Genetic Evidence for the Direction of Transcription of the *trfA* Gene of Broad Host Range Plasmid RK2

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For replication of broad host range plasmid RK2 in *Escherichia coli* two regions of the plasmid genome are essential, *oriV_{RK2}* between 12.0 and 12.7 kb on the genome (defined clockwise from the unique *EcoRI* site) and *trfA*, located between 16.0 and 17.4 kb, which provides a *trans*-acting product necessary for *oriV_{RK2}* function. The properties of an insertion mutant of a mini-RK2/ColE1 hybrid plasmid suggest that the *trfA* promoter lies clockwise from the 17.4 kb RK2 coordinate. Fusion of the *trfA* gene lacking its normal promoter to the *E. coli trpE* gene in a hybrid plasmid confirmed that *trfA* is transcribed anticlockwise, towards the Tc^r gene of RK2. Repression of *trpE* promoter activity in these fusions showed that replication of an RK2 replicon can be regulated by varying the level of *trfA* gene expression. The results also indicate the presence of a transcription unit running anticlockwise through the *trfB* region located between 54.0 and 56.0 kb on the RK2 genome.

INTRODUCTION

Interest in the plasmids RK2, RP4 and RP1, which belong to *Escherichia coli* plasmid incompatibility group IncP (*Pseudomonas* IncP-1), has arisen due to their ability to transfer between and maintain themselves stably in a wide range of Gram-negative bacterial species (Datta & Hedges, 1972; Olsen & Shipley, 1973; Beringer, 1974; Cho *et al.*, 1975). They are for this reason termed broad host range plasmids. RK2, RP4 and RP1 are indistinguishable (Burkardt *et al.*, 1979) and have been studied in some detail (for a review see Thomas, 1981*a*).

Recently it has been demonstrated that the uniquely broad host range properties of RK2 are due to its replication system, since the conjugal transfer system of the sex factor F can transfer an RK2 replicon from *E. coli* to *Pseudomonas aeruginosa* while even if mobilized by the RK2 transfer system a mini-F replicon will not establish itself and replicate in *P. aeruginosa* (Guiney, 1982). This further justifies the detailed study of those genes which RK2 utilizes for replication in a wide range of hosts.

RK2 (56.4 kilobase pairs, kb) carries genes conferring resistance to penicillin, kanamycin and tetracycline in addition to the genes required for conjugal transfer and plasmid maintenance (consisting of plasmid DNA replication and partitioning to daughter cells). In *E. coli* plasmid replication proceeds unidirectionally from a vegetative replication origin, *oriV_{RK2}*, located between coordinates 12.0 and 12.7 kb (defined clockwise from the unique *EcoRI* site) (Meyer & Helinski, 1977; Thomas *et al.*, 1980). Replication from this replication origin depends not only on host chromosome specified gene products, but also on the product, probably a protein, of the *trfA* gene that acts in *trans* (Figurski & Helinski, 1979; Thomas *et al.*, 1980; Thomas, 1981*b*). *trfA* maps between 16.0 and 17.4 kb on the RK2 genome (Thomas, 1981*b*). Genetic analysis of these regions has involved deletion analysis of RK2 to produce mini-replicons which have been further analysed (Thomas *et al.*, 1980, 1981; Thomas, 1981*b*). The interpretation of the results of deletion analysis has been complicated by the presence on RK2 of certain regions that are host

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lethal or inhibit plasmid maintenance (*kil* functions) when other RK2 segments (carrying *kor* *kil*-override functions) are not present in the same cell (Figurski & Helinski, 1979; Figurski *et al.*, 1981; Smith & Thomas, 1983). The mini-plasmid pRK248 is made up of two RK2 segments produced by *Hae*II partial digestion of pRK229, a deletion derivative of RK2 (Thomas *et al.*, 1980). These two segments run from 12.0 to 18.7 kb and 54.0 to 0.3 kb. The reason for the inclusion of the 54.0 to 0.3 kb segment (the 54.0 to 56.0 kb segment of which is referred to as the *trfB* region) is that it contains *korD*, which is required to suppress *kilD*, which maps between 17.5 and 18.7 kb and is therefore present in pRK248 (Smith & Thomas, 1983). Since pRK248 was obtained by *Hae*II partial digestion of pRK229 and *kilD* lies in the same *Hae*II fragment as part of the *trfA* gene it is not surprising that *kilD* remains in pRK248. However, the relationship of the *kil* and *kor* genes to RK2 plasmid maintenance is unclear.

