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Replication control of plasmid R1: RepA synthesis is regulated by CopA RNA through inhibition of leader peptide translation

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The replication frequency of plasmid R1 is post-transcriptionally controlled by an antisense RNA, CopA, that binds to the leader region in the RepA mRNA, CopT, and ultimately inhibits the synthesis of the replication initiator protein RepA. We present results demonstrating that CopA controls RepA synthesis indirectly. A reading frame for a 24 amino acid leader peptide (Tap, *t*ranslational *a*ctivator *p*eptide) is located in the region between the *copA* and *repA* genes. A translational fusion between the *tap* and *lacZ* genes was used to demonstrate that *tap* is translated and controlled by CopA. Stop codons (UAA, UAG and UGA) introduced at three different positions within the *tap* gene led to a severe decrease in *repA* expression. Specific suppression of the stop codons reversed the effect. This indicates that *tap* translation is required for RepA synthesis. Phylogenetic comparisons between IncFII-like plasmids, together with previous *in vitro* and *in vivo* results (Öhman and Wagner, 1989, 1991), suggest that a stable RNA stem–loop structure sequesters the *repA* ribosome binding site irrespective of CopA–CopT duplex formation. The results presented here show that ribosomes translating the *tap* reading frame have to terminate close to the start codon of *repA* to permit reinitiation (direct translational coupling), and that transient disruption of the inhibitory RNA stem–loop is insufficient for activation of *repA* translation. The possibility that direct translational coupling is required because of a suboptimal *repA* RBS cannot be excluded. A model accounting for the involvement of *tap* in copy number regulation of plasmid R1 is presented, and its implications are discussed in relation to studies of the IncFα plasmid ColIb-P9, in which a similar leader peptide has been described (Hama *et al.*, 1990; Asano *et al.*, 1991a,b).

Key words: antisense RNA/leader peptide/plasmid R1/replication control/translational coupling

Introduction

Plasmid R1 is a low copy number plasmid belonging to the IncFII group. The synthesis of the RepA protein is rate-limiting for initiation of replication. Regulation of RepA synthesis is accomplished by a small antisense RNA, CopA, which interacts with its target, CopT, in the RepA mRNA, resulting in the formation of an RNA–RNA duplex (Figure 1; for a review, see Nordström *et al.*, 1984). The structures and sequences required for duplex formation between the two key components, CopA and CopT, as well as the kinetics of the binding reaction, have been studied extensively (Persson *et al.*, 1988, 1990a,b). Binding between the two RNAs takes place ~80 nt upstream of the ribosomal binding site (RBS) of *repA*. Therefore, several mechanisms for indirect inhibition by CopA have been postulated. Dong *et al.* (1987) proposed that formation of the CopA–CopT duplex induces a subsequent secondary structure change in the RepA mRNA that sequesters the *repA* RBS. We have tested predictions from this model *in vitro* and *in vivo*, and were unable to find experimental support for such a mechanism (Öhman and Wagner, 1989, 1991; Berzal-Herranz *et al.*, 1991). A second possibility is that CopA, by binding to CopT during transcription, could induce the formation of a transcriptional terminator in the mRNA. Such a mechanism has been reported by Novick *et al.* (1989) for the *Staphylococcus aureus* plasmid pT181. However, mutational studies of the intergenic region between *copA* and *repA* (see Öhman and Wagner, 1991) and analyses of *in vivo* RNAs from plasmid-carrying cells (P.Blomberg, unpublished) failed to yield evidence for an attenuator mechanism in the regulation of R1 replication. A third possibility is that RNA duplex formation indirectly facilitates the decay of the RepA mRNA. We have previously shown that RNase III is involved in processing the RNA duplex, and indicated that this cleavage affects *repA* expression (Blomberg *et al.*, 1990). More recently, we have shown that complete duplex formation between CopA and CopT is not required for inhibition of RepA synthesis, which suggests that RNase III cleavage is not an obligatory event in the inhibitory pathway (Wagner *et al.*, 1992). In addition, changes in RepA mRNA stability due to duplex-dependent RNase III cleavage were investigated, but only a minor decrease in mRNA half-life (2- to 3-fold) was observed (P.Blomberg, unpublished).

Since our experimental data did not support any of the three models discussed above, we considered the possibility that the inhibition by CopA might occur by preventing activation of *repA* expression rather than by inhibiting RepA translation. This was emphasized by recent mutational studies of the region between the *copA* and *repA* genes. Öhman and Wagner (1991) isolated mutants whose phenotype suggested that the *repA* RBS might be translationally inert when buried in a specific local RNA stem–loop (see also below). In support of this, an *in vitro* analysis of RepA mRNAs, both free and in a duplex with CopA RNA, showed that two consecutive G residues within the *repA* Shine–Dalgarno (SD) sequence were completely inaccessible both to a structure-probing chemical, CMCT, and to ribonuclease T1 (Öhman and Wagner, 1989). In work reported by Berzal-Herranz *et al.* (1991), a seemingly paradoxical mutation was described. This mutation abolished *copA* expression due to an alteration in the −35 promoter element, but, contrary

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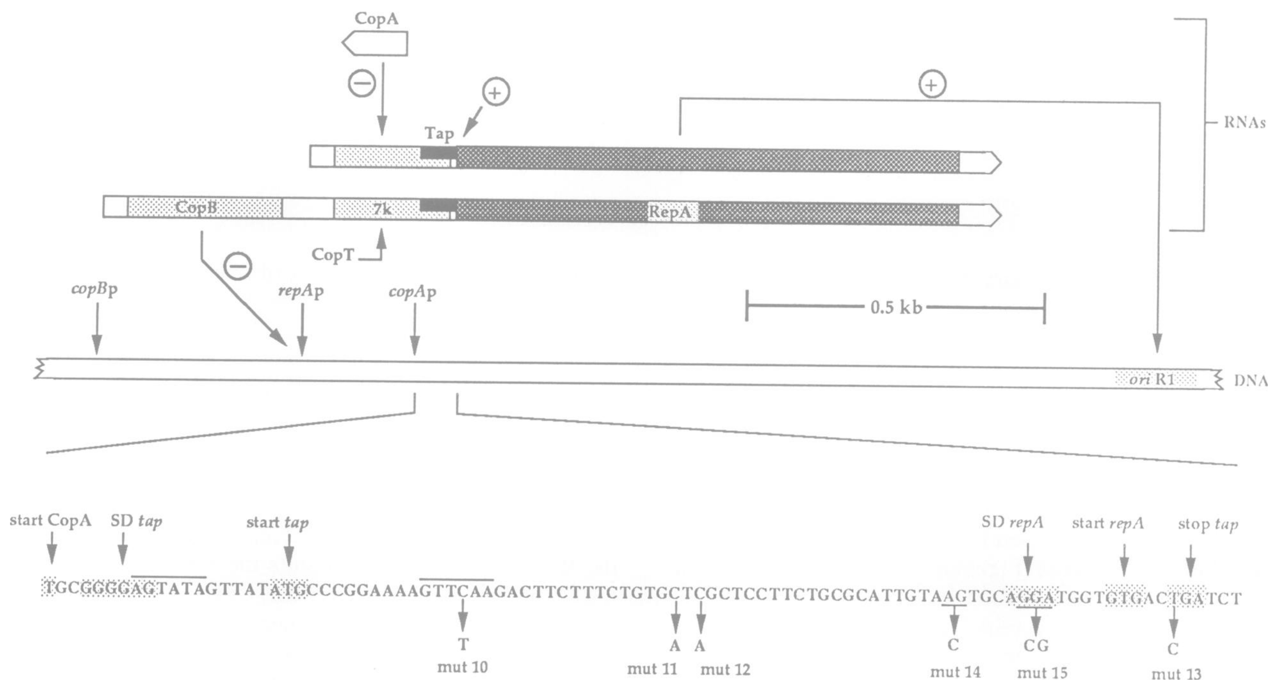

