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Post-transcriptional control of expression of the *repA* gene of plasmid R1 mediated by a small RNA molecule

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Fusions between the *repA* gene of plasmid R1 (required for autonomous plasmid replication) and the *lac* genes have been the basis for *in vivo* studies of regulation of *repA* expression. Two gene products – the CopA RNA and the CopB protein – act as inhibitors of *repA* expression. Comparison of the effects of addition in *trans* of the two Cop functions on transcription and translation of *repA-lac* gene fusions show that the CopB protein represses transcription of the *repA* gene, whereas the CopA RNA interferes with the RepA mRNA in such a way that effective translation is inhibited. The CopA RNA does not seem to have a direct effect on the transcription of the *repA* gene but, as a consequence of the post-transcriptional regulation, transcriptional polarity within the *repA* gene is observed. It is also shown that the CopA RNA interacts with its target (CopT) only when the region is transcribed to form RepA mRNA.

Key words: gene fusions/plasmid replication control/polarity/post-transcriptional control/RNA inhibitor

Introduction

Our analysis of the low copy number plasmid R1 has revealed that a major facet of the replication control system is regulation of expression of an essential replication function (Light and Molin, 1981, 1982). Here we present a further analysis of this system which reveals an interesting example of gene regulation in bacteria.

Replication of plasmid R1 is controlled by two *cop* gene products – a 90 nucleotide RNA molecule (the CopA RNA) (Stougaard *et al.*, 1981a), and an 86 amino acid polypeptide (the CopB protein) (Molin *et al.*, 1981), both of which act by inhibiting expression of a gene, *repA*, whose product seems to be required for replication (Light and Molin, 1981). The requirement for RepA polypeptide for R1 replication has not been demonstrated directly, but disruption of the *repA* structural gene results in inability of the plasmid to replicate from the R1 origin (Kollek *et al.*, 1978). The two replication inhibitors act at two separate sites independently of one another (Light and Molin, 1982): the CopB protein represses *repA* expression at a site close to the promoter, at the stage of initiation of transcription. The CopA RNA interferes with *repA* expression at a stage after transcription initiation. The target, *copT*, lies in the leader sequence between the *repA* promoter and the structural *repA* gene. These aspects of the control system are summarized in Figure 1. Based on analysis of the interaction between wild-type and mutant CopA RNA and their target sequences it was concluded that (1) *copT* overlaps with the *copA* gene, and (2) the action of CopA RNA at *copT* involves nucleic acid-nucleic acid interaction. However, it was not possible to assess how this interaction takes place. The two most likely possibilities would be either attenuation (termination) of *repA* transcription upstream of the *repA* structural gene, or post-transcriptional inhibition of *repA* expression (inhibition of translation of the RepA mRNA).

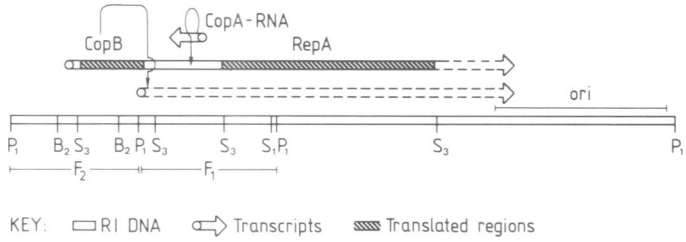

Fig. 1. Basic replicon of plasmid R1 – restriction sites, genes and regulatory loops. Restriction enzyme recognition sequences are indicated as follows: *Pst*I (P_1), *Bgl*II (B_2), *Sau*3A (S_3), *Sal*I (S_1). The *Pst*I fragments encoding the replication control genes are denoted F_1 and F_2 according to our standard nomenclature (Molin *et al.*, 1981). The replication origin is shown as ori. The products of the three structural genes *copB, copA* and *repA* are shown according to the Key. Transcription of the *copB* gene continues as indicated without terminating after the gene, apparently unaffected by the CopB function (Light and Molin, in preparation). Note that within the *copA* gene both DNA strands are being transcribed (in opposite directions) such that the CopA RNA is complementary to part of the leader sequence of the RepA mRNA. The arrows (↝) point to the sites of action of the two Cop functions, both of which inhibit expression of the *repA* gene (Light and Molin, 1982). See the text for details.

The present analysis was designed to distinguish between these two possibilities through the construction and analysis of gene fusions in which the effect of excess concentrations of Cop functions on transcription downstream of *copT* could be assayed. Inhibition at the level of transcription should severely reduce the rate of distal RepA mRNA synthesis, whereas post-transcriptional inhibition should only have a marginal effect on the extension of the *repA* transcript. In addition, we have made a series of hybrid plasmids which have been used to determine the molecular nature (DNA or RNA) of CopT.

