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Convergent transcription interferes with expression of the copy number control gene, *copA*, from plasmid R1

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The copy number control gene, *copA*, of plasmid R1 codes for an 80-nucleotide untranslatable RNA. In *Escherichia coli* minicells, some *copA* hybrid plasmids and R1 miniplasmids also express a transcript of ~200 nucleotides. Only the small RNA mediates the CopA phenotype. The switch between 80- and 200-nucleotide RNA synthesis is shown to be caused by convergent transcription; if transcription proceeds in both directions the inactive larger RNA is synthesised; the active small RNA is formed when *copA* transcription is not opposed by transcription from the other direction. The data presented indicate that convergent transcription interferes with *copA* expression by abolishing or reducing normal *copA* transcription termination.

Key words: E. coli minicells/oppositely directed transcription/plasmid incompatibility/transcription termination

Introduction

Replication of the IncFII antibiotic resistance plasmid R1 is controlled by two cytoplasmic inhibitors, CopA and CopB, which inhibit expression of a gene, *repA*, coding for a positively acting replication function (Light and Molin, 1981; Yoshikawa, 1974). The CopA inhibitor is small, untranslatable RNA molecule (Stougaard *et al.*, 1981a), and CopB inhibitor a small, basic polypeptide (Molin *et al.*, 1981). It has been argued that plasmid incompatibility is a consequence of the copy number control system, but it was recently shown that, although this is true for the CopA function (Molin and Nordström, 1980), the CopB function does not seem to play any role in incompatibility between wild-type plasmids (Molin *et al.*, 1981; Riise *et al.*, in press).

The CopA-determined incompatibility has also been questioned, since some hybrids carrying the *copA* gene fail to show displacement of certain IncFII replicons (Danbara *et al.*, 1980; Riise *et al.*, in press). The apparent conflicts in these observations prompted us to analyse the RNAs transcribed from the *copA* gene inserted in various genetic environments to see if sequences outside the gene could interfere with its expression. Also, we considered it interesting to analyse the effects of transcription reading against transcription from the *copA* gene. This is relevant, since it has been shown that transcription initiated from the *repA* promoter must read through the *copA* gene in the opposite direction to *copA* transcription (Light and Molin, 1981; cf. Figure 1). A system with similar properties has been analysed by Ward and Murray (1979), and the term convergent transcription was introduced to describe the phenomenon.

The analyses of RNAs from *Escherichia coli* minicells showed that some *copA* hybrid plasmids direct the synthesis of an 80-nucleotide RNA, which was identified as a *copA* transcript. However, other *copA* hybrid plasmids express a larger RNA molecule (~200 nucleotides), which also seemed to be correlated with the presence of the *copA* gene (Stougaard *et al.*, 1981a).

In this communication we first present evidence that the large RNA made from some *copA* hybrid plasmids is indeed a *copA* transcript. Then we describe the analysis of transcripts synthesised from various *copA* hybrid plasmids and show a correlation between patterns of transcription and incompatibility phenotype. Finally, we investigate *copA* expression influenced by different promoters reading against the *copA* gene. The data presented here strongly indicate that expression of the *copA* gene is affected by the presence of a strong, oppositely directed promoter, and it is suggested that the lack of incompatibility observed with some *copA* hybrid plasmids is caused by convergent transcription.

Results

Two copA transcripts

Using plasmid pBR322 as a cloning vector, two types of *copA* hybrid plasmids have been constructed: one by insertion of the *Pst*I fragment F_1 in the β-lactamase gene in both orientations (plasmids pKN317 and pKN423); and the other type by insertion of the *Sau*3A fragment containing the *copA* gene (cf. Figure 1) in the *Bam*HI site of the tetracycline resistance gene in the two possible orientations (plasmids pOU504 and pOU505). Transcripts encoded by such hybrid plasmids can be identified from plasmid-carrying minicells after labelling with [^{3}H]uridine, separation on polyacrylamide gels, and autoradiography.