Fig. 1. The basic replicon of plasmid R1 with the location of mutations used in this work. The figure shows the basic replicon of plasmid R1. The locations of important sites are indicated. Positions of the reading frames coding for proteins whose translation has been demonstrated are shown boxed within the wide arrows representing the RNAs. Factors affecting replication positively or negatively are indicated by (+) or (−). The lower part of the figure shows the sequence of the coding strand of the intergenic region between the *copA* and *repA* genes. The −10 and −35 *copA* promoter sequences are overlined. Start and stop codons for *tap* and *repA* are indicated. The mutational changes introduced for the present study are shown, and are denoted by mut 10 to mut 15.

to expectation, resulted in decreased *repA* expression. The base change is here shown to introduce a stop codon in a hitherto overlooked reading frame coding for a 24 amino acid leader peptide (designated Tap, *t*ranslational *a*ctivator *p*eptide), whose function is the subject of the present study.

Translation of leader peptide reading frames has previously been shown to be required for, or to enhance, the translation of downstream reading frames. Translational coupling between reading frames has often been identified as the mechanism behind the enhanced translation, but disruption of secondary structures that sequestered RBSs has also been demonstrated (for a review, see de Smit and van Duin, 1990). The location of the *7k* gene, overlapping the *copA* gene and preceding *repA* in plasmid R1, indicated that it might promote translational coupling to *repA*, but this was ruled out by the phenotype of a stop codon mutation in *7k* (Wagner *et al.*, 1987). Hama *et al.* (1990) have recently reported that the gene for a leader peptide (*repY*) in the IncIα plasmid ColIb-P9 is involved in Rep protein expression. They presented evidence for a long-distance RNA pseudoknot structure that enhances *repZ* expression; the formation of the pseudoknot is dependent on *repY* translation (Asano *et al.*, 1991a,b).

The location of the *tap* gene in plasmid R1, overlapping the sequences involved in the formation of an RNA hairpin that could render the *repA* RBS inactive, and the close proximity of the translation stop and start signals for these two reading frames, raises the question: by what mechanism does *tap* affects *repA* expression? The results described below address this question and bear on the function of the *tap* gene in R1 as well as in other IncFII-like plasmids. The results reported below are discussed in relation to results obtained with the ColIb-P9 plasmid.

Results

A reading frame for a 24 amino acid leader peptide is encoded in the intergenic region between copA and repA

A number of mutations that affect *repA* expression are located in the intergenic region between *copA* and *repA* (Berzal-Herranz *et al.*, 1991; Öhman and Wagner, 1991). In one of these, the *copA* promoter mutant mentioned in the Introduction (Berzal-Herranz *et al.*, 1991), the *copA* promoter is inactivated by a C to T base change (mutation 10 in Figure 1). The mutation unexpectedly led to a severe decrease in *repA* expression. This phenotype remained obscure until it was found that this mutation, in addition to abolishing *copA* promoter activity, changed a CAA to a UAA stop codon. This codon is the sixth codon in an open reading frame which encodes a putative 24 amino acid peptide, which we call Tap. The first nucleotide of the SD sequence of *tap* is located two bases downstream of the nucleotide complementary to the 5′-end of CopA RNA, and the stop codon is positioned two nucleotides downstream of the *repA* start codon (Figure 1). The position of the reading frame suggests that it might serve as a leader peptide in *repA* expression.

Translation of tap is inhibited by CopA

To test whether *tap* is translated *in vivo*, we fused the *lacZ* gene to the *tap* reading frame. The resulting plasmid (pGW233) contains R1 sequences from just upstream of the *repA* promoter to codon 10 in *tap*.

β-galactosidase activities were measured in cells harbouring the *tap*−*lacZ* fusion plasmid, and in cells harbouring a *repA*−*lacZ* translational fusion in the same vector background. The results of such measurements show

Table I. Expression of *tap–lacZ* and *repA–lacZ* in the presence or absence of CopA *in trans*

Plasmid(s)	CopA *in trans*[a]	Fusion	Relative β-galactosidase activity[b]
pGW133	–	*repA–lacZ*	1.00
pGW133 + pGW15	+	*repA–lacZ*	0.03
pGW233	–	*tap–lacZ*	10.50
pGW233 + pGW15	+	*tap–lacZ*	0.38

[a]CopA was provided *in trans* by plasmid pGW15 (Table IV).
[b]β-galactosidase activities of cells carrying *repA–lacZ* or *tap–lacZ* fusion plasmids were measured as described by Miller (1972). The activity measured in cells harbouring plasmid pGW133 was assigned the value 1.00.

Table II. Effect of mutations in the *tap* reading frame on RepA–LacZ synthesis

Plasmid	Mutation[a]	β-galactosidase activity	
		Relative values[b]	Miller units[c]
pGW177L	Wild-type	1.00	27.90
pGW177L-10	$CAA_6 \rightarrow UAA$	0.03	0.76
pGW177L-11	$UGC_{11} \rightarrow UGA$	<0.01	0.02
pGW177L-12	$UCG_{12} \rightarrow UAG$	<0.01	0.14
pGW177L-13	$UGA_{25} \rightarrow CGA$	0.02	0.43
pGW177L-14	$AGU_{19} \rightarrow CU$	2.23	62.13
pGW177L-15	$GGA_{21} \rightarrow CG$	2.10	58.70

[a]The subscript numbers refer to the codons of the *tap* reading frame (Figure 1).
[b]All β-galactosidase activities were normalized to those measured in XAc cells containing the wild-type plasmid pGW177-L.
[c]The values given represent an average of three or four independent measurements and are shown here in Miller units (Miller, 1972). Background values for plasmid-free cells were below the detection limit.