We have made use of a series of promoter cloning vehicles from which β-galactosidase synthesis is dependent on insertion of DNA fragments carrying a promoter directed towards the *lac* genes (Casadaban and Cohen, 1980; Casadaban *et al.*, 1980). These fusion vectors are of two types. (1) Vectors for transcriptional fusions which have the whole *lac* operon intact, except for the promoter/operator region. The normal translation start signal upstream of the *lacZ* gene is preserved in these plasmids. (2) Vectors for translational fusions which are deleted of the promoter, the translation start sequence, and the first seven and one third codons of the *lacZ* gene. Expression of the *lac* genes from such vectors requires a promoter and initation of translation from the inserted gene in such a way that a fused polypeptide is expressed (in-frame translation).

Another type of transcription fusion vector plasmid used was constructed by An and Friesen (1979). The gene responsible for tetracycline resistance was deleted of its promoter and suitable restriction enzyme recognition sites were introduced upstream of the *tet* gene for insertion of promoter-carrying DNA fragments. One of these plasmids, pGA46, was chosen

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Table I. Plasmids

Plasmid	Parent plasmid(s)	Relevant phenotype	Type of gene fusion[a]	Source/Reference
pKN1562	R1	Km, Cop$^+$		Molin *et al.* (1979)
pBR322	pMB1	Ap, Tc		Bolivar *et al.* (1977)
pGA46	p15	Cm		An and Friesen (1979)
pKB252	pMB9	Tc, imm$_\lambda$, Lac promoter		Backman and Ptashne (1978)
pVH17	pBR322	Ap, Deo promoter		Valentin-Hansen *et al.* (1982)
pMC81	ColE1	Ap, Lac$^-$		Casadaban and Cohen (1980)
pOU504	pBR322 + pKN1562	Ap, CopA$^+$		Stougaard *et al.* (1981a)
pOU16	pBR322 + pKN1562	Ap, Tc, CopB$^+$		Riise *et al.* (1982)
pOU565	pBR322 + pOU417	Ap, CopA-417, CopT$^+$		Stougaard *et al.* (1981a)
pJL207	pGA46 + pMC81	Cm, Lac$^-$		Light and Molin (1982)
pJL99	pMC874 + pKN1562	Km, CopA$^+$, Lac$^+$	*repA-lacZ* (trl)	Light and Molin (1981)
pJL99Δ5	pJL99Δ*Pvu*II	Km, CopA$^+$, LacZ$^-$, Y$^+$, A$^+$	*repA-lacZ* (trl)	This work
pJL99Δ6	pJL99Δ*Pvu*II	Km, CopA$^+$, LacZ$^-$, Y$^+$, A$^+$	*repA-lacZ* (trl)	This work
pJL99Δ14	pJL99Δ*Pvu*II	Km, CopA$^+$, LacZ$^-$, Y$^+$, A$^+$	*repA-lacZ* (trl)	This work
pJL111	pMC1403 + pKN1562	Ap, CopA$^+$, CopB$^+$, Lac$^{(+)}$	*repA-lacZ* (trl)	Light and Molin (1981)
pJL128	pGA46 + pJL99	Cm, CopA$^+$, Lac$^+$	*repA-lacZ* (trl)	Light and Molin (1981)
pJL234	pGA46 + pJL111	Cm, CopA$^+$, Lac$^+$	*repA-lacZ* (trl)	Light and Molin (1982)
pJL24	pGA46 + pKN1562	Cm, Tc, CopA$^+$	*repA-tet* (trc)	This work
pJL171	pJL24 + pMC81	Cm, CopA$^+$, Lac$^+$	*repA-lac* (trc)	This work
pJL263	pJL207 + pJL111	Cm, CopA$^+$, Lac$^+$	*repA-lac* (trc)	This work
pJL232	pKB252 + pOU565	Tc, CopA-417, CopT$^+$	*lacp-copT* (trc)	This work
pJL233	pJL232Δ*Eco*RI	Tc, CopA-417, CopT$^-$		This work
pJL242	pVH17 + pOU565	Ap, CopA-417, CopT$^+$	*deop-copT* (trc)	This work
pJL243	pJL242Δ*Eco*RI	Ap, CopA-417, CopT$^-$		This work

[a]The type of gene fusion established is indicated by (trc) for transcriptional and (trl) for translational fusions.

here as the vector for constructing fusions with the *repA* gene from plasmid R1.