Despite the presence of *kilD* close to *trfA* it has been possible to obtain *trfA* without *trfB* and *korD* (Thomas, 1981*b*). This was achieved by using sufficiently strong selection to pick up a mutant in which *kilD* has been inactivated in some way. It was thus possible to demonstrate that the product(s) of the *trfA* region along with *oriV_{RK2}* are sufficient for replication in *E. coli*. However, recently we reported that this was not sufficient for replication in *P. aeruginosa* (Thomas *et al.*, 1982), and that the *trfB* region needs to be introduced in *cis* to *trfA* and *oriV_{RK2}* in order to obtain a mini-plasmid that will be maintained in *P. aeruginosa*. Whatever the explanation is for this observation it appears that this *trfB* requirement can be removed by further deletions into the *trfA* region, which are likely to remove *kilD* (Schmidhauser *et al.*, 1983; this laboratory, unpublished). It can thus be demonstrated that *trfA* and *oriV_{RK2}* are sufficient for replication in at least part of the parental RK2 range of bacterial hosts, that is, *P. aeruginosa*, *Pseudomonas putida*, *Acinetobacter calcoaceticus*, *Rhizobium meliloti* and *Azotobacter vinelandii* (Schmidhauser *et al.*, 1983; this laboratory, unpublished).

While the *trfA* gene has been mapped to between coordinates 16.0 and 17.4 to 17.5 kb (Thomas *et al.*, 1980; Thomas, 1981*b*), it is unclear whether this defines simply the *trfA* coding sequence or the *trfA* promoter as well. The results presented in this paper demonstrate that in the deletion derivative pCT4.3 (Thomas *et al.*, 1980), which retains the RK2 sequences between coordinates 16.0 and 17.4 kb, the normal *trfA* promoter is deleted and this allows a demonstration of the direction of transcription of the *trfA* gene and implies that there is transcription in the *trfB* region in the same direction as the *trfA* gene.

METHODS

Bacterial strains, plasmids and growth conditions. The *E. coli* strains used were a K12 strain MV10 (C600 Δ *trpE5*, from the collection of D. R. Helinski, Dept of Biology, University of California, San Diego, USA) and *E. coli* C strains C2110 (*rha his polA1*, from M. Kahn, Dept of Bacteriology & Public Health, Washington State University, Pullman, USA) and C2110 Ch3/14, a C2110 derivative with RK2 regions *trfA* and *trfB* in the chromosome (Thomas *et al.*, 1981). Plasmids used in this study are listed in Table 1. Media used were L broth and M9 Casamino acids medium (M9.CAA) (Kahn *et al.*, 1979). For solid medium 1.5% (w/v) agar was added. For selection of antibiotic resistances carried by plasmids medium was supplemented with chloramphenicol (25 μ g ml⁻¹, for Cm^r), kanamycin (50 μ g ml⁻¹, for Km^r), penicillin (150 μ g ml⁻¹ in liquid; 300 μ g ml⁻¹ in agar, for Pn^r) and tetracycline (25 μ g ml⁻¹, for Tc^r). Plasmids carrying the *trpE*⁺ gene of *E. coli* were selected in MV10 by growth on M9.CAA medium. Bacteria were grown at 37 °C.

Transformation. Bacteria were made competent by CaCl₂ treatment as described in Meyer *et al.* (1977) but using L broth to grow the bacteria to exponential phase.