Table III. Codon-specific suppression of stop codons in the *tap* gene measured in cells carrying *repA–lacZ* fusion plasmids

Bacterial strain	Suppressor	Degree of suppression[a]			
		pGW177L	pGW177L-10	pGW177L-11	pGW177L-12
XAc	None	1.0	1.0	1.0	1.0
NHY317	Su6 (UAG)	0.9	n.d.	2.0	**92.9**
CDJ64	Su9 (UGA)	1.7	n.d.	**12.5**	1.1
XA10B	SuB (UAG+UAA)	2.2	**36.7**	n.d.	n.d.
XA102	Su2 (UAG)	2.1	1.7	n.d.	n.d.

[a]The degree of suppression was defined as the ratio between activities measured in the suppressor host strain and the activity in strain XAc (both harbouring the same plasmids). The values in boldface indicate specific suppression of the *tap* stop codons. For each plasmid, the β-galactosidase acvtivities shown in Table II were given the value of 1.00.

that *tap* was translated (Table I). In comparison, the β-galactosidase activity of cells harbouring the *tap–lacZ* fusion plasmid (pGW233) was significantly (10-fold) higher than that from cells carrying the *repA–lacZ* fusion plasmid (pGW133). The higher level of *tap* expression was not due to lower steady-state levels of CopA produced from plasmid pGW233 (data not shown).

Since CopA is known to inhibit *repA* expression, we tested whether *tap* expression was also subject to inhibition by the antisense RNA. A compatible CopA donor plasmid (pGW15) was introduced into cells carrying either pGW133 or pGW233. The synthesis of both fusion proteins was severely inhibited when CopA RNA was supplied *in trans* (Table I). This indicates that CopA, in addition to its inhibitory effect on *repA* expression, also inhibits *tap* translation, and that the degree of repression caused by CopA (a decrease by a factor of 30) is approximately the same for both Tap–LacZ and RepA–LacZ synthesis.

Translation of tap is required for RepA synthesis

If translation of the Tap leader peptide were required to activate expression of *repA*, then mutations in *tap* should decrease RepA synthesis. To test this prediction, we introduced UGA and UAG stop codons at positions 11 and 12 in the *tap* reading frame, respectively (see Figure 1). The effect of these mutations, and the previously characterized UAA mutation, on *repA* expression was then determined. All three mutations should cause premature termination of *tap* translation. As shown in Table II, expression of *repA–lacZ* was severely decreased by either one of the three stop codons. Hence, either *tap* translation or the *tap* gene product itself seem to be required for RepA synthesis.

Suppression of the stop codons in tap restores repA expression

If the low RepA–LacZ synthesis from the stop codon-mutant plasmids were due to premature termination of *tap*

translation, β-galactosidase activity should be partially restored in nonsense suppressor strains. Therefore, we analysed *repA−lacZ* expression from plasmids of the pGW177-L series (separately carrying the three different stop codons) in suitable host strains. The results, which are presented in Table III, show that the effect of all three stop codons was suppressed in a codon-specific manner. RepA−LacZ synthesis from cells carrying pGW177L-10 was increased ~37-fold when this plasmid was introduced into XA10B (UAA suppressor strain), and RepA−LacZ synthesis from plasmids pGW177L-11 (UGA) and pGW177L-12 (UAG) was ~12- and ~93-fold higher, respectively, when they were present in their cognate suppressor strains (CDJ64 and NHY317; Table III). No significant suppression was observed when the mutant plasmids were introduced into heterologous suppressor strains (Table III).

In conclusion, the results of the suppression tests suggest that premature termination of *tap* translation causes a low *repA−lacZ* expression. It also implies that changes in the metabolic stability of the mRNA or in its secondary structure are unlikely to account for the observed mutant phenotypes.

RepA is translationally coupled to Tap

Öhman and Wagner (1991) have recently suggested that the RBS of *repA* is sequestered in an RNA stem−loop. Certain mutants which showed an elevated level of *repA* expression were found to carry mutations that could destabilize this putative hairpin. The existence of this RNA structure suggests two possible functions of *tap*. Either, *tap* translation could activate *repA* translation by disrupting the inhibitory RNA structure, or the terminating ribosomes could transfer directly to the nearby *repA* start codon (direct translational coupling). To distinguish between these possibilities, we mutated the UGA stop codon of *tap* to CGA (mut 13, Figure 1), so that the *tap* reading frame was extended by 19 codons. This base change does not alter the predicted amino acid sequence of *repA*. When this mutation was present, a drastic decrease in *repA* expression was observed (pGW177-13, Table II). This result indicates that a transient disruption of the inhibitory RNA structure is insufficient for initiation of *repA* translation, and suggests that the *tap* stop codon has to be positioned near the *repA* start codon, so that direct translational coupling can occur by a reinitiation event.

Fusion of the tap and repA reading frames increases RepA−LacZ synthesis

Direct translational coupling requires that the number of ribosomes initiating at the *tap* RBS must be at least as high as the one subsequently initiating at the *repA* RBS. This agrees with *tap−lacZ* expression being ~10-fold higher than that of *repA−lacZ* (pGW233 versus pGW133, Table I).