Results

Expression of the lacY and lacA genes from translational repA-lacZ fusions

The *repA-lac* fusion hybrids used in our experiments have all the three *lac* genes present downstream of the fusion site. Thus, using translational *repA-lacZ* fusion plasmids (for a list of the plasmids used see Table I) (e.g., pJL99), from which β-galactosidase expression is known to be severely inhibited by CopA (Light and Molin, 1981), the effect of the inhibitor at the level of transcription can be analysed through measurements of permease *(lacY)* and acetylase *(lacA)* activities. These genes have their own translation start sequences and will therefore be expressed in proportion to the rate of transcription.

However, studies of transcription of the *lac* operon carrying amber mutations in the *lacZ* gene have shown that Lac mRNA, if untranslated, is terminated prematurely, due to the exposure of internal transcription termination sites in the *lacZ* gene (polarity) (Zipser, 1970). Therefore, inhibition of *repA* expression, both at the level of transcription and translation, would be expected to result in decreased transcription of the distal *lac* genes.

To circumvent this problem, we constructed a series of deletion mutants from pJL99 using the restriction enzyme *Pvu*II. This enzyme has three recognition sequences in the *lacZ* gene: one 102 bp downstream of the start codon, one 408 bp upstream of the stop codon, and one 45 bp before the stop codon (Berman, personal communication). As shown in Figure 2, we have made deletions covering almost the entire *lacZ* gene as well as one in which only the small fragment near the C-terminal end is missing. In all the plasmids the translational fusion site, the *copA/copT* region upstream of the *lac* genes and the *lacY* and *lacA* genes are preserved. Moreover, in all these plasmids, translation of the truncated *lacZ* gene is terminated at the natural stop codon. Strains were constructed which also harboured either pOU504 (pBR322-*copA*$^+$) or pOU16 (pBR322-*copB*$^+$), and the expression of the *lacY* and *lacA* genes was analysed. The results (Table II) show that deletions of the major part of the *lacZ* structural gene, expected to abolish polarity, greatly influences the pattern of gene expression: from all plasmids both *lacY* and *lacA* expression is inhibited by the presence of CopB, but an increased amount of CopA only has a severe inhibitory effect on *lacY* and *lacA* expression from plasmid pJL99 and the derivative carrying a small deletion near the end of the *lacZ* gene. Expression of *lacY* and *lacA* is almost totally insensitive

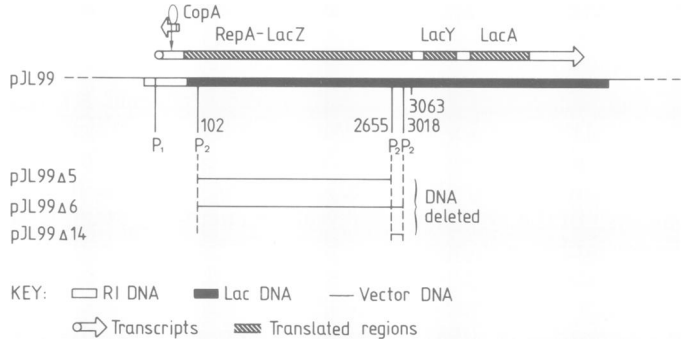

Fig 2. Deletion derivatives of the *repA-lacZ* translational fusion plasmid, pJL99. The map of pJL99 shows the R1 DNA insertion upstream of the *lac* genes (cf., Light and Molin (1981) for a complete map of pJL99). *Pst*I (P_1) and *Pvu*II (P_2) sites are shown. The numbers (102, 2655, 3018 and 3063) refer to nucleotide numbers of the *lacZ* gene (M.Berman, personal communication). The extent of the deletions in three *Pvu*II derivatives of pJL99 are shown.

Table II. Expression of the *lac* genes from pJL99 and deletion derivatives in the absence and presence of *copA*$^+$ and *copB*$^+$ chimeric plasmids

Plasmid	% of *lacZ* gene deleted[a]	Expression of β-gal in presence of[b]			Expression of permease in presence of[c]			Expression of transacetylase in presence of[d]		
		–	pOU504 (CopA$^+$)	pOU16 (CopB$^+$)	–	pOU504 (CopA$^+$)	pOU16 (CopB$^+$)	–	pOU504 (CopA$^+$)	pOU16 (CopB$^+$)
pJL99	0	100	3	1	+	–	–	100	12	9
pJL99Δ5	83	–	–	–	+	+	–	100	78	11
pJL99Δ6	95	–	–	–	+	+	–	100	80	9
pJL99Δ14	12	–	–	–	+	–	–	100	20	14

[a]Deletions created with *Pvu*II, cf. Figure 2.
[b]Specific activities of β-galactosidase relative to that expressed from pJL99 in the absence of other plasmids. In all the deletion derivatives parts of the *lacZ* gene have been removed, and no functional β-galactosidase is therefore synthesized.
[c]Expression scored on melibiose plates as growth (+) or no growth (–) at 42°C.
[d]Specific activities of transacetylase relative to that expressed from pJL99.

