The EMBO Journal vol.3. no.7 pp.1513–1519, 1984

The *korB* gene of broad host range plasmid RK2 is a major copy number control element which may act together with *trfB* by limiting *trfA* expression

Christopher M. Thomas and Atta A.K. Hussain[1]

Department of Genetics, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, UK

[1]Present address: Max-Planck-Institut für Molekulare Genetik, Abteilung Wittman, D-1000 Berlin 33 (Dahlem) FRG

Communicated by K.Nordström

For replication, plasmid RK2 encodes a vegetative replication origin, $oriV_{RK2}$, and a gene, *trfA*, whose polypeptide product(s) is essential for $oriV_{RK2}$ activity. The *trfA* gene is transcribed as part of a polycistronic operon which also includes *kilD*. Transcription of this operon is negatively regulated by the products of the *trfB/korD/korA* and *korB* loci. Mini replicons previously studied in detail lack the *korB* locus and have copy numbers significantly higher than RK2 itself. Here we report that *korB* in *trans* expresses incompatibility towards RK2 replicons either when the *korB* gene dosage is high or when it is expressed from a strong foreign promoter. This incompatibility can be largely overcome if a *trfA* gene which is expressed from a foreign promoter, and is therefore not regulated by *korB*, is supplied in *trans*. When *korB* is introduced in *cis* to mini RK2 replicons the copy number is reduced to within the range estimated for parental RK2. Deletions in the $oriV_{RK2}$ region which otherwise cause quite large increases in plasmid copy number have only a small effect when *korB* is present in *cis*. These results suggest that *korB* in combination with *trfB* may be the overriding copy number control element in RK2 reducing *trfA* expression to levels limiting for replication.

Key words: DNA replication/plasmid RK2/copy number control/plasmid incompatibility

Introduction

Plasmid RK2, Figure 1 (similar or identical to plasmids RP1, RP4, R68 and R18, Burkardt *et al.*, 1979; Stokes *et al.*, 1981) which belongs to the *Escherichia coli* incompatibility group IncP (*Pseudomonas* group IncP-1) is capable of conjugal transfer between and stable maintenance in most Gram negative bacterial species (for a general review, see Thomas, 1981a). Comparison of the replication and transfer systems of RK2 and narrow host range plasmid F in *E. coli* and *Pseudomonas aeruginosa* has suggested that it is the replication genes of RK2 which may be ultimately responsible for its broad host range (Guiney, 1982). Two plasmid loci are essential for RK2 plasmid replication. The vegetative replication origin, $oriV_{RK2}$, from which replication proceeds unidirectionally anti-clockwise, is located between coordinates 12.3 and 13.0 kb (defined clockwise from the unique *Eco*RI site according to coordinates based on the revised genome size of 60 kb, Lanka *et al.*, 1983, Figure 1) (Meyer and Helinski, 1977; Figurski and Helinski, 1979; Thomas *et al.*, 1980, 1981; Stalker *et al.*, 1981). The *trfA* gene, located between coordinates 16.0 and 17.3 kb, specifies two polypeptides of 43 and 32 kd by alternative translational starts within a single open reading frame of which the smaller, at least, provides a function which is essential for replication from $oriV_{RK2}$ (Figurski and Helinski, 1979; Thomas *et al.*, 1980; Thomas, 1981b; Shingler and Thomas, 1984a; Smith and Thomas, 1983a).

Plasmid RK2 is maintained at a copy number of five to seven copies per chromosome equivalent (Figurski *et al.*, 1979). While RK2 copy number regulation is not fully understood, the region of the vegetative replication origin has been identified as strongly expressing incompatibility (Meyer, 1979; Thomas *et al.*, 1980, 1981) which could be due either to its production of replication inhibitor(s) or to titration of a positively acting replication element. Recent studies have further identified at least two loci, *incA* and *incB*, in the $oriV_{RK2}$ region which coincide with copy number control elements (designated *copA* and *copB*) since the deletions defining *incA* and *incB* increase copy number (Thomas *et al.*, 1984). However, even with an apparently wild-type $oriV_{RK2}$ segment the mini replicon used has a copy number of 10–11 copies per chromosome equivalent. This prompted us to search for an extra copy number control element that this replicon lacks.