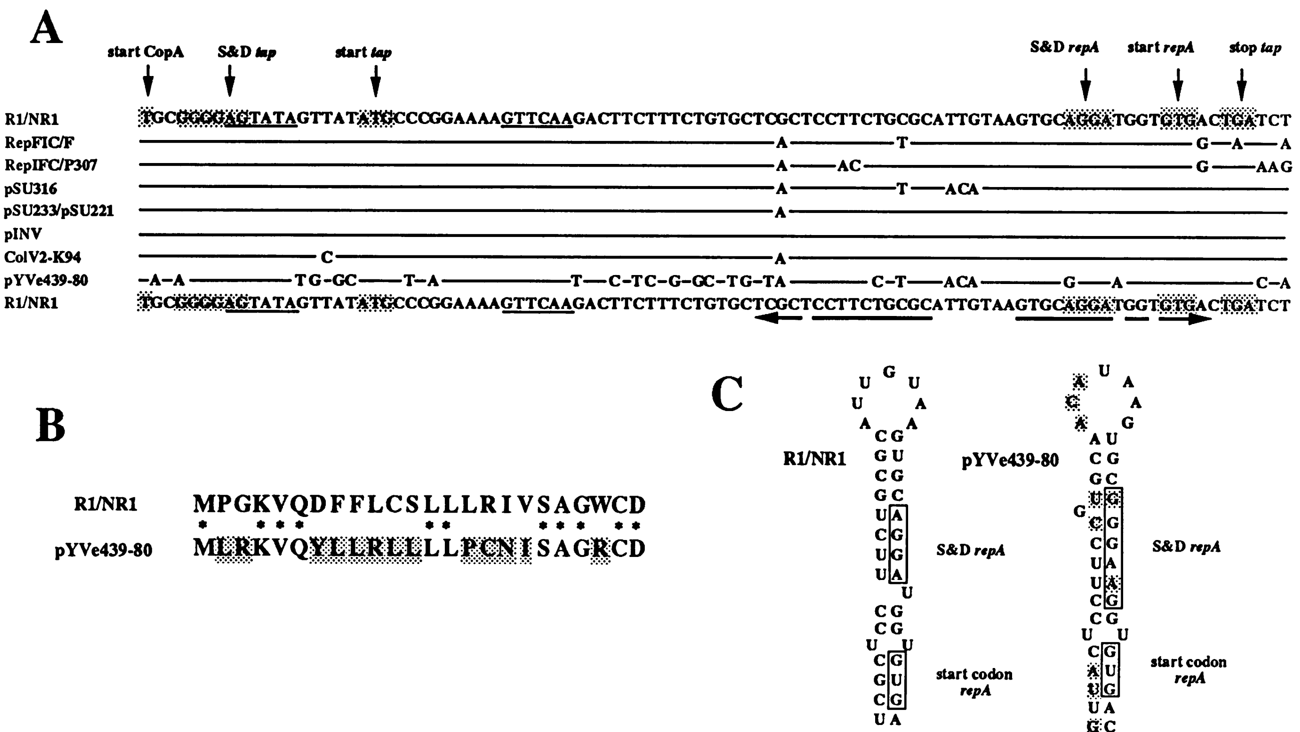

Fig. 2. (A) Comparison of the DNA sequences in the regions between the *inc* and *rep* genes in IncFII-like plasmids. The DNA sequences were compiled from published data (Ryder *et al.*, 1982; Weber and Palchaudhuri, 1986; Saadi *et al.*, 1987; Silva *et al.*, 1988; Lopéz *et al.*, 1989; Vanooteghem and Cornelis, 1990). The R1/NR1 sequence is shown both above and below the other sequences to facilitate comparison. Translation start and stop signals as well as the position complementary to the 5′-most nucleotide of CopA RNA are indicated. The positions of the −10 and −35 regions of the *copA* promoter are underlined. The location of the inverted repeat resulting in the putative inhibitory RNA stem−loop is shown by fat arrows under the DNA sequence (see text). Base changes in all plasmids relative to the R1/NR1 sequence are shown, and identical nucleotide sequences are indicated by horizontal lines. (B) Comparison of the predicted amino acid sequences of the *tap* reading frames in R1/NR1 and pYVe439-80. Amino acid sequences are given in the one-letter code. Identical amino acids are shown by asterisks between the sequences. Shaded letters highlight the amino acid changes between the two peptides. (C) Comparison of predicted RNA secondary structures in the translation start region of R1/NR1 and pYVe439-80. Folding of the local RNA secondary structure containing the Rep protein RBS (see Figure 2A and the text) was predicted using the algorithm of Zuker and Stiegler (1981) with the energy parameters defined as in Freier *et al.* (1986). The calculated ΔG° value for the R1/NR1 structure is −14.3 kcal/mol, and that for pYVe439-80 is −16.2 kcal/mol. The same analysis was performed for the corresponding RNA sequences of all the other plasmids, and no major changes in the structures of the stem−loops were predicted (data not shown).

However, polarity effects, changed mRNA stabilities or altered specific activities of fusion proteins could in part invalidate the expression ratio measured. We therefore constructed two mutants in which the *tap* frame was fused to the *repA* frame (see Figure 1). The resulting plasmids, therefore, carry *tap*–*repA*–*lacZ* fusions and differ from the wild-type *repA*–*lacZ* plasmid by only one nucleotide in length (mut 14 and 15, Figure 1). Mutation 14 leaves the wild-type *repA* SD sequence intact, wheras mutation 15 changes it. This provides an additional test for readthrough-activated *repA* translation, since ribosomes passing the *repA* RBS region out-of-frame might make the translation start site accessible in mutant 14, wheras in mutant 15 no consensus *repA* SD is present. Both mutants showed higher β-galactosidase activities than the wild-type construct, consistent with a coupling mechanism (Table II). Note that both mutant plasmids gave similar activity values, indicating that without termination near the *repA* RBS, reinitiation did not take place, even when an intact SD sequence was present.

Expression of repA does not involve ribosomal frameshifting

Since *tap* expression was substantially higher than *repA* expression, we had to consider the possibility that *repA* was translated by high level ribosomal frameshifting from the *tap* into the *repA* frame. An inspection of the DNA sequence showed that the 'frameshifting window' for such an event is small, and no suitable 'shifty' sequences are apparent (for a review on ribosomal frameshifting, see Atkins *et al.*, 1990). The potential shift from the *tap* to the *repA* frame would have to occur downstream of a UAA codon located 12 nt in front of the *repA* start codon, and upstream of the UGA stop codon of *tap*. If ribosomal frameshifting occured upstream of the *tap* stop codon, normal *repA*–*lacZ* expression would be expected in cells carrying plasmid pGW177-L13 (*tap* UGA stop codon mutated to CGA; Table II). This is in contrast to the result obtained. Furthermore, ribosomal frameshifting would lead to *repA* translation products of identical size from wild-type, mutant 14 and 15 plasmids, whereas a reinitiation event at the *repA* RBS would result in a predicted size difference of 2.4 kDa between the products from the wild-type and the two frameshift mutant plasmids. The sizes of the protein products were determined by gel analysis after translation in a cell-free transcription–translation system. The *repA* translation products of mutants 14 and 15 were larger than that from a wild-type plasmid (data not shown). We conclude that ribosomal frameshifting is unlikely to explain why *tap* is required for *repA* expression.

The tap reading frame is highly conserved in IncFII-like plasmids

Figure 2A shows a sequence comparison of the intergenic region between the *inc* genes and the *rep* genes in a number of IncFII-like plasmids. All plasmids contain identically positioned 24 codon ORFs. Figure 2B shows an amino acid sequence comparison between *tap* in R1 and the equivalent ORF in the distantly related *Yersinia* plasmid pYVe439-80. This comparison shows that although pYVe439-80 and R1 have different amino acid sequences (13/24 amino acid changes), the underlying DNA sequence differences do not alter the positions of the *tap* RBS in relation to the binding site of the antisense RNA, or the stop codon's position relative to the *repA* RBS. This strongly argues for the importance of translation of the reading frame and against the importance of its amino acid composition. Figure 2C shows the predicted secondary structures in the mRNAs of these two plasmids that are postulated to sequester the *rep* protein RBSs, with the sequence alterations highlighted. The strong conservation of the predicted local RNA structures, together with the conservation of the *tap* gene in all plasmids compared, suggests that the requirement for leader peptide translation is the same in these plasmids.