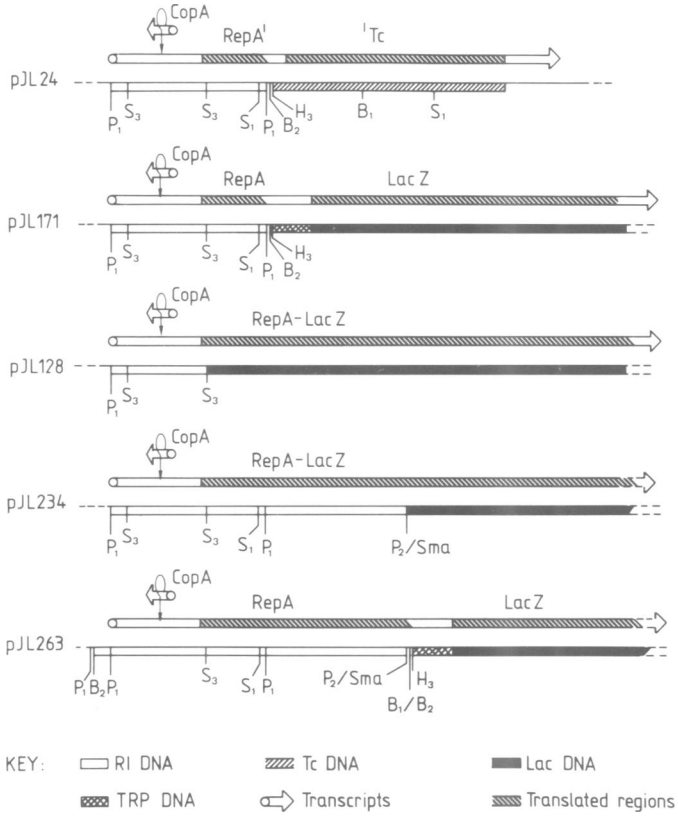

Fig. 3. Transcriptional and translational *repA* gene fusions inserted into plasmid pGA46. Restriction sites: *Pst*I (P_1), *Sau*3A (S_3), *Sal*I (S_1), *Bgl*II (B_2), *Hind*III (H_3), *Pvu*II-*Sma*I fusion (P_2/Sma), *Bam*HI-*Bgl*II fusion (B_1/B_2). Constructions of the plasmids are described in Materials and methods. Plasmid pGA46 is a promoter cloning vehicle with sites for *Pst*I, *Bgl*II and *Hind*III located just upstream of the Tc gene deleted of its promoter (An and Friesen, 1979). pJL24 has the *Pst*I-F_1 fragment from R1 (cf. Figure 1) inserted in pGA46 creating a transcriptional *repA-tet* fusion. pJL171 has a replacement of the Tc gene in pJL24 with the *lac* genes (minus promoter) from pMC81 (Casadaban and Cohen, 1980) resulting in a transcriptional *repA-lac* fusion. The TRP DNA refers to part of the *trp* operon which is placed upstream of *lac* in pMC81. pJL128 was made by inserting the translational *repA-lacZ* fusion from pJL99 (cf. Figure 2) on a *Pst*I fragment in pGA46 (Light and Molin, 1981). pJL234 was made by insertion of another translational *repA-lacZ* fusion from pJL111 in pGA46 (see Light and Molin, 1982). pJL263 carries a *Bgl*II-*Bam*HI fragment from pJL111 upstream of the *lac* genes inserted in pGA46 creating a transcriptional fusion.

to CopA if the major part of *lacZ* is deleted.

The data therefore suggest that the inhibition of *repA* expression mediated by the CopA RNA is exerted at a post-transcriptional level. In agreement with our previous findings (Light and Molin, 1982) the CopB protein represses transcription of the *repA* gene.