Preparation of plasmid DNA. Plasmid DNA was isolated from saturated cultures grown overnight in liquid medium with antibiotic selection by the Triton X-100 cleared method (Kahn *et al.*, 1979) or by a modification of the alkaline SDS extraction method of Birnboim & Doly (1979) (Smith & Thomas, 1983). This DNA was used for both gel electrophoretic analysis and *in vitro* recombination using DNA ligase.

Restriction enzyme digestion and *in vitro* recombination. Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories or Boehringer Chemicals and used under conditions similar to those recommended by the suppliers. Plasmid DNA was analysed by electrophoresis in 0.8 to 1.0% (w/v) agarose, in 0.5 $\times$ TBE buffer and photographed as previously described (Kahn *et al.*, 1979).

Table 1. Plasmids used in this study

Plasmid	Size (kb)	Relevant properties	Reference or source
pRK353	11.0	<i>trpE</i> ⁺ , R6K replicon	Kolter & Helinski (1978)
pDS3	2.5	Cm ^r , deletion of pACYC184 (P15A replicon)	D. Stalker, Calgene Inc., Davis, Calif., USA (unpublished)
pRK646	3 (approx.)	Pn ^r , R6K <i>ori</i> plasmid	R. Kolter, Harvard Medical School (unpublished)
pCT7	3.8	Km ^r , <i>oriV</i> _{RK2} , ColE1 replicon	Thomas <i>et al.</i> (1980)
pCT45	2.2	Km ^r , <i>oriV</i> _{RK2}	Thomas <i>et al.</i> (1981)
pCT60	16.1	Km ^r , <i>trfB</i> ⁺ , R6K + ColE1 replicons	C. M. Thomas (unpublished)
pCT85.3	13.1	Tc ^r , <i>trfA</i> ⁺ , R6K replicon	Thomas (1981 <i>b</i>)
pCT4	8.5	Km ^r , RK2 and ColE1 replicons	Thomas <i>et al.</i> (1980)
pCT4.3	7.2	as pCT4	Thomas <i>et al.</i> (1980)
pCT70	9.1	Pn ^r derivative of pCT4.3	This study (Fig. 1)
pCT71	9.1	as pCT70	This study (Fig. 1)
pCT170	14.6	Pn ^r , <i>trfA</i> ⁺ , R6K replicon	This study (Fig. 2)
pCT171	14.6	as pCT170	This study (Fig. 2)
pCT172	18.4	pCT170/pCT7 hybrid	This study (Fig. 3)
pCT174	18.4	pCT171/pCT7 hybrid	This study
pCT176	13.2	<i>Hind</i> III deletion of pCT172	This study (Fig. 3)
pCT178	13.2	<i>Hind</i> III deletion of pCT174	This study

RESULTS

Properties of pCT4.3 and its derivatives

The construction of pCT4, whose structure is shown in Fig. 1, has been described previously (Thomas *et al.*, 1980). pCT4.3 was obtained from a Km^r transformant which grew after MV10 had been transformed with pCT4 DNA partially digested with *Hha*I (Thomas *et al.*, 1980). Recent DNA sequence analysis data (Smith & Thomas, 1984*a*) indicate that, while the deletion in pCT4.3 might have been stimulated by *Hha*I partial digestion, it is not simply a removal of a DNA segment bounded by *Hha*I sites. The extent of the deletion is shown in Fig. 1. All the DNA deleted comes from the *trfA* region. The ease with which pCT4.3 was isolated may be due to its loss of *kilD*, which may give this plasmid an advantage over pCT4. pCT4.3 could still transform a *polA* strain, C2110 (Table 2), which is non-permissive for the ColE1 replicon in pCT4.3, and therefore *oriV*_{RK2} and *trfA* must still be functional in this plasmid. Thus at least the coding sequence for *trfA* must lie in the portion of the *trfA* region remaining in pCT4.3, i.e. between 16.0 and 17.4 kb (Fig. 1).

