA plates (Sambrook *et al.*, 1989). When appropriate, antibiotics were included at 50 μg/ml.

Bacterial strains

The *Escherichia coli* strain DH5α (F^-, *endA*1, *hsdR*17, *supE*44, *thy*-1, λ^-, *recA*1, *gyrA*96, *relA*1, Δ[*argF*$^-$*lacZYA*]U169, φ80Δ*lacZ*ΔM15) (Hanahan, 1985) was used for all experiments reported here.

Plasmids and plasmid constructions

The plasmids used are listed in Table III. Plasmid pGW58-SCA was constructed using a mutagenic 41-meric oligodeoxyribonucleotide, 5′-CATAT AACTA TACTC CCCGC AGGGC CCCGG TAATC TTTTC G-3′, using the method of Taylor *et al.* (1985a,b). We used dCTP-phosphorothioates (Pharmacia) for first strand synthesis and the enzyme *Ban*II for the nicking step. Candidate clones were identified by colony hybridization using the 5′-^{32}P-labelled mutagenic oligodeoxyribonucleotide as a probe. Two clones with mutant plasmids were obtained, and the correct deletion (see Figure 1) was verified by DNA sequencing. No additional mutations were found in the R1 insert of plasmid pGW58-SCA.

Plasmid pGW643-SCA was constructed by excising the *Sau*3A fragment of pGW58-SCA containing the mutated *copA* gene and inserting it into the *Bam*HI site of plasmid pSP64. The direction of the insert was identical to that of the *copA* fragment insert of pGW643 (Wagner *et al.*, 1987).

Plasmid pGW15 was built by ligating the *copA* gene-containing *Eco*RI–*Hin*dIII fragment of plasmid pGW643 into *Eco*RI–*Hin*dIII-restricted DNA of plasmid pSU2718.

Plasmid pGW15-SCA was constructed in the same way, except that the *copA* fragment was obtained from pGW643-SCA.

Incompatibility test

Incompatibility tests were performed essentially according to Nordström *et al.* (1980). *E.coli* cells harbouring the mini-R1 plasmid

pKN1562 (conferring resistance to kanamycin) were transformed with test plasmids either lacking R1 inserts, or with wild-type or mutant *copA* genes. These plasmids confer resistance to ampicillin to the host cells. Transformants were selected on LA plates containing the antibiotic appropriate for the incoming plasmid. After growth on the plates overnight, several colonies were resuspended in 0.9% NaCl, and dilutions were spread out on plates containing Km (to test for the presence of pKN1562) and on plates containing Ap (to obtain a measure of the number of cells tested). Plasmid loss in excess of the predicted statistical loss of the mini-R1 under non-selective conditions is indicated by a decrease in the ratio [number of cells on Km-plate/number of cells on Ap-plate].

β-galactosidase measurements

All measurements were performed as described by Berzal-Herranz *et al.* (1991).

In vitro transcriptions of CopT and CopA RNAs

Transcriptions of CopA or S-CopA were performed on appropriate plasmid DNA templates using *E.coli* RNA polymerase (Pharmacia, Sweden), following the protocols of Persson *et al.* (1988). CopT transcriptions were done on linearized plasmid pGW640 using SP6 polymerase (Promega Biotech) as described (Persson *et al.*, 1988). The CopT species used was 322 nt long and was the same as used previously (Persson *et al.*, 1990a). For uniform labelling, [α-^{32}P]UTP was included in the transcription mixtures to give a final UTP concentration of 40 μM, and for 'unlabelled' RNAs, [^{3}H]UTP was used at 100 μM. The use of ^{3}H-labelled nucleotides was needed in order to permit an exact determination of the RNA concentration, which in turn was required for calculating binding rate constants with accuracy.

In vitro CopA/CopT binding assays

Binding assays were performed as described in Persson *et al.* (1988, 1990a,b).