Expression of the repA gene monitored by transcriptional gene fusions

repA-tet transcriptional fusions. The *Pst*I-F_1 fragment from the basic replicon region of R1 carries the *repA* promoter, the *copA* gene and a small part of the *repA* structural gene (see Figure 1). This fragment was inserted in the *Pst*I site of pGA46 (see Materials and methods). Only in the orientation shown in Figure 3 (pJL24) did the hybrid mediate significant tetracycline resistance. The *Pst*I-F_1 fragment carries targets for both the CopA RNA and the CopB protein (Light and Molin, 1982). Hybrid plasmids carrying either of the two *cop* genes [pOU16 (*copB*$^+$) and pOU504 (*copA*$^+$)] were transformed to cells harbouring pJL24 and the level of tetracycline resistance determined for the double plasmid strains. As shown in Table III, the presence of the *copB*$^+$ hybrid reduced the resistance level to below 2 μg/ml, whereas the *copA*$^+$ hybrid had no significant effect.

repA-lac transcriptional fusions. The *lac* genes (without promoter) were inserted in pJL24, replacing most of the Tc gene (Figure 3). The resulting plasmid, pJL171, has a Lac$^+$ phenotype due to the creation of a *repA-lac* transcriptional fusion (see Materials and methods). The introduciton of *copA*$^+$ or *copB*$^+$ hybrid plasmids again showed that only the latter repressed transcription from the inserted *Pst*I-F_1 fragment; despite the presence of the CopA target site only a slight inhibition was exerted by extra copies of the *copA*$^+$ gene with this type of gene fusion (Table III). These results are in contrast to the severe inhibition by both CopA and CopB of *repA-lac* expression from a comparable translational *repA-lac* fusion hybrid, pJL128.

The lack of response of transcriptional gene fusions to an extra gene dosage of *copA*$^+$ once more indicates that the CopA RNA primarily inhibits translation of the RepA mRNA without causing transcription termination at the *copT* sequence.

Transcriptional polarity in the repA gene

The gene fusion plasmids described above all have a relatively small part of the *repA* gene fused to either the *tet* or the *lac* genes. To discover whether or not transcriptional polarity in the *repA* gene is significant in the absence of translation of the RepA mRNA, a plasmid, pJL263, was constructed which carries a transcriptional *repA-lac* fusion in which 85% of the *repA* gene is located upstream of the *lac* genes (see Figure 3). Plasmid pJL263 was used to test the ef-

Table III. Effects of *copA*$^+$ and *copB*$^+$ on expression of the *repA* gene monitored by transcriptional and translational fusions

Plasmids	Relevant phenotype of fusion plasmid[a]	Level of *repA* expression monitored as	
		Level of resistance to tetracycline (μg/ml)[b]	β-galactosidase activity[c]
pJL24	Tc (transcription)	20	
pJL24 + pOU504 (CopA$^+$)		20	
pJL24 + pOU16 (CopB$^+$)		<2	
pJL171	Lac$^+$ (transcription)		100
pJL171 + pOU504 (CopA$^+$)			62
pJL171 + pOU16 (CopB$^+$)			2
pJL128	Lac$^+$ (translation)		100
pJL128 + pOU504 (CopA$^+$)			5
pJL128 + pOU16 (CopB$^+$)			1
pJL263	Lac$^+$ (transcription)		100
pJL263 + pOU504 (CopA$^+$)			16
pJL263 + pOU16 (CopB$^+$)			1
pJL234	Lac$^+$ (translation)		100
pJL234 + pOU504 (CopA$^+$)			3
pJL234 + pOU16 (CopB$^+$)			1

[a]The indicated phenotypes (Tc or Lac$^+$) refer to properties expressed as a consequence of the construction of fusions with the *repA* gene. In brackets is indicated what type of gene fusion is obtained in each case (transcriptional or translational). All these plasmids have the same copy number (vector in all cases is pGA46).
[b]Resistance levels to tetracycline was determined by spreading 200–400 cells on plates containing increasing concentrations of the antibiotic (single cell resistance). The indicated concentrations represent the level allowing 100% survival of the bacteria.
[c]The β-galactosidase activities have for each individual fusion plasmid been normalized to 100 which thus represents the relative level expressed from each *repA-lac* fusion in the absence of additional plasmids carrying R1 *cop*$^+$ genes.

fect on transcription through the *repA* gene when translation of RepA mRNA was inhibited by excess amounts of the CopA RNA. For comparison, *repA* expression measured as synthesis of β-galactosidase from an analogous translational *repA-lacZ* fusion plasmid, pJL234, was determined under similar conditions.

The data in Table III show that although the rate of *repA-lac* expression in the presence of a pBR322-*copA*$^+$ chimeric plasmid (pOU504) is clearly reduced in both cases, the degree of inhibition is much stronger for the translational fusion which agrees with the observations described above (Tables II and III). It is important to note, however, that the post-transcriptional control exerted by the CopA RNA does interfere with transcription of the *repA* gene if a major part of the gene is present – presumably due to transcriptional polarity (premature termination in the absence of translation).

When the rate of transcription of *repA* is inhibited by the CopB protein the two types of fusion plasmids respond identically (Table III) showing that no internal promoter sequences have been created in the construction of plasmid pJL263.