To define further the location of essential RK2 functions in pCT4.3, DNA of this plasmid was digested partially with *Hae*II to produce on average a single cut per plasmid DNA molecule. This DNA was ligated with DNA of pRK646 (Table 1) that had been completely digested with *Hae*II (as a source of a *Hae*II fragment carrying a β -lactamase gene conferring resistance to penicillin) and transformed into MV10 selecting Km^r and Pn^r. A number of Km^r Pn^r plasmids were isolated by this method. One such plasmid, pCT70, had the Pn^r fragment inserted in the *trfB* region while a second, pCT71, had an insert between the *trfA* and *oriV*_{RK2} regions. Transformation of pCT70 and pCT71 plasmid DNA into C2110 indicated that while pCT71 replicated as pCT4.3, pCT70 was less able to transform C2110 (Table 2). When Pn^r was selected no transformants were obtained for pCT70 in C2110 but with Km^r selection only a quantitative difference between pCT70 and pCT71 was observed. This probably reflects the lower gene dosage necessary to give Km^r compared to Pn^r.

Contrary to initial expectations the defect in pCT70 could not be complemented by the *trfB* region in *trans* on plasmid pCT60 in C2110 but it could be complemented by the presence in the chromosome of both *trfA*⁺ and *trfB*⁺ regions (C2110 Ch3/14). Similarly pCT70 could be complemented when the plasmid pCT85.3 carrying the *trfA* region and only 140 bp of the *trfB* region was present. It seemed, therefore, that the insertion in pCT70 was affecting *trfA* gene function. A possible explanation for this was that in pCT4.3, expression of the *trfA* gene was

Fig. 1. Structure of pCT4 and of pCT4.3 and its derivatives. A linear map of pCT4 is shown with the unique *EcoRI* site at each end. Restriction sites are shown as B, *Bam*HI; E, *Eco*RI; H, *Hae*II; Hd, *Hind*III; P, *Pst*I; Ss, *Sst*II; X, *Xho*I. *Hae*II fragments are labelled according to Thomas *et al.* (1980). The genetic regions shown are the RK2 vegetative DNA replication origin, *oriV_{RK2}*; a gene *trfA* that is necessary for *oriV_{RK2}* activity; *kilD*, a function that inhibits plasmid maintenance when *korD* is absent; *korA*, which suppresses the host lethal effect of RK2 gene *kilA* not present in pCT4; *trfB*, which is defined as the gene complementing a temperature sensitive mini-RK2 plasmid replication mutant pRK2501ts3; Km^r, a gene conferring kanamycin resistance; and ColE1 *ori*, the mini-replicon of ColE1. The segments of pCT4 present in the deletions are shown and the sites of insertion of *Hae*II Pn^r fragments in pCT70 and pCT71.

Table 2. Transformation frequencies of pCT4.3 and its derivatives

The experiments with Km^r selection were done on different occasions from the Pn^r experiments. Both sets of experiments were performed twice in duplicate. Each set gave qualitatively the same results. The averages from a single experiment for each selection are presented. Standard deviations for plates with 300 to 800 colonies varied from 30 to 130, and for plates with 50 to 200 colonies from 8 to 15. Differences between pCT70 and both pCT4.3 and pCT71 were consistently significant. A likely reason for differences in the results of Km^r and Pn^r selection is that Km^r can be conferred by a single copy of a plasmid while this level of Pn^r requires multiple copies. Strain C2110 is *polA*.

