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Control of pMB1 replication: inhibition of primer formation by Rop requires RNA1

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The *rop* gene participates in the control of plasmid copy number by interfering with transcripts originating from the primer promoter. Here we show that this inhibition mechanism requires RNA1 *in trans*. Mutations in the RNA1 coding sequence that result in plasmids with altered incompatibility properties do not affect the ability of the molecule to participate in the Rop inhibition mechanism. Furthermore we show that the target of the Rop-RNA1 inhibitory mechanism is located, at least in part, after the 52nd nucleotide of the sequence encoding the primer transcript.
Key words: plasmid replication/RNA processing/transcription termination/ColE1

Introduction

Initiation of DNA replication of plasmids of the ColE1 family requires the synthesis of an RNA transcript which starts ~555 nucleotides upstream from the origin (Itoh and Tomizawa, 1980). This transcript, after having been processed by the enzyme RNase H, serves as primer for the initiation of DNA synthesis by DNA polymerase I. Regulation of initiation of replication is achieved at the level of primer formation. Two plasmid encoded elements have been shown to regulate plasmid copy number (Figure 1). The first is an RNA molecule of 108 nucleotides (RNA1) which is complementary to the 5′ portion of the RNA primer. RNA1 specifically inhibits the processing of the primer precursor (Conrad and Campbell, 1979; Muesing *et al.*, 1981; Tomizawa and Itoh, 1981; Tomizawa *et al.*, 1981; Lacatena and Cesareni, 1981).

A second region, encoding a *trans*-acting negative regulatory element, was originally identified in deletion mutants with increased copy number (Twigg and Sherratt, 1980). We have shown that the region identified by these deletions contains a gene (*rop*) which negatively regulates β-galactosidase synthesis under the control of the promoter of the primer. The *rop* gene is located ~500 nucleotides downstream from the replication origin and encodes a protein of 63 amino acids whose amino acid sequence is conserved in the two related plasmids pMB1 and ColE1 (Cesareni *et al.*, 1982). These results have been recently confirmed by Som and Tomizawa (1983) who used the galactokinase promoter-probe system. Furthermore the same authors have proved that the levels of galactokinase messenger synthesized under the control of the primer promoter are decreased in the presence of *rop*, thus ruling out any explanation based on possible artefacts at the level of translation.

Rop is not a basic protein nor could we detect any *in vitro* activity of the purified protein (Lacatena *et al.*, 1983). This suggests that the function of the *rop* gene product is somewhat distinct from that of a classical repressor, and that more different factors might be involved in the inhibitory mechanism. Here we present genetic evidence that the Rop-mediated inhibitory activity on primer transcription requires the presence of RNA1. Furthermore we show that at least part of the Rop-RNA1 target is located between nucleotides 52 and 134 of the primer.

Results

We have previously shown that plasmids derived from ColE1 and pMB1 code for an element which can inhibit *in trans* β-galactosidase synthesis when the *lacZ* gene is transcribed from the primer promoter. Deletion analysis indicated that a DNA fragment of 309 nucleotides located downstream from the replication origin contained information essential for this function (Cesareni *et al.*, 1982). Our deletion analysis however would have failed to detect other elements involved in this mechanism if these had been encoded in a region essential for DNA replication.

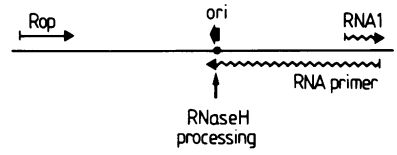

Fig. 1. Schematic representation of the region essential for pMB1 replication and its control. The thick arrow at the origin points toward the direction of replication, wavy and straight lines represent transcripts and protein coding sequences, respectively.