Discussion

In this communication, we report that a previously undescribed reading frame is located in the intergenic region between the *copA* and *repA* genes of plasmid R1, and is involved in the expression of the *repA* gene. This reading frame codes for a leader peptide, Tap, which is translated, and the expression of the *tap* gene is regulated by CopA RNA (Table I). Translation of *tap* is required for the synthesis of a RepA–LacZ fusion protein, as shown by the severe effects of three different stop codon mutations in three different *tap* codons (Table II). The decrease in *repA*–*lacZ* expression in these mutants could be partially reversed in suppressor strains, indicating that the mutations affect *tap* translation rather than mRNA structure or stability (Table III). A comparison of the R1 DNA sequence in this region with the sequences found in other IncFII-like plasmids indicates that the location of this reading frame is completely conserved, even though the amino acid sequence of Tap may vary considerably (Figure 2A and B). Therefore, translation of *tap*, rather than the protein product itself, seems to be involved in *repA* expression. A possible function of *tap* is suggested by the overlap between its stop codon and the RBS region of *repA* (see Figures 1 and 2A). Since in all the plasmid sequences shown in Figure 2A, the UGA (or UAA in RepIFC/F) codon of *tap* lies only two nucleotides dowstream of the GUG start codon of *repA*, translational coupling is a likely explanation for the requirement of *tap* expression for RepA synthesis. In addition, all sequences shown contain an imperfect inverted repeat even though the primary nucleotide sequences vary between plasmids. We propose that this repeat leads to the formation of an RNA stem–loop that sequesters the RBS of *repA* (Figure 2C). The high predicted stability of this stem–loop (Figure 2C) together with the effects of mutations introducing stop codons in the *tap* reading frame (Table II) suggest that in the absence of *tap* translation, the *repA* RBS is inaccessible for initiating ribosomes. To activate the *repA* RBS, either ribosomes translating the *tap* reading frame could disrupt the RNA structure, or ribosomes terminating at the *tap* stop codon could reinitiate at the nearby *repA* start codon (direct translational coupling). In plasmids carrying a mutation that converts the *tap* UGA codon to CGA, resulting in an extension of the *tap* reading frame by 19 codons, *repA*–*lacZ* expression was drastically decreased (mut 13, Table II). This indicates that disruption of the RNA stem–loop is not sufficient, and that translation of *tap* must terminate in the vicinity of the *repA* translation start signals to permit efficient coupling. In addition, the possibility that *repA* expression is accomplished by ribosomal frameshifting from the *tap* into the *repA* frame, is not supported by our results. We conclude that RepA synthesis is indirectly controlled by regulation of *tap* translation.

Our current view is summarized in the model presented

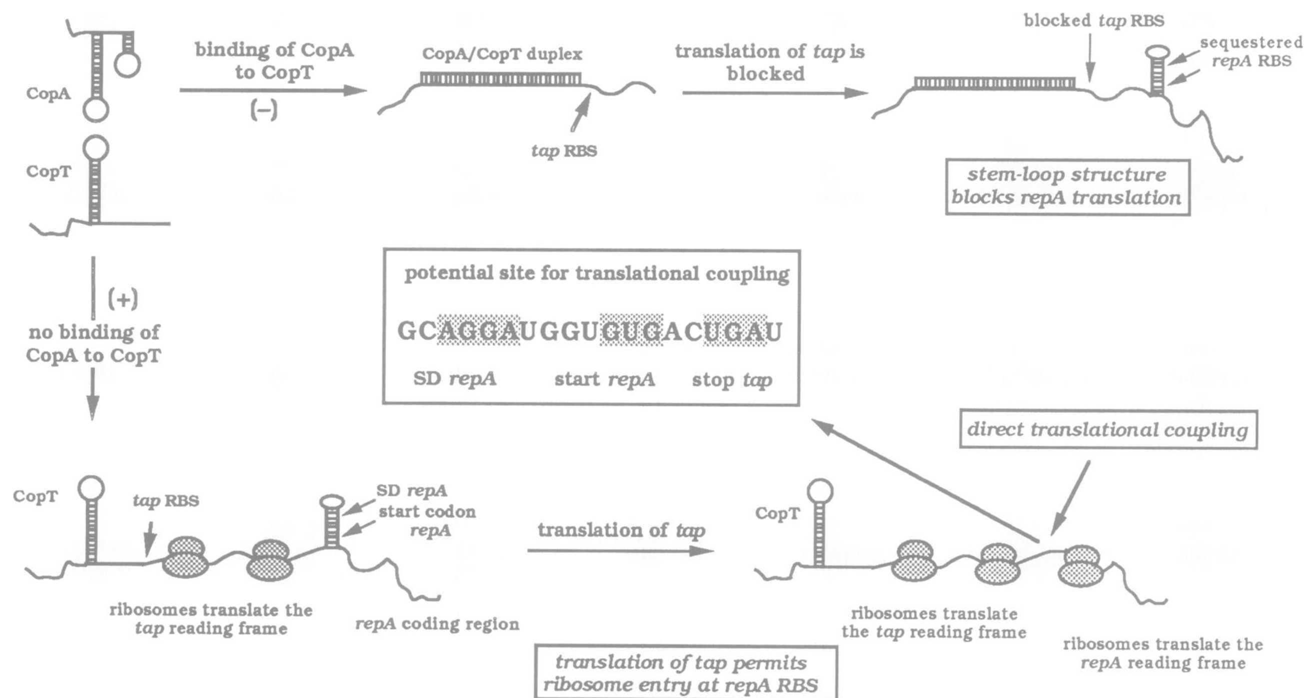

Fig. 3. A model for the involvement of *tap* in replication control of plasmid R1. The model is described in the Discussion. The signs (−) and (+) define the inhibitory and the productive pathway, respectively (see text for details).

in Figure 3. The replication frequency of plasmid R1 is controlled by CopA RNA. The intracellular concentration of CopA (which is linearly correlated with, and therefore represents a measurement of, the plasmid copy number) and the binding rate constant of CopA to its target, CopT, determine the kinetics of RNA duplex formation (see Persson *et al.*, 1988, 1990a,b). The probability of 'escape' versus binding determines the relative flows through either the active (+) or the inhibitory (−) pathway. If CopA fails to bind, ribosomes translate the *tap* reading frame, terminate at the UGA stop codon and reinitiate at the *repA* RBS (direct translational coupling). RepA protein synthesis is permitted and the *de novo* synthesized protein acts to promote initiation of replication at *oriR*. If CopA binds to CopT, a duplex is formed. This prevents translation of the Tap reading frame. Under these conditions, the stable RNA stem−loop that sequesters the *repA* RBS prevents translation of the *repA* reading frame. Consequently, no RepA protein is synthesized and initiation of replication does not occur.