Figure 2 shows the transcripts synthesised from the hybrid plasmids. The position of the 80-nucleotide (80n) RNA_{copA} (the *copA* gene product) is indicated. The identification of this RNA_{copA} transcript from plasmids pOU504 and pOU505 was described recently (Stougaard *et al.*, 1981a). In contrast to these *Sau*3A hybrid plasmids, the *Pst*I hybrids make no (pKN317) or only small amounts (pKN423) of the 80n RNA_{copA}. Instead, several larger transcripts appeared, and one of these RNAs with an approximate size of 200 nucleotides (200n) was shown to be a *copA* transcript. This identification was based on hybridization experiments using single-stranded *Pst*I F_1 fragment DNA as described previously (Stougaard *et al.*, 1981a). Moreover, this 200n RNA is not made from the hybrid plasmid pOU417, which contains a *copA* promoter mutation (Figure 2A). Thus, both the 80n and the 200n RNAs are *copA* transcripts, and it appears that *copA* transcription can be influenced by the presence of promoters in the vector plasmid.

The two different *copA* transcripts have also been observed in minicells carrying the plasmids pJL127 and pJL128. The

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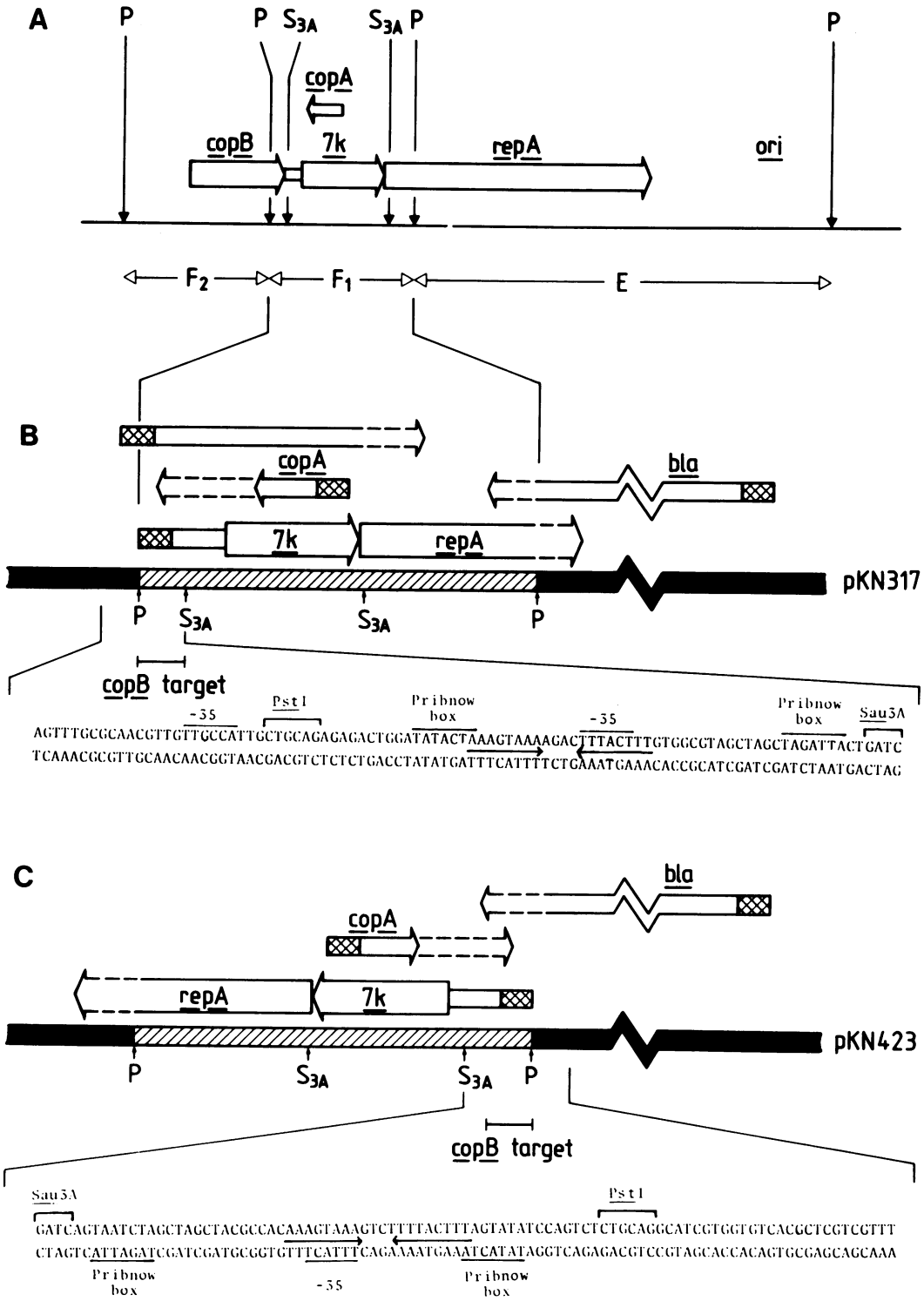