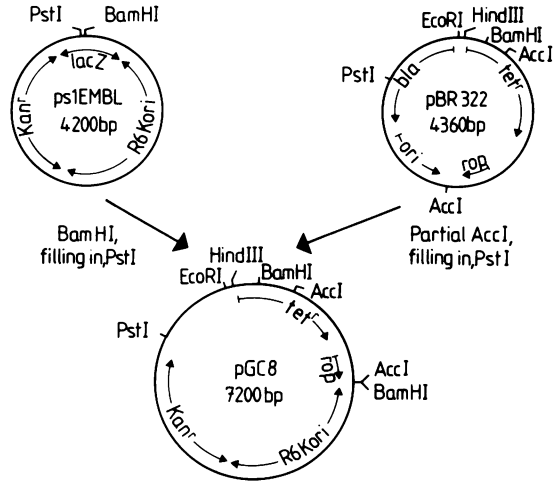

Fig. 2. Construction of plasmid pGC8. *lacZ* is the region encoding the α-peptide of β-galactosidase. *bla*, *Kan*^{r} and *tet*^{r} represent the genes that confer resistance to ampicillin, kanamycin and tetracycline. Filling in of the 5′ protruding ends generated by restriction endonuclease was obtained by incubation in the presence of the large fragment (Klenow) of DNA polymerase 1.

To assess whether any other plasmid-encoded elements participate in the inhibitory function we have cloned the *Pst*I-*Acc*I DNA fragment of pBR322 that contains *rop* into plasmid p1sEMBL (Figure 2). This plasmid is a small derivative of R6K which does not share any sequence homology with ColE1 (Poustka *et al.*, 1984). In *Escherichia coli* minicells, plasmid pGC8, the derivative of p1sEMBL containing the *rop* gene, synthesizes the 7-K protein which we suggested to be the product of the *rop* gene itself (not shown). When we transfected pGC8 into an *E. coli* strain which expresses β-galactosidase under the control of the primer promoter, however, we could measure only a minor effect (10% decrease) on β-galactosidase synthesis.

This result shows that the 7-K protein by itself is not sufficient to interfere with transcription originating from the primer promoter. pGC8, however, can complement the *rop* deficiency of a plasmid which, like pAT153, has been deleted of the *rop* gene. All the ColE1 or pMB1 plasmid derivatives that we have tested can be complemented by pGC8 (not shown). The only sequence that all these plasmids have in common is a DNA fragment of ~700 nucleotides upstream from the replication origin.

RNA1 is necessary for Rop inhibition

The results presented in the previous section prove that two plasmid-encoded inhibitory elements cooperate in regulating *in trans* transcription from the primer promoter. One is encoded in a fragment of 309 nucleotides which we have identified as the *rop* gene. The second is synthesized from the region essential for DNA replication.

To identify this second element we have inserted a series of DNA fragments originating from the replication origin region of pMB1 into the *Hind*III site of pACYC184. This plasmid is a derivative of p15A which is poorly active in the Rop complementation assay. Of all these constructions the one that contains the smallest pMB1 fragment that is still able to inhibit β-galactosidase synthesis in the presence of Rop is pac163 (described in Materials and methods). Plasmid pac163 contains a fragment of the pMB1 replication origin region which includes the primer promoter and part of the downstream region up to the *Hpa*II restriction endonuclease site at position 2855 in the pBR322 sequence (Sutcliffe, 1979). No polypeptide is encoded by this fragment in either reading direction. Both strands however are transcribed, starting either from the promoter of the RNA primer or from the promoter of RNA1.

Plasmid pac163pII2 differs from plasmid pac163 in having a single base transition in the −35 box of the primer promoter, which makes the promoter itself inactive (Cesareni, 1982). As a consequence, this plasmid can only synthesize RNA1 and not the fragment of RNA primer which is synthesized by the wild-type pac163. Despite this mutation, however, pac163pII2 maintains its ability to inhibit the synthesis of β-galactosidase when the *lacZ* gene is fused to the primer promoter (Figure 3). We conclude that RNA primer transcription is not involved in the inhibition mechanism and that RNA1 is the molecule that either directly or indirectly cooperates with the product of the *rop* gene. This conclusion is confirmed by the result of the last experiment in Figure 3. pACR1 is a plasmid construction equivalent to pac163 that contains a deletion from the *Hae*III site at position 2952 to the *Hpa*II site at position 2855 in the pBR322 sequence (Sutcliffe, 1979). This deletion removes the −35 box of the RNA1 promoter and makes this promoter inactive. pACR1 is