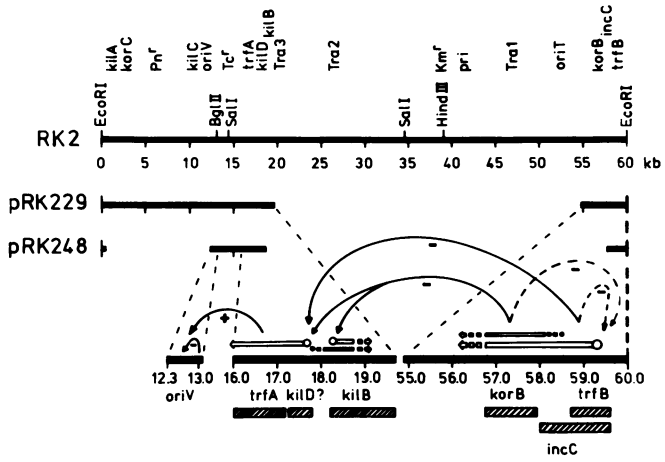

Fig. 1. Genetic map of RK2, pRK229 and pRK248 showing recently discovered gene control circuits. The size of RK2 (RP4) has recently been revised to 60 kb (Lanka *et al.*, 1983). The kb coordinates of replication and maintenance loci have been accordingly revised here simply by using key restriction sites (in particular *Sal*I at 14.4 kb and *Eco*RI at 0/60 kb) as reference points to align the old and new maps. Clearly some loci, but not others, have moved. The loci shown on RK2 are all defined in the Introduction except: Pn^r, Km^r, Tc^r, genes conferring resistance to penicillin, kanamycin and tetracycline, respectively; Tra1, Tra2 and Tra3, blocks of genes required for conjugal transfer (Barth and Grinter, 1977; Barth *et al.*, 1978; Barth, 1979); *oriT*, the proposed origin of DNA replication during conjugal transfer (Guiney and Helinski, 1979; Guiney and Yakobsen, 1983); *pri*, gene coding for a primase which facilitates conjugal transfer (Lanka and Barth, 1981). On the expanded segments of *trfA* and *trfB* regions, circles and open block arrows represent promoters and transcripts; discontinuous arrows indicate regions of uncertainty. The 3′ ends of transcripts are not known. Our present best estimates of segments coding for individual loci are shown by hatched boxes. Positive (+) and negative (−) circuits are indicated by curved arrows, discontinuity indicating only tentative proposals.

Table I. Elimination of pRK248 by plasmids carrying various segments of the *trfB* and *korB* region[a]

Relevant RK2 genotypes	pMB1-derived incoming plasmid	Proportion of colony retaining pRK248		pCT407(IncQ)-derived incoming plasmid	Proportion of colony retaining pRK248	
		+tryptophan	−tryptophan		+tryptophan	−tryptophan
−	pKO4	46/50,38/50	42/50,44/50			
trfB$^+$ *incC*$^+$ *korB*$^-$	pCAS107	44/50,46/50	47/50,43/50	pCT407	N.T.	42/50,42/50
trfB$^+$ *incC*$^+$ *korB*$^+$	pCAS155	0/50, 8/50	5/50, 0/50	pCT415	N.T.	41/50,46/50
trfB^0 *incC*$^-$ *korB*$^\pm$	pCAS156	30/50,34/50	38/50,23/50			
trfB^0 *incC*$^-$ *korB*$^+$	pCAS157	2/50,33/50[b]	1/50, 0/50[b]	pCT417	35/50,36/50	16/50,27/50
trfB^0 *incC*$^-$ *korB*$^-$	pCAS158	47/50,48/50	48/50,47/50	pCT418	N.T.	35/35,50/50

[a]C600 (pRK248) was transformed with DNA of the plasmids shown and penicillin-resistant (pMB1-derived plasmids) or streptomycin-resistant (pCT407-derived plasmids) transformants were selected on M9.CAA medium with and without tryptophan (200 μg/ml). Four transformants were streaked to single colonies on the same medium and on L agar + tetracycline to screen for pRK248. If pRK248 was present the single colonies were replica plated onto L agar + tetracycline to determine what proportion of each of two transformant colonies had retained pRK248. N.T. = Not tested.
[b]In this experiment, of transformants on medium + tryptophan 28/50 retained a significant proportion of resident as estimated by direct replica plating while only 5/50 transformants did so on medium − tryptophan. In a second experiment on medium + tryptophan the proportion of resident retention was 22/50 and 28/50.