Fig. 1. Genetic and physical map of the replication control region of plasmid R1. **Broad arrows:** Translation products or open reading frames. **Thin arrows:** RNA transcripts. **(A)** The basic replicon of plasmid R1. The direction of transcription and translation is shown for the *copA* and *copB* genes (copy number control), the 7 K gene (a potential 7000 dalton polypeptide) and the *repA* gene (positively acting function); *ori* (the origin of replication). Restriction enzyme sites: P:*Pst*I; S_{3A}:*Sau*3A. E, F_1, and F_2: *Pst*I fragments. **(B)** Extended map of *Pst*I fragment F_1 cloned in plasmid pBR322. Restriction enzyme sites and transcription and translation products are indicated as in **A.** promoter sequences; fragment F_1 sequences; pBR322 sequences. Also shown is the DNA sequence around the left border of the *Pst*I fragment F_1 and the target for the *copB* gene product. Pribnow boxes and −35 sequences are underlined. **(C)** As in **B** except for the orientation of the F_1 fragment.

former makes both types, whereas the latter only makes the larger RNA (Figure 2B). These two plasmids were constructed by inserting into the promoter cloning vector pGA46 (An and Friesen, 1979) a *Pst*I fragment, containing part of the F_1 fragment with the *repA* gene fused to the *lacZ* gene, in the two orientations. As described previously (Light and Molin, 1981), β-galactosidase synthesis is directed by the *repA* promoter which is located near the end of the *Pst*I frag-