Recipient strain	Selection	RK2 markers in host	Average no. of transformants per µg DNA		
			pCT4.3	Plasmid: pCT70	pCT71
MV10	Km ^r	—	6 × 10 ⁵	5 × 10 ⁵	3 × 10 ⁵
C2110	Km ^r	—	8 × 10 ³	2 × 10 ³	1.5 × 10 ⁴
C2110	Pn ^r	—	ND	0	1.7 × 10 ³
C2110 Ch3/14	Pn ^r	<i>trfA</i> ⁺ <i>trfB</i> ⁺	ND	5 × 10 ²	1.3 × 10 ³
C2110(pCT60)	Pn ^r	<i>trfB</i> ⁺	ND	0	1.4 × 10 ³
C2110(pCT85.3)	Km ^r	<i>trfA</i> ⁺	1.0 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴

ND, Not done because pCT4.3 is Pn^r.

dependent on a promoter in the *trfB* region clockwise from the *Hae*II site between the B₂ and B₁ fragments. In this case the pCT4.3 *Sst*II fragment carrying *trfA* should not show *trfA* expression unless an external promoter is provided. This was tested by cloning the Pn^r *Sst*II fragment of pCT71 (Fig. 1) downstream from the *E. coli trpE* promoter (*ptrpE*).

Fig. 2. Construction of pCT170 and pCT171. pCT71 and pRK353 were digested to completion with *Sst*II and then ligated. Pn^r colonies obtained by transformation of MV10 with this DNA were screened for Km^s, and Pn^r Km^s colonies were examined for plasmid DNA. Two sorts of plasmids differing by the orientation of *Sst*II fragments were found. These were designated pCT170 and pCT171. Restriction sites are shown as B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Ss, *Sst*II. Genes are designated as in Fig. 1.

Cloning *trfA* downstream from the *trpE* promoter

The *E. coli trpE* gene has an *Sst*II site close to the 3' end of its coding sequence (Yanofsky *et al.*, 1981). This gene is conveniently cloned in the R6K mini-replicon pRK353, where this is the only *Sst*II site. DNA of pRK353 and pCT71 was digested with *Sst*II, ligated and transformed into MV10 selecting Pn^r. Transformants were screened for Km^s (Km^r is the other marker on pCT71) and Pn^r Km^s plasmid DNA was analysed. Plasmids with both orientations of the expected hybrid were obtained, pCT170 and pCT171 (Fig. 2). The expectation was that possibly only pCT170 might show *trfA* activity. Both plasmids were tested for their ability to express *trfA*, by transformation of MV10(pCT170) and MV10(pCT171) with pCT45 plasmid DNA. pCT45 consists of a *Hae*II fragment with *oriV*_{RK2} joined to a *Hae*II Km^r fragment. Its replication is dependent on *trfA* provided in *trans* (Thomas *et al.*, 1981). It was found that pCT45 gives about 10⁴ transformants per µg plasmid DNA in both MV10(pCT170) and MV10(pCT171) indicating that both pCT170 and pCT171 provided enough *trfA* gene product for pCT45 to replicate. If indeed the expression of the *trfA* gene on the Pn^r *trfA Sst*II fragment from pCT71 is dependent on an external promoter then there must be transcription in both directions at the *Sst*II site in pRK353. Further attempts to show orientation-dependent *trfA* expression by insertion of the Pn^r *trfA Hind*III fragments from pCT170 and pCT171 into other high copy number vectors were unsuccessful. All such helper plasmids made to date are well able to support pCT45 replication. One reason for these results might be that when present on a high copy number plasmid (pRK353 is maintained at 15 to 20 copies per chromosome equivalent) very little transcription per *trfA* gene is necessary to give a *trfA* gene product concentration sufficient to allow replication of the *trfA* dependent plasmid pCT45. Therefore, plasmids were constructed in order to test for *trpE* promoter requirement when *trfA* is at low copy number.