From recent studies described below it appeared that *korB* may represent such an element.

RK2 carries a series of *kil* loci which either inhibit host bacterial growth (*kilA, kilB* and *kilC*) or may simply block plasmid replication (*kilD* or *kilB'*) when other RK2 functions, *kor* loci, are absent from the cell (Figurski and Helinski, 1979; Figurski *et al.*, 1982; Smith and Thomas, 1983; Pohlman and Figurski, 1983a, 1983b), Figure 1. The mini RK2-derived plasmids (pRK2501 and derivatives) used in the copy number experiments described above contain *kilD*, which is adjacent to *trfA*, and therefore necessarily also *korD* which lies in the *trfB* region and has not been separated from the loci *korA* and *trfB* (Smith and Thomas, 1983; Bechhofer and Figurski, 1983), Figure 1. For simplicity we shall refer to this locus as *trfB* in the rest of this paper except when it is more helpful to use one of the other designations. The results of recent studies on transcription and its regulation in the *trfA* and *trfB* regions are summarized in Figure 1. A product of the *trfB* locus negatively regulates the *trfA* operon, of which *trfA* is the second cistron, but does not regulate clockwise transcription in the *trfA* region. A product of *korB* has an additional negative effect on *trfA* operon transcription and is also required for negative regulation of the clockwise transcription which is therefore tentatively assigned to *kilB* expression, while *kilD* is proposed to be part of the *trfA* operon (Shingler and Thomas, 1984b; Smith *et al.*, 1984). In the *trfB* region *trfB* and *korB* are expressed from a single promoter which may be autoregulated by products of both loci, although a weaker secondary promoter also gives some *korB* expression that would not be regulated in this way (Smith and Thomas, 1984b; Smith *et al.*, 1984). This *trfB, korB* operon contains an additional locus, *incC*, which expresses incompatibility towards parental plasmids similar to RK2 but not mini replicons like pRK248 (Meyer and Hinds, 1982; K.Ellis and P.Barth, in preparation). The *incC* locus appears to overlap *trfB* either due to *trfB* activity residing in an N-terminal subsegment of the *incC* polypeptide or due to two different overlapping reading frames (Bechhofer and Figurski, 1983; Smith and Thomas, 1984b).

These regulatory circuits may well represent the mechanism by which a set of maintenance functions are controlled. In particular the presence of *korB* in RK2 replicons may further decrease *trfA* operon expression compared with that in pRK248 and pRK2501 and could reduce the *trfA* protein concentration to a level limiting for replication. To test whether