The CopA RNA acts by binding to the RepA mRNA

An important prediction which can be made from the conclusion that the CopA function inhibits *repA* translation is that the target sequence, CopT, must be part of the RepA mRNA rather than one or both of the corresponding DNA strands. Since the dosage of the *copA* gene, and therefore by

Table IV. Titration of CopA RNA from pJL99

copT$^+$ chimeric plasmid added to pJL99	Promoter present upstream of *copT*	Relative specific activity β-galactosidase expressed from pJL99[a]
None	–	1.0
pOU565	*tetp*	3.5
pJL232	*lacp*	6.2
pJL233	–	1.1
pJL242	*deop*	5.3
pJL243	–	0.9

[a]Specific activities of β-galactosidase are expressed relative to that measured from pJL99 without any added plasmid.

inference, also the concentration of the CopA RNA seems to be important in controlling the rate of *repA-lac* expression, it was thought possible that the presence of a high concentration of the target site for the CopA function (CopT) might be able to titrate out CopA from a plasmid such as pJL99, thus increasing the rate of β-galactosidase expression.

Titration of replication of plasmid R6-5 was recently demonstrated by Danbara *et al.* (1981), although these experiments did not allow any conclusion about which of the Cop functions was titrated. We have chosen to use a mutant *copA* hybrid, pOU565, to screen for such a titration effect. The cloned mutant *copA* allele in plasmid pOU565 is *copA*-417, which has a mutation in the *copA* gene promoter resulting in an undetectable level of *copA* expression, but has a wild-type *copT* sequence (Stougaard *et al.*, 1981a). In the presence of pOU565 (pBR322 + a single *Sau*3A fragment carrying *copA*-417) the specific activity of β-galactosidase produced from pJL99 in the CSH50 background was increased relative to the normal level from pJL99 alone (see Table IV). The simplest explanation for the observed stimulation of *repA-lac* expression is titration of CopA RNA expressed from pJL99 through binding to CopT of pOU565.

A further analysis of the basis for the titration effect was attempted by insertion of the *copA*-417-*Sau*3A fragment downstream of a strong promoter. Plasmid pVH17 has the *deo* promoter on a small *Eco*RI fragment upstream of a *Bgl*II site towards which transcription is directed. Plasmid pKB252 has the *lac* promoter on an *Eco*RI fragment upstream of a *Bgl*II site towards which transcription is directed. In both cases the *Bgl*II site was used for insertion of the *copA-Sau*3A fragment from pOU565. Both the resulting hybrids (pJL242 and pJL232), having the mutant *copA-Sau*3A fragment inserted such that transcription is towards *repA* (cf., Figure 1), greatly stimulate *repA-lac* expression from pJL99. Deletion of the *Eco*RI fragments from pJL242 and pJL232 remove the promoters resulting in reduction of transcription through *copT*, and the level of titration is accordingly reduced (Table IV). These deletion plasmids titrate the CopA RNA even less effectively than pOU565 itself. This is not unexpected, since in pOU565 there is presumably some expression of CopT from the *tet* promoter of pBR322. Thus, maximal stimulation of *repA-lac* expression from pJL99 is obtained when the *copT* fragment is downstream of a strong promoter; a good indication that the CopA RNA expressed from the test plasmid (pJL99) binds to the transcript carrying the *copT* sequence (the RepA mRNA) rather than to the *copT* DNA sequence.

Discussion

We have analysed the regulatory pathways involved in controlling expression of the *repA* gene from the resistance plasmid R1. The results obtained suggest that this gene is regulated at two levels: (1) the CopB protein represses initiation of *repA* transcription, and (2) the CopA RNA inhibits translation of the RepA mRNA. This conclusion is obtained from measurements of gene expression using various gene fusions which monitor the level of transcription initiated from the *repA* promoter. It was found that introduction of *copA*$^+$ chimeric plasmids to strains harbouring such *repA* gene fusions had very little effect on transcription of any of the three *lac* genes (activities of β-galactosidase, permease and transacetylase) and of the *tet* gene (single cell resistance to tetracycline). This lack of effect of CopA RNA despite the presence of its target, CopT, contrasts strikingly with the strong inhibition of β-galactosidase expression observed with translational *repA-lacZ* gene fusions (Light and Molin, 1981, 1982).