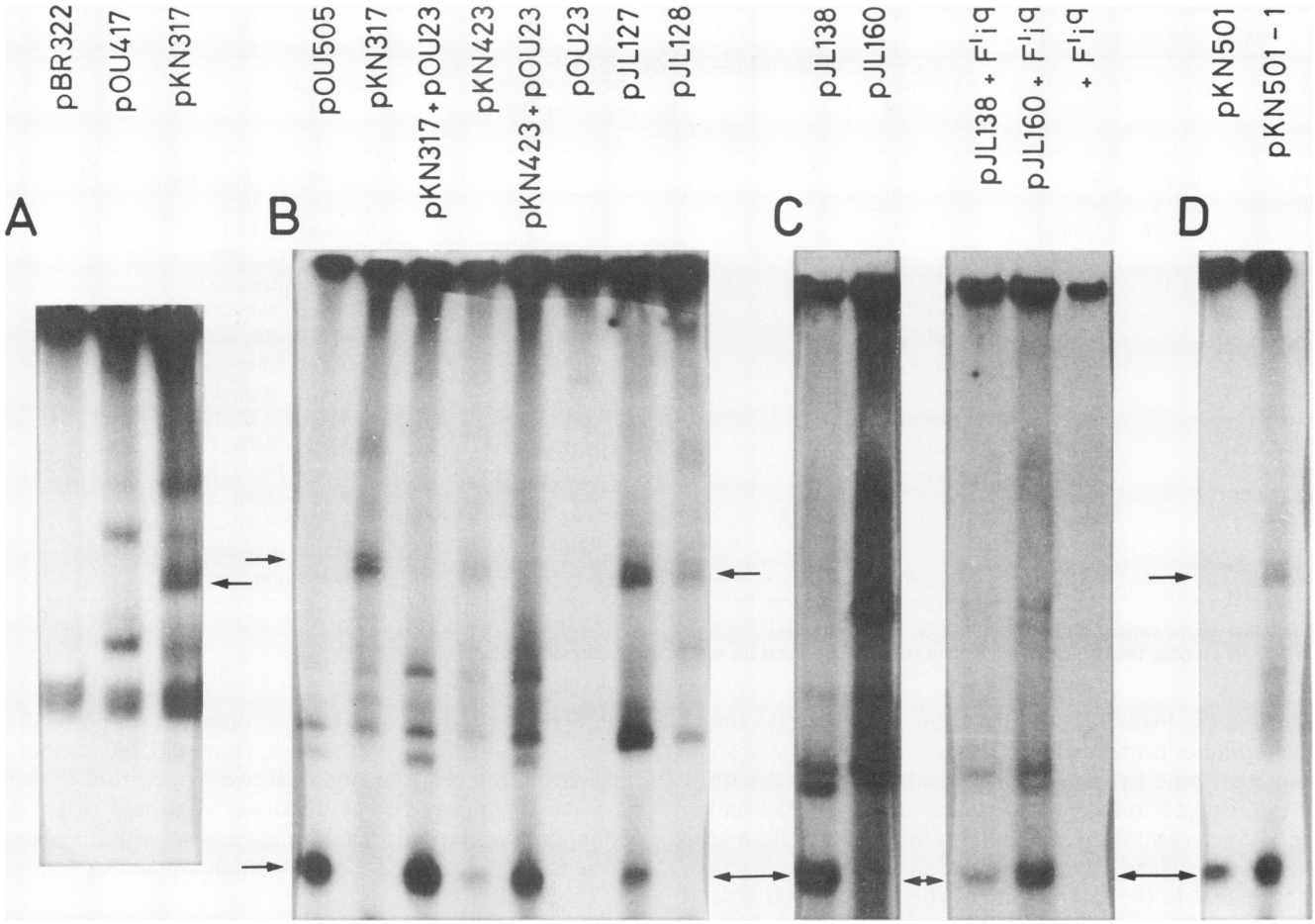

Fig. 2. Autoradiogram of plasmid-specific [^{3}H]uridine-labelled RNA molecules separated on polyacrylamide gels. Arrows indicate the position of the 200n and 80n RNA_{copA} transcripts.

ment F_1 (cf. Figure 1). In these hybrids, transcription proceeds in the anti-sense direction through the *copA* gene before entering the *repA-lacZ* fusion. Plasmid pJL127 mediates a weak Lac$^+$ phenotype (low level of transcription), whereas pJL128 is strongly Lac$^+$ (high level of transcription) as discussed by Light and Molin, 1981. Thus, strong convergent transcription from the *repA* promoter in pJL128 results in the complete disappearance of the small RNA_{copA}, and the formation of the 200n transcript. The nucleotide sequence upstream of the *repA* promoter is the same in pJL128 as in pKN317, since pGA46 has the promoter-distal end of the *bla* gene (from the *Pst*I site) and the orientation of the inserted fragment brings the *repA* promoter next to this (see Figure 1).

The effect of CopB on copA expression

Although *copA* hybrid plasmids (pKN317, pKN423, pOU504, and pOU505) all express incompatibility against R1 it has been shown (Danbara *et al.*, 1980; Riise *et al.*, in press) that if R100 is used as test plasmid, no incompatibility is observed from hybrids with the *Pst*I-F_1 fragment from R1 oriented as in pKN317 (Figure 1 shows the orientation of the F_1 fragments in pKN317 and pKN423 relative to the *bla* gene of pBR322). We have recently presented evidence (Riise *et al.*, in press) that this lack of incompatibility can be complemented by introduction of the R1 *copB* gene (which itself expresses no incompatibility activity). Strains were constructed that harbored R100 together with pKN317 or pOU23, or both, and incompatibility was only observed from the strain harboring all three plasmids.

The effect of the presence of the CopB function on *copA* expression was analysed in minicells. Transcripts from pKN317 and pKN423 in the absence and presence of plasmid pOU23 (a mini-F replicon carrying the *copB* gene) were labelled and separated on polyacrylamide gels and, as shown in Figure 2B, the presence of the CopB function results in a complete shift from the large to the small RNA_{copA}. These results indicate that the Inc$^+$/Cop$^+$ phenotype is connected to the small RNA_{copA} only, and since R100 and R1 express different CopB polypeptides [as shown by the nucleotide sequence analysis (Rosen *et al.*, 1980; Stougaard *et al.*, 1981b)] the difference in incompatibility behaviour of pKN317 towards the two IncFII plasmids suggests that the presence of an R1 CopB function is required for the expression of the 80n RNA_{copA} from pKN317.