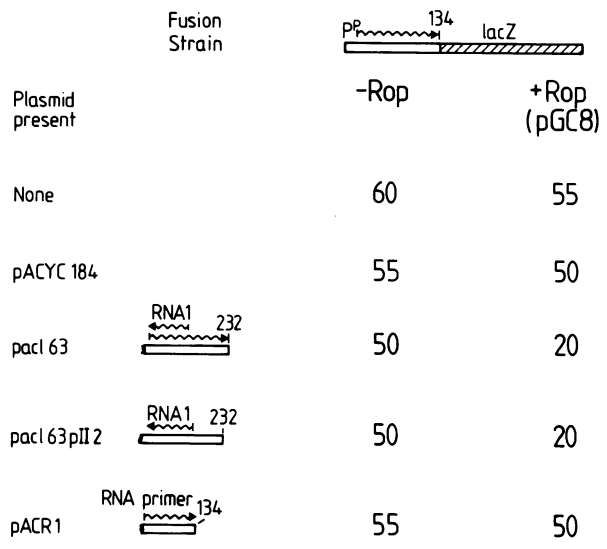

Fig. 3. Rop-RNA1 complementation assay. All the β-galactosidase assays were done in a bacterial strain (71/18) containing the phage φBG34. Numbers represent β-galactosidase enzymatic activities. Units are as defined by Miller (1972). Background levels (20 units), measured in a strain which contains the *lacZ* λ vector deleted of its promoter, have already been subtracted. Numbers in the small diagrams represent the last nucleotide of the primer sequence present in the pACYC184 derivative used in the complementation assay. Errors, measured as deviations from the average of at least three experiments, are of the order of 15%.

not able to inhibit β-galactosidase synthesis in the Rop complementation assay, supporting the conclusion that RNA1 is essential for this inhibition mechanism.

The RNA1-Rop target comprises sequences downstream from the primer promoter region

RNA1 inhibits the processing of the primer precursor by the enzyme RNase H by pairing with part of the complementary sequence in the primer precursor (Tomizawa *et al.*, 1981; Lacatena and Cesareni, 1981, 1983). If this interaction is essential also for the Rop-mediated control mechanism we would predict that these target sequences on the primer should be present in order for the inhibition to occur. To test this hypothesis we constructed the phage φBG46 which carries the β-galactosidase structural gene fused to the primer promoter and downstream sequences up to the *Alu*I site located 52 nucleotides from the start of primer transcription. As a consequence this fusion lacks part of the RNA1 target. The levels of β-galactosidase in bacteria carrying phage φBG46 are twice as high as those obtained with phages carrying fusions extending up to the *Hae*III site at position 134 (φBG34) (Figure 4). Furthermore, β-galactosidase synthesis is insensitive to inhibition by Rop and RNA1 suggesting that part of the target of Rop-RNA1 inhibitory activity lies after the *Alu*I site at position 52. This result confirms the finding of Som and Tomizawa (1983) who reported a similar experiment for the plasmid ColE1.

Analysis of mutations which affect the ability of RNA1 to interfere with primer formation

We have isolated a large number of pMB1 mutants which are compatible with a wild-type plasmid (Lacatena and Cesareni, 1983). The majority of these mutants synthesize an RNA1 which cannot interact with the wild-type RNA primer. It was, therefore, of interest to test whether these altered RNA1s could still participate in the Rop-mediated inhibition of β-galactosidase synthesis in the fusion strain. We have tested a

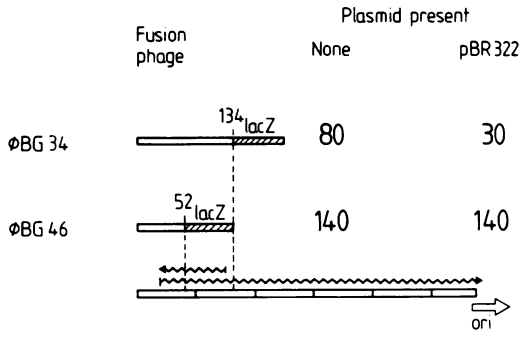

Fig. 4. Deletion analysis of the target of Rop-RNA1 inhibitory activity. Numbers in the table represent β-galactosidase activities measured as described in the legend to Figure 3 and in Materials and methods. In this case the background was not subtracted. Numbers in the small diagrams refer to the last nucleotide of the primer coding sequence present in the fusion phage. Wavy lines are the RNA1 and RNA primer transcripts.