In support of the proposed post-transcriptional regulation by the CopA RNA, an *in vivo* assay to study the interaction between the inhibitor RNA and its target was established. The rationale was that excess dosage of the target sequence CopT should titrate the inhibitor expressed from the fusion hybrid plasmid resulting in an increased level of *repA* expression. Analogous titration effects have been described for other regulated genes [e.g., *lac* (Gelfand *et al.*, 1978)]. The important observation made was that titration of the CopA RNA was dependent on transcription through CopT in the direction towards the *repA* gene. Transcription in the opposite direction had no significant effect (not shown). A simple interpretation of this result would be that the CopA RNA binds to a complementary sequence of the RepA mRNA rather than to one of the DNA strands, and such an interaction is in accordance with regulation at the level of translation. In parallel experiments stimulation of replication of R1 plasmid derivatives by such titration plasmids has been observed, and was analogously found to depend on the extent of transcription through CopT (not shown).

It could be argued that the reason why there is no or only small inhibitory effects of the CopA RNA on *repA-lac* expression from the transcriptional gene fusions is that DNA sequences downstream of the target site are different from those present in the translational fusion plasmids which do respond strongly. This argument is difficult to reconcile with the behaviour of two different translational *repA-lacZ* fusions. One, pJL99, has only two *repA* codons fused to the *lacZ* gene, whereas the other, pJL234, has 85% of the *repA* gene fused to *lacZ*. Yet they both respond strongly to extra CopA RNA (Light and Molin, 1981). Also, we use three different gene fusion systems to demonstrate lack of termination of transcription as a result of CopA RNA action: (1) expression of the *tet* gene (pJL24); (2) expression of *lacZ* (pJL171); and (3) expression of the *lacY* and *lacA* genes (deletion derivatives of pJL99). In the first two examples, only 60 bp downstream of the inserted R1 DNA are identical, followed by (1) the *tet* gene and (2) the *lac* genes. In the third case (deletion derivatives of pJL99) the sequence immediately downstream of the inserted R1 DNA is unaltered relative to the parent pJL99 (from which RepA-β-galactosidase expression is strongly affected by excess CopA RNA).

We therefore find it highly unlikely that the inhibition exerted by the CopA RNA is dependent on specific sequences downstream of the *repA* translation start sequence. We can also exclude creation of new internal promoter sequences in any of the fusion hybrids since the repression exerted by CopB on all fusions is as strong as that found for plasmids carrying only the *repA* promoter (60 bp) fused to the *lac* genes (Light and Molin, 1982).

The intriguing question raised by a post-transcriptional control circuit is how the inhibition of *repA* expression is transmitted over a distance of more than 100 nucleotides from the CopA RNA target site (CopT) to the start of the *repA* structural gene without interfering directly with transcription. One possibility is that the secondary structure of the mRNA near the ribosome binding site is important for initiation of translation, and that this structure is affected by the upstream interaction between CopA RNA and CopT. Alternatively, translation of the putative 64 amino acid polypeptide (Stougaard *et al.*, 1981b) (whose coding sequence contains CopT), from the leader region of the RepA mRNA, might be directly affected by an interaction between CopA RNA and its target. Such an effect might also influence the rate of initiation of translation of the *repA* structural gene. A third possibility is the involvement of a nuclease which makes a site-specific cleavage in the RepA mRNA mediated by the CopA RNA. Although there is evidence from the studies presented here that the mRNA is not rapidly broken down, such a cleavage could still be involved in the repression of *repA* translation.

There are other examples in *Escherichia coli* where gene regulation involves 'transfer' of a regulatory signal from a primary binding site to a distant site on an RNA molecule. In the case of plasmid ColE1 binding of a small RNA inhibitor (RNA I) at one site on the replication primer RNA affects processing of the primer several hundred nucleotides away (Tomizawa and Itoh, 1981). If initiation of *repA* translation is considered to be a special case of RNA processing there is a striking analogy in the function of the two regulatory RNA molecules. Control of ribosomal protein synthesis in *E. coli* is at least partly at the level of translation (Lindahl and Zengel, 1982), and in one case, the L10 operon, there is evidence that binding of a ribosomal protein to specific sequences on the mRNA represses initiation of translation ~100 nucleotides downstream on the mRNA (M.Johnsen, personal communication). The precise molecular mechanisms involved have not been clarified in these cases either.

The implications of a post-transcriptional control of *repA* expression by CopA-RNA for the understanding of the replication control system operating for plasmid R1 are primarily concerned with the role of the RepA protein. Since it was previously shown that the CopA RNA is the major replication control function (Molin *et al.*, 1981; Riise *et al.*, 1982) the conclusions derived here suggest that the RepA protein is the limiting factor for initiation of plasmid replication. This important role of the RepA protein is in accordance with both *in vivo* and *in vitro* experiments showing that initiation of replication is strictly dependent on *de novo* protein synthesis (Uhlin and Nordström, 1978; Diaz *et al.*, 1981). It is also striking that the two related plasmids, R1 and R100, which have been isolated independently in different parts of the world, show a strong conservation of the amino acid sequence of the RepA protein (Ryder *et al.*, 1982). However, if the RepA mRNA is also the primer for replication initiation, an indirect regulation of *repA* transcription may be exerted by the CopA RNA inhibitor as a polarity effect. It remains to

be seen whether the RepA mRNA is the primer for replication initiation, and, if it is, whether the transcriptional polarity is relevant for replication control.