Demonstration of trpE promoter requirement for trfA expression

The strategy adopted to demonstrate a requirement for the *trpE* promoter was to construct plasmids which could be tested for a functional RK2 replicon in which it was thought that the *trfA* gene was being expressed either from the *trpE* promoter, as proposed for pCT170, or from no known promoter, as proposed for pCT171. This was achieved by replacing the R6K (*polA*-independent) replicon in pCT170 and pCT171 by a *ColE1* (*polA*-dependent replicon carrying *oriV_{RK2}*) as well. These hybrids could be tested for *trfA* activity by transformation into the *polA* strain C2110. The vector replacement was carried out by inserting *EcoRI* digested pCT7 into *EcoRI* digested pCT170 and pCT171, and then by using partial *HindIII* digestion followed by ligation to delete the R6K replicon (Fig. 3). The two plasmids constructed in this way, pCT176 from pCT170 and pCT178 derived similarly from pCT171, differ only in the orientation of the *Pn^r trfA SstII* fragment relative to the *trpE* promoter. Transformation into C2110 showed that only pCT176 had a functional *trfA* gene (Table 3). pCT178 plasmid DNA had a normal transformation frequency in MV10 but gave virtually no *Km^r* transformants in C2110. That this reflects a deficiency in *trfA* gene product was indicated by the ability of pCT178 to give many *Km^r* transformants in C2110(pCT85.3), pCT85.3 being an R6K replicon carrying the *trfA* region of RK2 which can therefore provide sufficient *trfA* gene product in *trans*. The ability of pCT176 to replicate in C2110 is what we would expect from the orientation of RK2 DNA relative to *trpE* in pCT170. Consistent with the idea that *trfA* in pCT176 is expressed by transcription from the *trpE* promoter was the finding that the transformation frequency of pCT176 into C2110 was higher on M9.CAA medium (lacking tryptophan) where the *trp* genes would be induced. On M9.CAA medium with 50 µg tryptophan ml⁻¹ the transformants were fewer and very variable in size – consistently smaller than on M9.CAA medium. Here the *trp* genes should be repressed and *trfA* therefore less well expressed. The absence of a very large difference in the transformation frequency in the presence and absence of tryptophan, given the well established repression ratio for the *trpE* promoter, suggests that only a relatively modest level of *trfA* expression is required for plasmid establishment at quite reasonable efficiency. From other experiments it appears that even in the presence of tryptophan, the transformation frequency of pCT176 may be only fourfold lower than the maximum expected giving little scope for a dramatic enhancement by full *trpE* promoter induction. It is also possible that there may be sufficient tryptophan in M9.CAA medium to prevent full induction of the *trpE* promoter.

To investigate further the effect of external tryptophan concentration on replication of pCT176 in C2110, *Km^r* transformants on M9.CAA plates with tryptophan (50 µg ml⁻¹) were used to inoculate M9.CAA medium supplemented with kanamycin to select for continued presence of pCT176. The inoculated bacteria were evenly suspended in the medium (10 ml) which was then split into two and tryptophan added to a final concentration of 200 µg ml⁻¹ to one of each pair of cultures (four transformants were studied). The bacterial cultures were grown overnight at 37 °C with shaking and then plasmid DNA extracted by the alkaline SDS method. The cultures were also serially diluted and plated on medium with and without kanamycin to determine the proportion of bacteria still carrying pCT176. The cultures grown with tryptophan showed a 30% higher number of c.f.u. ml⁻¹ than cultures grown without tryptophan. Of the bacteria grown without tryptophan 89% carried pCT176 compared to 23.5% for bacteria grown with tryptophan. Overall the number of plasmid positive bacteria after growth with tryptophan was 34% of the number after growth without tryptophan. From comparison of plasmid DNA yield estimated by band intensity after agarose gel electrophoresis it appeared that growth in the presence of tryptophan severely inhibited plasmid DNA production. By running serially diluted samples of the pCT176 DNA from growth in the absence of tryptophan it was estimated that there was a 5- to 10-fold decrease in plasmid DNA yield as a result of growth in the presence of tryptophan. That this was not a non-specific effect on mini-RK2 replication was indicated by the fact that C2110(pCT71) grown in M9.CAA with kanamycin, with and without tryptophan, showed no significant difference in plasmid yield. pCT71 has *trfA* expressed from an RK2 promoter and should not therefore be affected by induction or repression of the tryptophan operon. The threefold difference in the number of plasmid positive bacteria in cultures of C2110(pCT176) grown with and without tryptophan is not sufficient to explain fully the 5- to 10-