The effect of controlled convergent transcription on the expression of the copA gene

The orientation-independent expression of the small RNA_{copA} from the *Sau*3A hybrid plasmids pOU504 and pOU505 indicates that the promoter for the Tc^R-gene in pBR322 is too weak to affect *copA* expression, and that no significant convergent transcription is initiated from a promoter within this *Sau*3A fragment. The latter is in agreement with the recent mapping of the *repA* promoter (Light and

Table I. Properties of plasmids used

Plasmid (*cop* genes)	Parent plasmid	Cloned fragment	Incompatibility[a] and presence of 80n RNA_{copA}			Reference
			No addition	+CopB	+F′i^q	
R1*drd-19*(*copA*$^+$, *copB*$^+$)	R1		+			Meynell and Datta, 1967
pKN501(*copA*$^+$, *copB*$^+$)	R1		+			Molin *et al.*, 1979
pKN501-1(*copA*$^+$, Δ*copB*)	pKN501		+			Molin *et al.*, 1981
pBR322	pMB1		−			Bolivar *et al.*, 1977
pGA46	p15		−			An and Friesen, 1979
pKN317(*copA*$^+$)	R1*drd-19*	*Pst*I-F_1	−	+		Molin and Nordström, 1980
pKN423(*copA*$^+$)	R1*drd-19*	*Pst*I-F_1	+	+		Riise *et al.*, in press
pOU417(*copA*$^-$)	pKN317	*Pst*I-F_1	−	−		This work (Stougaard *et al.*, 1981)
pOU504(*copA*$^+$)	pKN317	*cop*A-*Sau*3A	+	+		Stougaard *et al.*, 1981a
pOU505(*copA*$^+$)	pKN317	*cop*A-*Sau*3A	+	+		Stougaard *et al.*, 1981a
pJL127(*copA*$^+$)	pKN1562	*Pst*I-F_1[b]	+	+		Light and Molin, 1981
pJL128(*copA*$^+$)	pKN1562	*Pst*I-F_1[b]	−	+		Light and Molin, 1981
pJL138(*copA*$^+$)	pKN1562	*cop*A-*Sau*3A	+	+	+	Light and Molin, in preparation
pJL160(*copA*$^+$)	pKN1562	*cop*A-*Sau*3A + *lac*PO	−	−	+	Light and Molin, in preparation
F′i^q,Δz	F		−	−	−	Heidecker *et al.*, 1980
pOU23(*copB*$^+$)	pKN232	*cop*B-*Hpa*II	−	−	−	Riise *et al.*, in press

[a]Incompatibility against various IncFII plasmids (see text for details).
[b]Plasmid pGA46 carrying the *Pst*I-*Sau*3A fragment (CopB target) and the *cop*A-*Sau*3A fragment fused to the *lacZ* gene.

Molin, 1981; cf. Figure 1). By insertion of a strong, controllable promoter (unrelated to R1) next to the *copA-Sau*3A fragment, the same hybrid plasmid could be analysed with respect to the *copA* transcription pattern in the presence and absence of convergent transcription. For this purpose plasmid pJL138 was constructed by insertion of the *copA*-containing *Sau*3A fragment in the *Bam*HI site of the translational gene fusion vector, plasmid pMC1403 (Casadaban and Cohen, 1980). This insertion, resulting in a *repA-lacZ* gene fusion expressing a very low level of β-galactosidase synthesis, shows that the level of transcription reading towards *copA* must be low. Upstream of this insertion an *Eco*RI fragment with the *lac*PO region [derived from plasmid pKB252 (Backman and Ptashne, 1978)] was inserted such that transcription initiated from the *lac* promoter reads against *copA* transcription and into the *repA-lacZ* fusion (plasmid pJL160). This results in a much higher level of β-galactosidase synthesis. The analysis of *copA* transcripts from these two plasmids (Figure 2C) once more shows that the 80n RNA_{copA} is formed in the absence of strong convergent transcription (pJL138), whereas the 200n RNA_{copA} is predominantly made if there is strong convergent transcription (pJL160).