The above model suggests that Tap can be regarded as a leader peptide. Leader peptide reading frames have been previously demonstrated to be required for efficient translation of reading frames located downstream. Different classes of translational coupling can be distinguished. In some cases, inhibitory RNA structures are implicated in preventing leader peptide-independent translation of the second reading frame (e.g. Kastelein *et al.*, 1983; Berkhout *et al.*, 1987), whereas in others, translational coupling is required to activate a translation start site that is inherently ineffective in initiation of translation (Ivey-Hoyle and Steege, 1989). In a comparison between several similar killer gene systems, Gerdes *et al.* (1990) showed that most of them appear to require out-of-frame translation of long reading frames in order to activate translation from the overlapping, shorter reading frames coding for the killer proteins. Further examples of coupled genes were described by de Smit and van Duin (1990).

Recently, a requirement for leader peptide translation was demonstrated in the Inclα plasmid ColIb-P9 (Hama *et al.*, 1990), the first example of a plasmid in which expression of a *rep* gene requires translation of a leader peptide. This plasmid appears to be unrelated to the IncFII-like plasmids, but shares several features of interest, in particular concerning the possible mechanism of regulation. Like plasmid R1, ColIb-P9 replication requires synthesis of a Rep protein, RepZ, whose translation is indirectly controlled by an antisense RNA, Inc RNA. In contrast to R1, ColIb-P9 lacks an equivalent of the *copB* transcriptional repressor gene, and its antisense RNA is ~20 nt shorter [the structure corresponding to stem−loop I of CopA RNA (Wagner and Nordström, 1986) is absent]. The intergenic regions where the *tap* (R1) and *repY* (ColIb-P9) genes are located show few similarities, with the exception of the distance between the site where the antisense−target RNA duplex is formed and the presumed SD sequence of the leader peptide reading frame. The predicted leader peptides differ in size (29 versus 24 amino acids) and have no amino acid homology. Like in plasmid R1, the RNA sequence near the RBS of *repZ* is proposed to fold into an inhibitory, stable stem−loop structure. This structure, however, is different from the one shown in Figure 2C, in that the *repZ* SD is located on the 5′-half of the stem. Furthermore, the GUG (*repZ*) and UGA (*repY*) codons are 7 nt apart instead of 2 (spacing in R1, see Figures 1 and 2), and the spacing between the SD sequence and the start codon is suboptimal (4 nt) in R1, but optimal (9 nt) in ColIb-P9.

Are ColIb-P9 and R1 regulated by the same mechanism? The analysis of ColIb-P9 indicates that *repY* translation is required for *repZ* expression (Hama *et al.*, 1990). As for R1 (mut 13, Table II), ColIb-P9 leader peptide translation

Table IV. Bacterial strains and plasmids

E.coli strains	Genotype		Source/reference
DH5α	F⁻ *endA1 hsdR17 supE44 thy-1 recA1 gyrA96 relA1* Δ(*argF−lacZYA*)*U196* φ*80*Δ*lacZ*Δ*M15*		Hanahan (1985)
XAc	F⁻ *ara argE* (*UAG*) Δ(*lacproB*) *gyrA rpoB thi*		Coulondre and Miller (1977)
NHY317	F− *ara argE* (*UAG*) Δ(*lacproB*) *gyrA rpoB thi leuX* (*Su6*) *zic-501*::Tn*10*		Kirsebom *et al.* (1988)
CDJ64	F⁻ Δ(*lacproB*) *ara gyrA rpoB thi trpT* (*Su9*)		Coulondre and Miller (1977)
XA10B	F⁻ *ara argE* (*UAG*) Δ(*lacproB*) *met B gyrA rpoB thi* (*SuB*)		Miller and Albertini (1983)
XA102	F⁻ *ara argE* (*UAG*) Δ(*lacproB*) *metB gyrA rpoB thi ginV*(*Su2*)		Miller and Albertini (1983)
Plasmids	**Description**	**Parent plasmid(s)**	**Source/reference**
pACYC177	p15A plasmid, cloning vector	p15A	Chang and Cohen (1978)
pMC1403	pMB1-based *lacZ* fusion vector		Casadaban *et al.* (1980)
pGW15	p15A vector with cloned *copA* gene	pSU2718 + pGW643	Wagner *et al.* (1992)
pGW58	Cloned R1 control region, −296 to +596[a] (Δ*Bgl*II−*Bgl*II)	pMa5-8 + pJL133	Blomberg *et al.* (1990)
pGW58-10	Like pGW58, but with *copA* promoter down-mutation and UAA_6 stop codon in *tap*	pGW58	Blomberg *et al.* (1990)
pGW58-11	Like pGW58, UGA_{11} stop codon in *tap*	pGW58	This paper
pGW58-12	Like pGW58, UAG_{12} stop codon in *tap*	pGW58	This paper
pGW58-13	Like pGW58, UGA_{25} → CGA change in *tap*	pGW58	This paper
pGW58-14	Like pGW58, AGU_{19} → CU frameshift in *tap*	pGW58	This paper
pGW58-15	Like pGW58, GGA_{21} → CG frameshift in *tap*	pGW58	This paper
pGW58-L	*repA−lacZ* fusion derivative of pGW58	pGW58 + pGW133	Berzal-Herranz *et al.* (1991)
pGW177-L	*repA−lacZ* translational fusion in p15A vector	pACYC177 + pGW58-L	This paper
pGW177-10L	Like pGW177-L, but with *copA* promoter down-mutation and UAA_6 stop codon in *tap*	pGW177-L + pGW58-10L	This paper
pGW177-11L	Like pGW177-L, UGA_{11} stop codon in *tap*	pGW177-L + pGW58-11L	This paper
pGW177-12L	Like pGW177-L, UAG_{12} stop codon in *tap*	pGW177-L + pGW58-12L	This paper
pGW177-13L	Like pGW177-L,UGA_{25} → CGA change in *tap*	pGW177-L + pGW58-13L	This paper
pGW177-14L	Like pGW177-L, AGU_{19} → CU frameshift in *tap*	pGW177-L + pGW58-14L	This paper
pGW177-15L	Like pGW177-L, GGA_{21} → CG frameshift in *tap*	pGW177-L + pGW58-15L	This paper
pGW133	*repA−lacZ* translational fusion, pMB1 replicon	pJL133	Berzal-Herranz *et al.* (1991)
pGW233	*tap−lacZ* translational fusion, pMB1 replicon	pMC1403 + pGW58	This paper
pGW277	deletion of *lacZ* sequences	pGW177-L	This paper
pGW277-14	deletion of *lacZ* sequences	pGW177-14L	This paper
pGW277-15	deletion of *lacZ* sequences	pGW177-15L	This paper

[a]The nucleotide positions are given according to Ryder *et al.* (1982).