Fig. 3. Construction of pCT176 and pCT178. To construct pCT176, pCT170 and pCT7 were digested with *Eco*RI, ligated and MV10 transformed with this DNA. Plasmid DNA in Pn^r Km^r transformants was analysed. Both orientations of the *Eco*RI fragments relative to each other in hybrid plasmids were obtained (pCT172 and pCT173). One of these, pCT172 was digested with *Hind*III, ligated and transformed again into MV10 selecting Km^r Pn^r. Plasmid DNA in transformants was analysed and checked by *Hind*III and *Ssr*II digestion. pCT176 has only the two *Hind*III fragments dictated by selecting Km^r (since this Km^r is interrupted by a *Hind*III site). pCT178 was constructed from pCT171 and pCT7 in the same way so that it differs from pCT176 only by the orientation of the Pn^r *trfA* *Ssr*II fragment relative to the *trpE* promoter. Restriction sites are shown as in Fig. 2.

Table 3. Transformation frequencies in plasmids carrying *oriV_{RK2}* and *trfA* in two orientations relative to the *trpE* promoter

All selections were for Km^r. The experiment was performed twice in duplicate and while absolute transformation frequencies varied between experiments the absence of transformants with pCT178 in C2110 and the approximately two-fold difference with pCT176 in C2110 on M9.CAA.Trp and M9.CAA was reproducible with maximum standard deviations of 17 for plates with 200 to 400 colonies.

Recipient strain	Selective medium	Average no. of transformants per µg DNA	
		Transforming plasmid:	
		pCT176	pCT178
MV10	L agar	9×10^3	1×10^4
C2110	M9.CAA.Trp	6.7×10^2 (tiny)	0
C2110	M9.CAA	1.2×10^3	0
C2110(pCT85.3)	L agar	ND	5×10^3

ND, Not done.

fold difference in plasmid yield. There is therefore likely to be an effect on plasmid copy number per bacterium as a result of repressing *trpE* promoter activity, probably by limiting the concentration of *trfA* gene product. This inhibition of mini-RK2 plasmid replication may be the primary reason for pCT176 instability during growth in the presence of tryptophan.

DISCUSSION

The results described in this paper indicate that the structural gene for *trfA* lies between RK2 kilobase coordinates 16.0 and 17.4 kb and is transcribed from the 17.4 kb end. The normal *trfA* promoter, by definition, should be capable of giving sufficient *trfA* expression to allow establishment of the RK2 replicon after transformation. The 16.0 to 17.4 kb segment clearly lacks such a promoter. However, the inability to show a requirement for a defined external promoter when this DNA is attached to a high copy number plasmid raises the possibility that the fragment contains a weak promoter which is sufficient for *trfA* expression at high copy number although *trfA* expression under these circumstances could also be due to weak transcription from the vector DNA. If the 16.0 to 17.4 segment does have a weak promoter then it is conceivable that elsewhere on RK2 there are functions which are positively required to allow it to transcribe *trfA* sufficiently strongly. However, this seems less likely than that the normal *trfA* promoter lies clockwise from 17.4, probably between coordinates 17.4 and 18.7 kb, which is consistent with recent results (Thomas, 1981*b*; Shingler & Thomas, 1984).

In pCT4.3 a deletion has made *trfA* expression dependent on transcription from a promoter in the *trfB* region. Since the Pn^r fragment insertion in pCT70, into the *Hae*II site at 55.0 kb, has a polar effect on *trfA* expression, the *trfB* region promoter to which *trfA* has been linked must be clockwise from 55.0 kb. Recent experiments in this laboratory (Smith & Thomas, 1984*b*) have located a promoter in the 55.0 to 56.0 kb *Hae*II B₁ fragment which may be responsible for *korA*, *korD* and *trfB* expression. This could be the same promoter as that responsible for *trfA* expression in pCT4.3. This transcription unit appears to continue right through the *trfB* region as far as, and probably past, the *Hae*II site at 54.0 kb. The evidence suggests that there is only weak transcription originating in either the *Hae*II B₂ fragment of the *trfB* region or in the inserted Pn^r *Hae*II fragment, but neither of these segments is well enough characterized with respect to transcription initiation and termination signals to be sure.