The presence of Lac repressor molecules in cells harboring these plasmids should cause reduced transcription from the *lac*PO fragment in plasmid pJL160. An F′*lac* plasmid with the *lac* genotype i^q, Δz (F′i^q) which overproduces the *lac* repressor was conjugated to minicell producing strains harboring pJL138 and pJL160, respectively. The presence of the F′i^q plasmid had no effect on *copA* expression from pJL138, but from pJL160 the 80n RNA_{copA} is made instead of the larger transcript observed in the absence of F′i^q (Figure 2C). The incompatibility properties of plasmids pJL138 and pJL160 in the presence or absence of the F′i^q plasmid were shown to reflect the presence or absence of convergent transcription and thus of the 80n RNA_{copA}. Accordingly, in the absence of F′i^q plasmid, pJL160 is completely compatible with R1, whereas in the presence of F′i^q pJL160 exerts strong incompatibility towards R1. Plasmid pJL138 is incompatible with R1 also in the absence of F′i^q (Table I). It is important to note that in contrast to what was observed for pKN317 the lack of incompatibility expressed from pJL160 cannot be reversed by the presence of CopB (expressed from the wild-type R1 test plasmid). As discussed elsewhere (Light and Molin, in preparation), this is in agreement with the absence of the CopB target on the *copA*-containing *Sau*3A fragment (cf. Figure 1).

copA transcription from mini R1 replicons

The transcripts from two miniplasmid derivatives of plasmid R1 were analysed. Figure 2D shows that minicells containing the wild-type miniplasmid pKN501 direct the synthesis of the 80n RNA only, whereas the CopB mutant derivative, pKN501-1, makes both the 80n RNA_{copA} and the 200n RNA_{copA}.

Discussion

The experiments presented in this paper show that the copy number control gene, *copA*, of plasmid R1 codes for two transcripts, one of the size of ~200 nucleotides and the other of ~80 nucleotides. Hybridization of the large *copA* transcript to the *Pst*I-F_1 DNA showed that the coding strand was the same as that for the small RNA_{copA}. By comparison with a *copA* promoter mutant which makes no detectable amounts of either of the two RNAs, it was strongly indicated that the larger transcript is initiated from the *copA* promoter but terminated ~100 nucleotides downstream of the termination site for the 80n RNA_{copA}. The relative amounts of these transcripts depend on the genetic environment around the *copA* gene.

Expression of the *copA* gene is influenced by the level of transcription in the opposite direction (convergent transcription). This was, for example, shown for a plasmid in which *lacP*-promoted transcription against the *copA* transcription resulted in a simultaneous loss of the 80n RNA_{copA} and the expression of incompatibility. In the presence of Lac repressor molecules (inhibition of convergent transcription) both the 80n RNA_{copA} and the Inc$^+$ phenotype reappeared.

Several investigations have shown that the *Pst*I-F_1 frag-

ment of R1 (cf. Figure 1) cloned in the *bla* gene on pBR322 only expresses incompatibility against the related plasmid R100 when in one orientation (Danbara *et al.*, 1980, 1981; Riise *et al.*, in press). The hybrid plasmid which is Inc$^-$ in such tests (pKN317) was shown here to express no significant amounts of the 80n RNA$_{copA}$ – only the large *copA* transcript could be detected. The nucleotide sequence of the *bla* gene and the *Pst*I-F$_1$ fragment indicates that a good "hybrid"-promoter is by chance constructed in this plasmid (cf. Figure 1) resulting in a high level of transcription from the *repA* promoter. The same "hybrid" *repA* promoter is created in plasmid pJL128, resulting in a very high rate of *repA-lac* expression, and a lack of expression of the small RNA. The presence of the *copB* gene product changes both the Inc phenotype and the transcription pattern from these hybrid plasmids: expression of *repA-lac* is inhibited (Light and Molin, 1981; Light and Molin, in preparation), the 80n RNA$_{copA}$ is made and incompatibility observed. From these results we conclude that of the two transcripts expressed from the *copA* gene, only the 80n RNA has CopA activity (switch off of *repA-lac* expression and incompatibility). The larger *copA* transcription product is made as a consequence of convergent transcription, the level of which influences the relative amounts of the two RNAs made.

Since the *copB* gene from R1 and the analogous gene from R100 (and R6-5) based on the nucleotide sequences seem to be quite different (Danbara *et al.*, 1980; Rosen *et al.*, 1980; Stougaard *et al.*, 1981b), the different responses of these two plasmids to plasmids like pKN317 can be understood in terms of *copA* expression. Moreover, the complete lack of incompatibility, even against R1 from plasmid pJL160, can be explained, since it was recently shown that the target site for the *copB* gene product is located very near the *repA* promoter and thus outside the *Sau*3A fragment present in pJL160 (Light and Molin, in preparation).