must terminate close to the Rep protein RBS (Asano *et al.*, 1991b). However, the effect of *repY* is proposed to be primarily a transient disruption of the inhibitory RNA hairpin, whereupon a pseudoknot can form between the target loop sequences and a four nucleotide sequence immediately in front of the *repZ* SD. An analysis of *repZ*-defective mutants and second-site revertants supported the hypothesis that this RNA pseudoknot is formed *in vivo* (Asano *et al.*, 1991a,b). It is suggested that this structure prevents the inhibitory structure from refolding and enhances *repZ* translation. It is not clear whether a similar mechanism applies in the regulation of *repA* expression in plasmid R1. The recent finding that formation of a 'kissing complex' between CopA and CopT, without complete duplex formation, inhibits *repA* expression, is compatible with a pseudoknot model (Wagner *et al.*, 1992). On the other hand, many different mutants with increased copy number have been found to have base changes within the major CopA/CopT loop. Most of these mutations should affect the formation of a pseudoknot with putative, complementary RNA sequences as in the case of ColIb-P9, and therefore decrease *repA* expression. In contrast, all the mutants have elevated copy numbers, ranging from 2- to 8-fold higher than that of the wild-type plasmid (for a review, see Nordström *et al.*, 1984). The binding rate constants for CopA and CopT were determined *in vitro* for some of these mutants, and the decrease correlated quantitatively with the copy number increase of the mutant plasmids (Persson *et al.*, 1988, 1990b; and unpublished). This suggests that these mutations affect primarily or exclusively the binding between CopA and CopT. Interestingly, a mutation in the target loop sequence, UUGGCG to UUGGC*A*, in plasmid ColIb-P9 (mutant *rep*2041 in Asano *et al.*, 1991a), led to a >60-fold decrease in *repZ* expression, whereas an identical base change in plasmid R1, (plasmid pKN104, Givskov and Molin, 1984) resulted in a copy number increase by a factor of 8. This indicates that pseudoknot formation might not play a role in the regulation of *repA* expression in plasmid R1.

The need for translational coupling in the two plasmid systems might also be different, as suggested by the difference in spacing between the SD sequences and GUGs of the *rep* proteins, and between the leader peptide UGA and *rep* GUG codons. The suboptimal spacing in the *repA* RBS of R1 might render this translation start signal inherently ineffective. Further experiments, in which mutations are introduced in order to destabilize the inhibitory hairpin, will permit us to distinguish between the effect of this RNA structure versus RBS strength on the very low *tap*-independent RepA translation.

The results reported above indicate that the antisense

RNAs in the IncFII-like plasmids do not function by inhibiting translation of the Rep protein reading frames. Instead, the normal state for *rep* translation is 'off' and an activation event is required for RepA synthesis, such as translation of a leader peptide reading frame with subsequent translational coupling to the *repA* coding frame. It is this activation event which is inhibited by the antisense RNA. Thus, the effect of CopA should be reinvestigated in the light of these data. Considering the short distance (two nucleotides) between the end of the CopA–CopT RNA duplex and the RBS of *tap*, it is possible that this structure prevents ribosomes from initiating. An RNA duplex covering the RBS for a transposase has been shown, by toe-printing analysis, to prevent ribosome access (Ma and Simons, 1990). So far, blockage of an RBS by a nearby RNA duplex has not been reported, but in one study the close proximity of a stable RNA secondary structure to a ribosome entry site has been shown to result in reduced protein synthesis (Blumer *et al.*, 1987). We intend to analyse whether formation of the RNA duplex between CopA and CopT does prevent ribosome access to the *tap* RBS.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used are shown in Table IV.

The mutant plasmids pGW58-10 to pGW58-15 were constructed from pGW58 (Blomberg *et al.*, 1990) by oligonucleotide-directed mutagenesis (see below). The mutational alterations are shown in Figure 1.

Plasmid pGW177-L is a p15A replicon containing the same translational *repA–lacZ* fusion segment as pGW58-L (Berzal-Herranz *et al.*, 1991). The plasmid was constructed by cleaving pACYC177 DNA with *Hin*cII and inserting the DNA fragment carrying the wild-type *repA–lacZ* fusion bordered by *Eco*RI (rendered blunt-ended with Klenow enzyme and dNTPs) and *Nae*I sites. After ligation and transformation, we obtained a plasmid, pGW177-L, which conferred β-galactosidase activity, kanamycin resistance and ampicillin sensitivity (the *bla* gene was inactivated by insertion of the gene fusion fragment into the *Hin*cII site) to the host cells. Restriction mapping and sequencing were done to verify the correct fusion and to determine the orientation of the insert (opposite to that of the inactivated *bla* gene).

The mutant *repA–lacZ* fusion plasmids pGW177-10L to pGW177-15L were constructed by replacing the *Bgl*II–*Sal*I fragment of the wild-type fusion plasmid pGW177-L with the corresponding mutant fragments from the pGW58 series. The mutations were verified by DNA sequencing.

Plasmid pGW233 was constructed by cleaving pGW58 DNA with *Hgi*AI, creating flush ends using T4 DNA polymerase and dNTPs, and then recleaving with *Eco*RI, whereupon the ~0.4 kb *Eco*RI-blunt end fragment was inserted between the *Sma*I and *Eco*RI sites of the fusion vector pMC1403 (Casadaban *et al.*, 1980). The resulting plasmid was sequenced to confirm the correct in-frame fusion of the tenth codon of the *tap* reading frame with the *lacZ* frame.

For the experiment where the effect of CopA was assayed *in trans* (Table I) we used plasmid pGW15 (Wagner *et al.*, 1992).

For *in vitro* protein synthesis, we constructed the pGW277 series of plasmids. Here, pGW177-L (wild-type or mutant) plasmid DNA was digested with *Pvu*II. The largest fragment (~5.9 kb) was eluted from gels and religated. The resulting plasmids carry deletions of most of the *lacZ* sequences. The religation results in a UGA stop codon positioned so that termination of translation of the *repA* reading frame of the wild-type plasmid pGW277 should yield a protein product of 21.7 kDa.

Enzymes and chemicals

Restriction endonucleases and other enzymes used for cloning procedures and sequencing were bought from Pharmacia, unless otherwise stated. The methods used were as in Sambrook *et al.* (1989). Chemicals used were of the highest purity available.

Cell growth and media

Cells were grown in LB medium (Bertani, 1951) supplemented with 0.2% glucose. The solid medium, LA, was LB medium with 1.5% (w/v) agar. When appropriate, antibiotics (50 μg/ml) were included.

Oligonucleotide-directed mutagenesis

Mutations in the *tap* gene resulting in the plasmids of the pGW58 series were introduced into pGW58 using the Amersham oligonucleotide-directed *in vitro* mutagenesis kit version 2.1 (Taylor *et al.*, 1985a,b). Single-stranded (+) strand of pGW58 served as the template and S-dCTP phosphorothioate analogues were used in second-strand synthesis. The restriction enzyme *Ban*II was used for the nicking step. The mutagenic deoxyoligoribonucleotides used for mutants pGW58-11 to pGW58-15 are listed below.