These proposed locations of promoters are consistent with the results of *E. coli* RNA polymerase binding studies carried out on RP4 DNA (Burkardt & Wohlleben, 1981). RNA polymerase binding sites reveal the location but not the direction of possible promoters. The results for the *trfB* region show a peak of binding at coordinate 55.3 kb (worked out by multiplying fractional length on their graphs by 56.4 kb, the total size of RK2) and another at about 52.0 kb. This would fit with a promoter between coordinates 55.0 and 56.0 transcribing anticlockwise and there not being another one in the 54.0 to 55.0 kb region. The results for the *trfA* region show peaks at between 16.8 and 17.0 kb and at 17.7 kb although only the 17.7 kb coordinate peak is present in both their sets of data (with respect to *Eco*RI and *Hind*III sites). This binding site location at 17.7 kb is consistent with our mapping of the *trfA* promoter and its deletion in pCT4.3 which lacks the 17.4 to 18.7 kb sequences. Thus this is certainly consistent with the normal *trfA* promoter lying in the *trfA* region present in mini-RK2 plasmids such as pRK248 and pCT4 which have the RK2 segment from coordinates 16.0 to 18.7 kb. There do not seem to be any good RNA polymerase binding sites a short distance clockwise from the 18.7 kb coordinate, again reinforcing the above conclusions.

The inability to demonstrate a requirement for a known external promoter for *trfA* gene expression when *trfA* is on a high copy number plasmid suggests that only low levels of transcription across each copy of *trfA*, under these circumstances, are required to give a high enough *trfA* gene product concentration to allow *oriV*_{RK2} to function. However, reconstruction of RK2 replicons, with the *trpE* promoter transcribing in either direction with respect to the pCT4.3 *trfA* gene, clearly demonstrates the requirement for the *trpE* promoter for *trfA* expression in low copy number plasmids. In pCT176, which has the correct orientation of *trfA*, repression of the *trpE* promoter is sufficient to make *trfA* expression limiting for plasmid replication in a *polA* strain (where the ColE1 replicon is non-functional). This effect of *trpE* promoter repression and induction on pCT176 copy number is likely to be due to altered *trfA* protein levels since a non-specific effect on copy number through increased transcription would be more likely to show reduced copy number at high transcription levels. A specific effect on copy number control elements in *oriV*_{RK2} is also unlikely since the direction of transcription from the *trpE* promoter in pCT176 is more likely to inhibit *oriV*_{RK2} function than to stimulate it

(S. Warne & C. M. Thomas, unpublished). Further experiments are clearly needed to characterize this effect more fully but it seems likely that this sort of replication control could have applications in the development of IncP plasmid vectors whose copy number could be varied from very low up to that of the highest mutant copy number (35 copies per chromosome equivalent: Thomas *et al.*, 1984) by simply changing the tryptophan concentration in the medium. This might be useful in the cloning and expression of genes whose products may be host lethal when produced from multiple copies of the gene not only in *E. coli* but also in other Gram-negative bacterial species. The *trpE* promoter is probably able to function in other species (Nagahari *et al.*, 1977, 1979) but normal regulation is lacking so that it may be necessary to introduce the *E. coli trpR* gene into vectors for use in other species. The construction of such a plasmid is underway at present.

The results described in this paper are further examples of the power of operon fusions (for a review see Franklin, 1978) in genetic analysis. The particular fusions described establish the direction of transcription of *trfA* and the presence of promoters in *trfA* and *trfB* regions, and may allow control of mini-RK2 plasmid copy number by regulation of tryptophan concentration.

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