Deletion of the promoter-proximal end of the *copB* gene of R1 has been shown to result in a 10-fold increased replication rate of the plasmid (Molin *et al.*, 1981). Since CopB acts as a repressor of *repA* transcription (Light and Molin, 1981; Light and Molin, in preparation), this effect on the plasmid copy number might be solely a consequence of derepressed *repA* transcription. However, as shown here, the increased rate of *repA* transcription from *copB* deletion mutants also affects *copA* expression, resulting in reduced levels of the small RNA$_{copA}$ (Figure 2D), and this would be assumed also to play a role in the setting of the copy number level. At present we cannot say whether the increased *repA* transcription rate or the reduced level of RNA$_{copA}$ is the main cause of the higher copy number. The decreased amounts of RNA$_{copA}$ made from *copB* mutants were also reflected in complementation experiments in which a small extra gene dosage of *copA* was found to have a strong effect on the copy number in contrast to the seemingly proportional effect of extra *copA* genes on wild-type copy number (Light and Molin, 1981; Molin and Nordström, 1980).

The observations made here represent a new example of convergent transcription. Other examples have shown how two oppositely directed transcriptions influence each other (Ward and Murray, 1979) but, to our knowledge, this is the first direct demonstration of an effect at the level of transcription termination. The observed anti-termination caused by convergent transcription could be a result of either a change of secondary structure of the DNA at the termination sequence or an interaction between the two transcripts, which will have complementary primary structures. It should, however, be added that the total level of *copA* transcription also seems to be reduced as a result of convergent transcription. This can be seen from the gels in Figure 2 by comparing the RNA$_{copA}$ bands with vector plasmid transcripts.

An important assumption behind the suggested interaction between the two opposing transcripts is that the probability that they meet in the *copA* coding sequence is high. It was observed that the stronger the promoter directed against the *copA* gene, the stronger was the effect on the CopA-RNA (compare the effects exerted by the promoters for the *tet, bla,* and *lac* genes). Although we have no precise knowledge about the frequency of transcription of the *copA* gene, it is likely to be relatively high since the CopA-RNA is easily detected despite its rapid degradation. It therefore seems reasonable to assume that transcription of both strands of the *copA* gene occurs simultaneously in those cases where the large CopA-RNA is expressed.

As discussed above, *copB* mutant plasmids of R1 synthesise reduced levels of RNA$_{copA}$, which shows that convergent transcription is involved in regulation of the replication rate of such plasmids. For wild-type plasmids it is possible that convergent transcription exerted on *repA* expression by *copA* transcription could be an element in the replication control system of plasmid R1.

Materials and methods

Bacterial strains and plasmids

The *E. coli* K12 strain used is the minicell producing M2141 (Dougan and Sherratt, 1977) containing the plasmids listed in Table I.

Growth of cells and plasmid transfer

The cells were grown as described by Molin *et al.* (1979), and transformation of bacteria was essentially as according to Cohen *et al.* (1972).

Preparation of plasmid DNA

Preparation of plasmid DNA was as described by Clewell and Helinski (1969) with the modification that the lysozyme solution contained 100 μg/ml RNase that had been heated to 90°C for 10 min to destroy DNase activity. Nucleic acids were concentrated according to Humphreys *et al.* (1975). Preparative dye buoyant density gradient centrifugation was performed in a vertical rotor for 2 h (Stougaard and Molin, 1981).

Purification and labelling of minicells

Minicells of *E. coli* K12 strain M2141 were separated from growing cells essentially as described by Kennedy *et al.* (1977). Labelling of minicells with [^{3}H]uridine (10 μCi/ml) (Amersham, UK) and purification of RNA was as described by Stougaard *et al.* (1981a).

Polyacrylamide gel electrophoresis

RNA molecules were separated on polyacrylamide gels containing 10% acrylamide, 0.2% bisacrylamide, and 0.1% (w/v) SDS in a Tris-borate buffer system. RNA was eluted from the gels as reported previously for DNA fragments (Stougaard *et al.*, 1981a), and RNA-DNA hybridization was carried out essentially as described by Southern (1975).

Enzyme digestions and agarose gel electrophoresis

Restriction enzyme reactions and agarose gel electrophoresis were as reported by Molin *et al.* (1979). T4 polynucleotide kinase and S1 endonuclease reactions were performed as recommended by the manufacturer (Boehringer, Mannheim, GmbH).

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