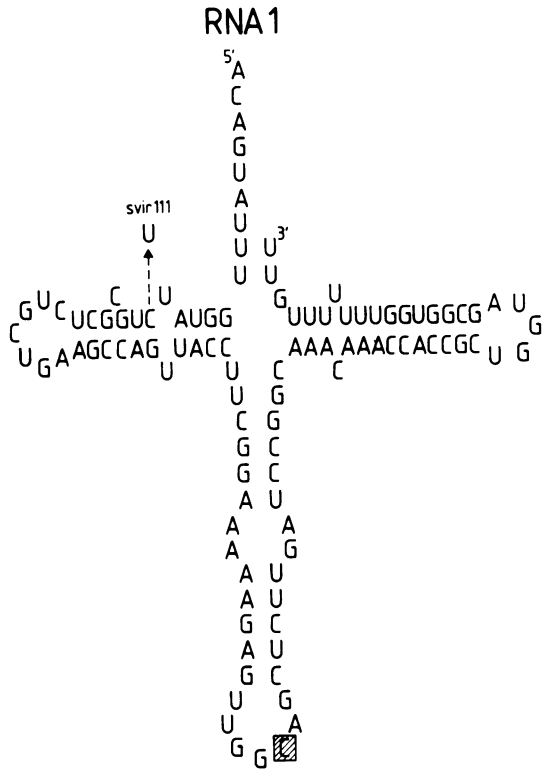

Fig. 5. Primary sequence and secondary structure of RNA1. The sequence of RNA1 synthesized by the mutant *svir*11 is shown. The single base change with respect to the wild-type sequence is enclosed in a hatched box. *svir*111 is a derivative of *svir*11 which differs in a single base of the first stem of the RNA1 molecule.

sub-set of our collection of mutants carrying single, double or triple mutations in loop I or loop II and in stem I of the RNA1 cloverleaf structure. This sub-set includes *svir*002, *svir*015, *svir*017, *svir*020, *svir*038, *svir*102, *svir*111, *svir*201a, *svir*203a, *svir*301, *svir*312, *svir*318, *svir*216, *svir*2036 (Lacatena and Cesareni, 1983).

The majority of these mutant RNA1s could inhibit β-galactosidase synthesis as efficiently as wild-type in the presence of Rop, as judged by the colour of the relevant strains on lac McConkey plates. When *svir*111, however, was transformed into strain 71/18 carrying the fusion prophage and plasmid pGC8, no inhibition of β-galactosidase synthesis was obtained. *svir*111 has a C→T transition which results in destabilization of the first stem of RNA1 (Figure 5). Since we have not been able to purify RNA1 from cells which contain *svir*111 we conclude that the RNA1 synthesized by plasmid *svir*111 is unstable and that its instability is the cause of its defectiveness in the Rop inhibition test.

Discussion

We have shown that β-galactosidase synthesis under the control of the promoter of the primer for initiation of pMB1 replication is regulated *in trans* by the product of the *rop* gene and RNA1. Both elements have to be present for the inhibition to occur. It is likely that this mechanism, which we have studied by looking at its effect on the expression of β-galactosidase synthesis in fusion phages, is relevant for the control of plasmid copy number. While this has been clearly shown for what concerns the participation of the *rop* gene (Twigg and Sherratt, 1980), RNA1 involvement is more difficult to prove unambiguously. Since the target of Rop-RNA1 lies in the RNA1 coding sequence any RNA1 mutation results in an alteration of the target of this inhibitory mechanism. As a consequence it is not straightforward to prove genetically that RNA1 is essential for the *rop*-mediated regulation of plasmid copy number. The assay with the *lacZ* fusion phage provides a convenient tool to separate on different vectors the elements (target and inhibitors) of this regulation mechanism and allow us to overcome the problems that we have just mentioned. Recent *in vitro* experiments from our laboratory (Lacatena *et al.*, 1984) have shown that RNA1 is absolutely required for inhibition of plasmid replication by purified Rop in an *in vitro* system. These results parallel the results obtained *in vivo* with the *lacZ* fusion system and suggest that both ways of assaying Rop are actually measuring the same activity. Since we have shown that the target of the Rop-RNA1 replication mechanism lies, at least in part, >52 nucleotides downstream from the primer promoter, we can exclude the possibility that Rop and RNA1 affect the initiation of transcription of the primer precursor in a quasi-classical way.