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized in our own laboratory using an Applied Biosystems 394 DNA/RNA synthesizer. The oligodeoxyribonucleotides used for site-specific mutagenesis were: 5'-AAGGA GCGAT CACAG AAA-3' (mutation 11); 5'-GAAGG AGCTA GCACA GA-3' (mutation 12); 5'-TGAAG ATCGG TCACA CC-3' (mutation 13); 5'-CAGTC ACACC ATCCT GCAGT ACAAT GCGCA GAAG GA-3' (mutation 14); 5'-AAGAT CAGTC ACACC ACGTG CACTT ACAAT GCG-3' (mutation 15).

β-galactosidase measurements

The β-galactosidase measurements shown in Tables I, II and III were done according to Miller (1972).

In vitro translation assay

Translation of proteins encoded by plasmids was performed in a cell-free transcription–translation system (Zubay, 1973) with a kit from Promega Biotech. Supercoiled plasmid DNA (~1.0 μg) was used as template in 25 μl reaction mixtures. The proteins were labelled with [^{35}S]methionine (New England Nuclear). Protein products were electrophoresed using the Pharmacia Phast-gel system, and the dried gels were then autoradiographed on Kodak X-Omat AR film or analysed with a PhosphorImager (Applied Biosystems).

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References

Asano,K., Kato,A., Moriwaki,H., Hama,C., Shiba,K. and Mizobuchi,K. (1991a) *J. Biol. Chem.*, **266**, 3774–3781.

Asano,K., Morikawi,H. and Mizobuchi,K. (1991b) *J. Biol. Chem.*, **266**, 24549–24556.

Atkins,J.F., Weiss,R.B. and Gesteland,R.F. (1990) *Cell*, **62**, 413–423.

Berkhout,B., Schmidt,B.F., van Strien,A., van Boom,J., van Westrenen,J. and van Duin,J. (1987) *J. Mol. Biol.*, **195**, 517–524.

Bertani,G. (1951) *J. Bacteriol.*, **62**, 293–300.

Berzal-Herranz,A., Wagner,E.G.H. and Díaz-Orejas,R. (1991) *Mol. Microbiol.*, **5**, 97–107.

Blomberg,P., Wagner,E.G.H. and Nordström,K. (1990) *EMBO J.*, **9**, 2331–2340.

Blumer,K.J., Ivey,M.R. and Steege,D.A. (1987) *J. Mol. Biol.*, **197**, 439–451.

Casadaban,M.J., Chou,J. and Cohen,S.N. (1980) *J. Bacteriol.*, **143**, 971–980.

Chang,A.C.Y. and Cohen,S.N. (1978) *J. Bacteriol.*, **134**, 1141–1156.

Coulondre,C. and Miller,J.H. (1977) *J. Mol. Biol.*, **177**, 525–576.

de Smit,M.H. and van Duin,J. (1990) *Progr. Nucleic Acids Res. Mol. Biol.*, **38**, 1–35.

Dong,X., Womble,D.D. and Rownd,R.H. (1987) *J. Bacteriol.*, **169**, 5353–5363.

Freier,S.M., Kierzek,R., Jaeger,J.A., Sugimoto,N., Caruthers,M.H., Neilson,T. and Turner,D.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9373–9377.

Gerdes,K., Poulsen,L.K., Thisted,T., Nielsen,A.K., Martinussen,J. and Hove-Andreasen,P. (1990) *New Biol.*, **2**, 946–956.

Givskov,M. and Molin,S. (1984) *Mol. Gen. Genet.*, **194**, 286–292.

Hama,C., Takizawa,T., Moriwaki,H. and Mizobuchi,K. (1990) *J. Biol. Chem.*, **265**, 10666–10673.

Hanahan,D. (1985) In Glover,D.M. (ed.), *DNA Cloning—A Practical Approach*. IRL Press, Oxford, Vol. I, pp. 109–135.

Ivey-Hoyle,M. and Steege,D.A. (1989) *J. Mol. Biol.*, **208**, 233–244.
Kastelein,R.A., Berkhout,B. and van Duin,J. (1983) *Nature*, **305**, 741–743.
Kirsebom,L.A., Baer,M.F. and Altman,S. (1988) *J. Mol. Biol.*, **204**, 879–888.
Lopéz,J., Crespo,P., Rodríguez,J.C., Andrés,I. and Ortiz,J.M. (1989) *J. Gen. Microbiol.*, **135**, 1763–1768.
Ma,C. and Simons,R.W. (1990) *EMBO J.*, **9**, 1267–1274.
Miller,J.H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Miller,J.H. and Albertini,A.M. (1983) *J. Mol. Biol.*, **164**, 59–71.
Nordström,K., Molin,S. and Light,J. (1984) *Plasmid*, **12**, 71–90.
Novick,R.P., Iordanescu,S., Projan,J., Kornblum,J. and Edelman,I. (1989) *Cell*, **59**, 395–404.
Öhman,M. and Wagner,E.G.H. (1989) *Nucleic Acids Res.*, **17**, 2557–2579.
Öhman,M. and Wagner,E.G.H. (1991) *Mol. Gen. Genet.*, **230**, 321–328.
Persson,C., Wagner,E.G.H. and Nordström,K. (1988) *EMBO J.*, **7**, 3279–3288.
Persson,C., Wagner,E.G.H. and Nordström,K. (1990a) *EMBO J.*, **9**, 3767–3775.
Persson,C., Wagner,E.G.H. and Nordström,K. (1990b) *EMBO J.*, **9**, 3777–3785.
Ryder,T.B., Davison,D.B., Rosen,J.I., Ohtsubo,E. and Ohtsubo,H. (1982) *Gene*, **17**, 299–310.
Saadi,S., Maas,W.K., Hill,D.F. and Bergquist,P.L. (1987) *J. Bacteriol.*, **169**, 1836–1846.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
Silva,R.M., Saadi,S. and Maas,W.K. (1988) *Infect. Immun.*, **56**, 836–842.
Taylor,J.W., Ott,J. and Eckstein,F. (1985a) *Nucleic Acids Res.*, **13**, 8765–8785.
Taylor,J.W., Schmidt,W., Cosstick,R., Okruszek,A. and Eckstein,F. (1985b) *Nucleic Acids Res.*, **13**, 8749–8764.
Vanooteghem,J.-C. and Cornelis,G.R. (1990) *J. Bacteriol.*, **172**, 3600–3608.
Wagner,E.G.H. and Nordström,K. (1986) *Nucleic Acids Res.*, **14**, 2523–2538.
Wagner,E.G.H., von Heijne,J. and Nordström,K. (1987) *EMBO J.*, **6**, 515–522.
Wagner,E.G.H., Blomberg,P. and Nordström,K. (1992) *EMBO J.*, **11**, 1195–1203.
Weber,P.C. and Palchaudhuri,S. (1986) *J. Bacteriol.*, **166**, 1106–1112.
Zubay,G. (1973) *Annu. Rev. Genet.*, **7**, 267–287.
Zuker,M. and Stiegler,P. (1981) *Nucleic Acids Res.*, **9**, 133–148.

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