Northern blot analysis

Northern analyses were done on total RNA preparations from strains harbouring appropriate plasmids, according to the protocols of Blomberg *et al.* (1990). For the analysis of CopA RNAs we used an 8% sequencing gel. The probe for detection of CopA sequences was the oligodeoxyribonucleotide COP (5′-TAATC TTCTT CAACT-3′). The oligodeoxyribonucleotide was labelled using T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (New England Nuclear) as described by Öhman and Wagner (1989).

S1 nuclease mapping

The protocol for the RNA 3′-end determination (Figure 3A) was essentially as described by Blomberg *et al.* (1990). The probe was an oligodeoxyribonucleotide: 5′-dCTTTT CCGGG CATAT AACTA TACTC CCCGC ATAGC TGAAT TGTTG GCTAT ACGGT TTAAG TGGGC CCCGG TAATC TTTTC GTACT CGCCA AAGTT GAAGA AGATT ATCGG GGTTT TTGCT TTTCT GG-3′ (S1-1). The 3′-end of this oligo was labelled using a second oligodeoxyribonucleotide, complementary to the 3′-end of S1-3: 5′-GGAGC CAGAA AAGCA AAAAC CCCG-3′ (S1-2). The two oligodeoxyribonucleotides were annealed and the resulting 3′-recessed end of S1-1 was filled in using Klenow enzyme in the presence of [α-^{32}P]dCTP and [α-^{32}P]dTTP according to Blomberg *et al.* (1990). The protected DNA fragments were electrophoresed on an 8% polyacrylamide gel containing 7 M urea, alongside a sequencing reaction (Maxam and Gilbert, 1980) performed on the probe. Note that the S1 nuclease experiment was carried out with RNA extracted from *E.coli* DH5α cells harbouring plasmid pGW58 and either one of three plasmids: a control plasmid (pSU2718), a CopA-donor plasmid (pGW15) or a S-CopA-donor plasmid (pGW15-SCA). The reason for using cells containing pGW58 instead of the *repA−lacZ* fusion plasmids was that at large CopA/CopT RNA ratios the antisense RNA interfered with the hybridization of the probe. This competition led to very weak signals. The bands obtained with RNAs extracted from cells containing the fusion plasmids were qualitatively identical to the ones shown here (see Figure 3A, and data not shown).

Primer extension analysis

Primer extensions were carried out as described by Blomberg *et al.* (1990). For the analysis we used the oligodeoxyribonucleotide BOX-A (5′-dGCAGA AGGAT CGAGC ACAG-3′) and AMV reverse transcriptase (Pharmacia). Sequence markers were synthesized from R1-DNA with the same primer as employed in the primer extension reactions using a T7 sequencing Kit (Pharmacia). The samples were analysed on an 8% sequencing gel.

RNase III cleavages in vitro

In vitro synthesized and purified RNAs (CopA and CopT) were incubated in 10 μl TMN_{150} buffer (20 mM Tris−Cl pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT) for 5 min at 37°C. The concentrations were chosen such that for the wild-type CopA species almost complete duplex formation should occur within ~3−4 min. All samples also contained 10 μg of bulk tRNA (Sigma). About 0.005 μg of purified RNase III (gift from D.Court) was added to all incubations, and after continued incubation for 1 min at 37°C, the reactions were terminated by the addition of 1.5 vol of stop buffer (formamide 93%, Na_2EDTA 30 mM, xylene cyanol 0.05%, bromophenol blue 0.05%, SDS 0.5%). For the samples where RNA duplexes were artificially formed, the first incubation step in the protocol above was changed as follows: the RNAs were heated for 3 min at 85°C, followed by slow cooling to room temperature in order to permit annealing of the complementary RNA species. Finally, after boiling for 3 min, the RNA samples were electrophoresed on 8% sequencing gels. In the experiment reported here, we used denatured, ^{32}P-end-labelled pBR322 *MspI* fragments as size markers. The gels were dried and subsequently autoradiographed on Kodak X-Omat AR film with Dupont intensifying screens at −70°C.

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