We have considered three hypothetical molecular mechanisms by which Rop might act. (i) Rop increases the ability of RNA1 to inhibit the processing of the primer precursor at the origin. (ii) Rop and RNA1 together affect the primer formation by causing the premature termination of transcription of the primer precursor. (iii) The interaction of Rop and RNA1 with the primer precursor affects the stability of the latter against ribonuclease degradation.

Mechanism (i) assumes that the primer precursor is transcribed past the origin even in the presence of inhibitory concentrations of Rop and RNA1. This is inconsistent with the *in vivo* results described above which show that genes fused to a fragment of DNA containing the primer promoter and only 120 nucleotides of the downstream pMB1 sequences are not expressed in the presence of Rop and RNA1. The possibility remains that the decrease pf β-galactosidase activity observed is due to artefacts at the level of translation. This, however, seems improbable because it would be difficult to explain why such artefacts should also be observed in fusions with genes as different as *lacZ* (Cesareni *et al.*, 1982), *galK* (Som and Tomizawa, 1983) and the tetracycline resistance gene (unpublished observation). Furthermore Som and Tomizawa (1983) have demonstrated a decrease of the actual levels of the

Table I. Bacteria, phages and plasmids

Strain	Genotype	Reference	Use
Bacteria			
71/18	△[*lac-pro*]F′ *lac*I^{q}Z△M15*pro*$^+$	Messing *et al.* (1977)	*lac*Z host
L41	71/18 (λϕBG34)	Cesareni *et al.* (1982)	test strain for primer transcription
Phages			
132	*h*λ*lacZi*21*nin*5	Maurer *et al.* (1980)	promoterless *lacZ*
ϕBG34	(b)	Cesareni *et al.* (1982)	primer (134)[a]-*lacZ* fusion
ϕBG46	(b)	this work	primer (52)[a]-*lacZ* fusion
Plasmids			
pACYC184	p15A*ori*, *tet*^{r}, *chl*^{r}	Cozzarelli *et al.* (1968)	vector
pac163	p15A*ori*, *chl*^{r}, *amp*^{r}, RNA1$^+$	this work	synthesizes RNA1
pac163pII2	p15A*ori*, *chl*^{r}, *amp*^{r}, RNA1$^+$	this work	as pac163 but defective in primer synthesis
pACR1	p15A*ori*, *chl*^{r}	this work	defective in RNA1 synthesis
ps1EMBL	R6K*ori*, *Kan*^{r}, α*lacZ*	Poustka *et al.* (1984)	vector
pGC8	R6K*ori*, *Kan*^{r}, *tet*^{r}, *rop*$^+$	this work	synthesizes Rop

[a]Refer to the last nucleotide of the primer transcript present in the fusion strain.
[b]Derivatives of λ132 carrying different insertions.

primer RNA in the presence of Rop. Experiments are in progress to determine the mechanism of Rop inhibition at the molecular level.

Materials and methods

Strains and bacteriological techniques

Bacteria, phages and plasmids used in this work are shown in Table I. Standard bacteriological techniques have been used as described by Miller (1972). β-galactosidase assays have been carried out according to Miller (1972) after lysing the bacterial cells with one drop of toluol.

Bacteria were made competent for transformation by growing them up to an O.D. 600 of 0.6, washing them in 30 mM $CaCl_2$ and concentrating them 20 times in 30 mM $CaCl_2$ 10% glycerol. Competent cells were kept frozen at −70°C.