Materials and methods

Bacterial strains and plasmids

The *E. coli* K12 strain used in all experiments was CSH50 (Miller, 1972) which has the genotype *Δlac-pro, rpsL*. The plasmids used are listed in Table I.

Growth of cells

The cells were grown in LB medium (Bertani, 1951), and growth was monitored using a Gilford Stasar II spectrophotometer at a wave-length of 450 nm. LA plates contain LB medium with 1.5% agar. McConkey-lactose plates were prepared as described by the manufacturer (Difco). Antibiotics were dissolved in the plate media at the following standard concentrations: ampicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (50 μg/ml) and tetracycline (10 μg/ml).

Transfer of plasmids to bacteria

Plasmids were introduced into cells by transformation (Cohen *et al.*, 1972).

Preparation of plasmid DNA, restriction enzyme treatment and ligation of DNA fragments

Plasmid DNA was prepared as described previously (Stougaard and Molin, 1981). Digestion of DNA with restriction enzymes and ligation of DNA fragments using T4 polynucleotide ligase was as described by Molin *et al.* (1979).

Activities of β-galactosidase

Measurements of β-galactosidase expressed from fusion plasmids were made essentially as described by Miller (1972). From an exponentially growing culture samples were taken for OD_{450} reading, and duplicate samples taken for activity measurements. Usually, 10 such samples were taken between OD_{450} 0.1 and 0.5, the activities plotted against OD_{450} and expressed as OD_{420}/min/OD_{450}/ml culture.

Activities of transacetylase

Duplicate samples of 10 ml from exponentially growing cultures were harvested at OD_{450} = 0.2–0.4. The cells were resuspended in 0.5 ml TA-buffer (50 mM Tris, 10 mM EDTA, pH 7.9), sonicated for 20 s, and heated to 70°C for 5 min. The precipitate was spun out in a microcentrifuge and the supernatant transferred to a new tube. Assays of activities were carried out as described by Miller (1972). Activities were expressed as OD_{412}/OD_{260}/h.

Plate assay for lactose permease activities

The strains were streaked on plates containing A+B minimal medium (Clark and Maaløe, 1967) supplemented with 0.5% melibiose, 20 μg/ml proline and antibiotics to select for the plasmids harboured by the strains. After incubation for 2 days at 42°C the plates were read: full growth indicates a high level of expression of the *lacY* gene, poor growth, low expression and no growth, no expression (Beckwith, 1970).

Construction of chimeric plasmids carrying transcriptional repA-lac fusions

Plasmid pJL24. Plasmid pGA46 is a promoter cloning vehicle constructed by An and Friesen (1979) in which insertion of promoter carrying DNA fragments upstream of the tetracycline resistance gene (deleted of its own promoter) results in resistance towards tetracycline. The *PstI*-F_1 fragment from a mini-R1 plasmid (pKN1562) was inserted in the *PstI* site of pGA46 (cf. Figure 3), and the orientation verified by mapping with *SalI*. Selection was for 5 μg/ml tetracycline.

Plasmid pJL171. The *Hind*III-*Bam*HI fragment from pMC81 (Casadaban and Cohen, 1980) carrying the *lac* genes deleted of the *lac* promoter region was inserted in pJL24 such that most of the *tet* gene (from *Hind*III to *Bam*HI) was replaced by *lac* (Figure 3). Selection was for Cm^R on McConkey lactose screening for Lac^+ and tetracycline sensitivity.

Plasmid pJL263. We have recently described the construction of a promoter cloning vector, pJL207, in which the *lac* gene (without promoter) of pMC81 (*Hind*III-*Bgl*II fragment) were inserted in pGA46 replacing the *Hind*III-*Bam*HI fragment covering most of the *tet* gene (Light and Molin, 1982). In the *Bgl*II site upstream of the *lac* genes was inserted a *Bgl*II-*Bam*H1 fragment from pJL111 carrying the promoter distal end of the *copB* gene, the *repA* promoter, the $copA^+$ gene, and 85% of the structural *repA* gene. Screening was for Cm^R and Lac^+ phenotype. The map shown in Figure 3 was verified by restriction enzyme mapping.

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