Enzymes

Most of the restriction enzymes have been purchased from BRL. Enzymatic reactions have been carried out according to the supplier's instructions. T4 DNA ligase was a gift of F.Winkler. Supercoiled plasmid DNA was prepared by the alkaline lysis method (Birnboim and Doly, 1979) and was purified by centrifugation to equilibrium in a density gradient of CsCl in the presence of ethidium bromide.

Plasmid and phage constructions

pGC8. pGC8 is a kanamycin-resistant plasmid which replicates under the control of an R6K origin and carries a functional *rop* gene. 0.5 μg of pBR322 DNA was partially digested by *Acc*I. After filling in the 5′ protruding ends using the large fragment of DNA polymerase 1 (Klenow), the DNA was digested to completion with the enzyme *Pst*I. The mixture of resulting fragments was ligated to 0.3 μg of plasmid ps1EMBL, previously digested by *Pst*I and *Bam*HI and filled in with DNA polymerase I. The ligation reaction was carried out at a DNA concentration of 10 μg/ml. A portion of the ligated mixture (~0.1 μg of total DNA) was used to transform strain 71/18. Transformants carrying recombinant plasmids were selected on plates containing kanamycin (5 μg/ml) and tetracycline (10 μg/ml). Plasmids carrying genes conferring resistance to the two drugs were analysed by restriction enzyme digestion.

pac163. Phage ϕBG10 (Cesareni, 1982) digested with *Hind*III and the fragment carrying pBR322 DNA from nucleotide 2854 to nucleotide 29 was inserted into the *Hind*III site of plasmid pACYC184 (Figure 6). The desired recombinants were selected on L-plates containing 20 μg/ml of chloramphenicol and 100 μg/ml of ampicillin. pac163pII2, which carries a mutation in the −35 region of the primer promoter was constructed in a similar way using the appropriate mutant phage (Cesareni, 1982).

pACR1. Plasmid pac129 (Brenner *et al.*, 1982) was digested by *Hae*III and the DNA fragment carrying the primer promoter was inserted into the *Bam*HI site of plasmid pACYC184 after addition of *Bam*HI linkers (BRL). Transformants carrying recombinant plasmids were selected on chloramphenicol plates (20 μg/ml) and recognized by replica plating on tetracycline plates (10 μg/ml).

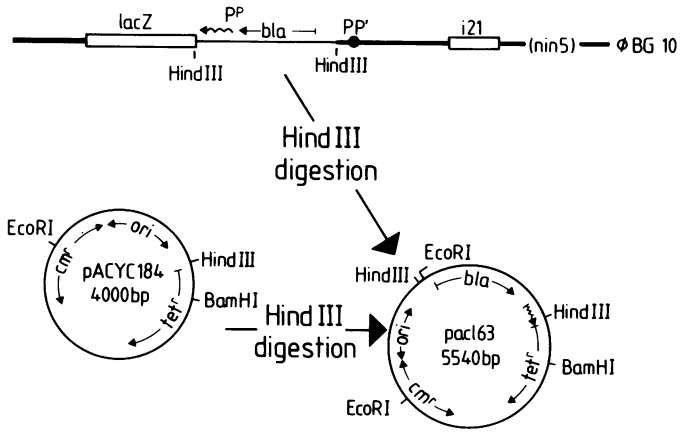

Fig. 6. Construction of pac163. Thick and thin lines represent λ phage and plasmid sequences, respectively. Wavy lines are transcripts originating from the primer promoter.

φBG46. pBR322 was digested by *Alu*I and the fragment carrying the primer promoter (nucleotides 3035−3556) was purified by electrophoresis on 6% polyacrylamide gel. After addition of *Hind*III linkers the fragment was ligated to phage λ132 digested by *Hind*III. The ligated DNA was packaged *in vitro* and the phages were recovered on lac McConkey plates on a lawn of *E. coli* K12 71/18. Phages forming red plaques were purified and verified by digesting their DNA with the enzyme *Hind*III.

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