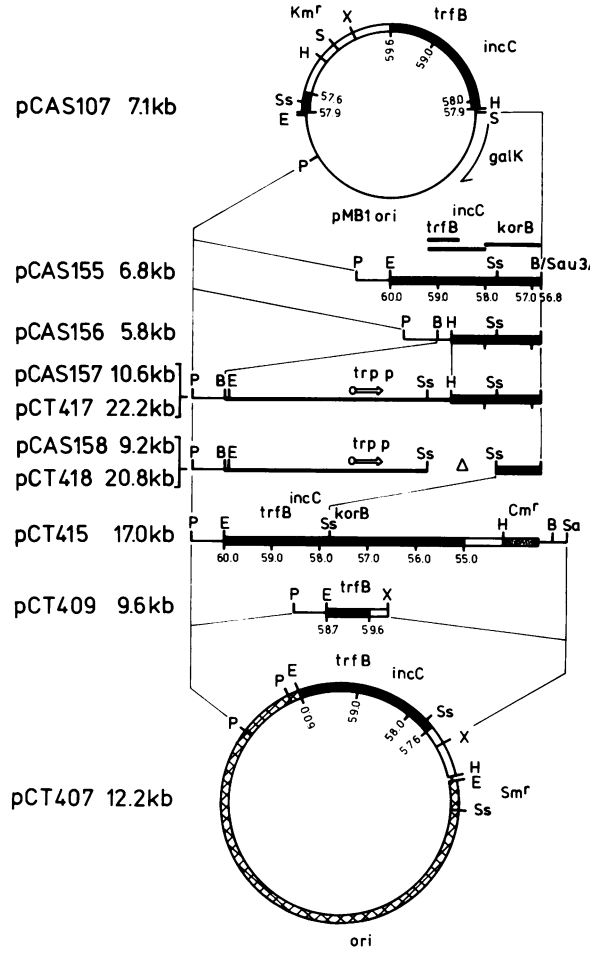

Fig. 2. Structure of plasmids containing segments of the *trfB* and *korB* region which were used for incompatibility tests. Restriction endonuclease cleavage sites are shown by: *Bam*HI, B; *Eco*RI, E; *Hind*III, H; *Pst*I, P; *Sma*I, S; *Sal*I, Sa; *Sst*II, Ss; *Xho*I, X. Plasmids pCAS155, pCAS156, pCAS157 and pCAS158 have a structure similar to pCAS107 the *Pst*I to *Sma*I Km^r *trfB* region segment being replaced by the *Pst*I to *Bam*HI/*Sau*3A segment as shown. Similarly pCT409 and pCT415 are related to pCT407 by a *Pst*I to *Xho*I or *Sal*I substitution while pCT417 and pCT418, related to pCT415, have segments substituted from pCAS157 and pCAS158 as shown. RK2-derived DNA ■ is shown with kb coordinates. Also distinguished is: Tn903-derived DNA, □ ; pRK353 (Kolter and Helinski, 1978)-derived DNA, ▬ ; pBR322 or pKO4-derived DNA, —— ; R300B-derived DNA, ▨

or not *korB* does play a role in copy number control we have investigated its expression of incompatibility and its effect in *cis* on copy number.

Results

The korB locus expresses incompatibility towards mini RK2 plasmids

In re-examining *incC* incompatibility (see Introduction) towards mini plasmids, various plasmids (previously described) based on the high copy number pMB1 replicon, and carrying either the *trfB* region (*trfB*$^+$ *incC*$^+$ *korB*$^+$) or *korB* alone (*trfB*$^-$ *incC*$^-$ *korB*$^+$) were tested for their ability to cause loss of pRK248, which carries wild-type $oriV_{RK2}$, *trfA* and *trfB* regions of RK2. The results (Table I) confirmed that pRK248 is not eliminated by the *trfB* region in *trans* (pCAS107, *trfB*$^+$ *incC*$^+$, Figure 2) but that the presence of *korB* in *trans* in addition to *trfB* (pCAS155, *trfB*$^+$, *incC*$^+$ *korB*$^+$, Figure 2) does result in a high rate of segregation of pRK248. From previous studies it appears that a large proportion of the *korB* gene transcription is as part of a polycistronic unit where *trfB* is the first cistron (Smith and Thomas, 1984b, Figure 1). In plasmid pCAS156 (*trfB*° *incC*$^-$ *korB*$^\pm$, Figure 2) the promoter and first part of this transcriptional unit are deleted, resulting in loss of at least part of the coding sequence for the polypeptides associated both with *trfB* and *incC* phenotypes. In pCAS157 (*trfB*° *incC*$^-$ *korB*$^+$, Figure 2) strong transcription of *korB* is restored by addition of the *trpE* promoter in correct orientation. Table I shows that pCAS156 may possibly show some incompatibility, consistent with the low residual expression of *korB* polypeptide observed (Smith and Thomas, 1984b), while pCAS157 shows significant incompatibility, the strength of which is increased by derepressing *trp* operon transcription, in contrast to the other plasmids where *trp* operon derepression has no effect. Deletion up to the *Sst*II site in the *trfB* region, giving pCAS158, (*trfB*° *incC*° *korB*$^-$, Figure 2), eliminates the incompatibility effect, consistent with our knowledge that the *korB* gene starts before this *Sst*II site. Therefore, *korB* alone in *trans* is sufficient for this incompatibility phenotype towards mini plasmids like pRK248 containing both *trfA* and *trfB* regions.

A series of plasmids similar to those used above but based on the IncQ replicon of R300B were used to test expression of incompatibility from relatively low copy number plasmids. Some of these plasmids have already been described elsewhere (Shingler and Thomas, 1984b). They are all derivatives of pCT407 (Thomas *et al.*, 1982, Figure 2) which contains the *trfB* region. A *trfB*$^+$ *incC*$^+$ *korB*$^+$ derivative (pCT415, Figure 2) contains RK2 DNA from coordinates 55.0 to 60.0 kb while pCT417 (*trfB*° *incC*$^-$ *korB*$^+$, Figure 2) and pCT418 (*trfB*° *incC*° *korB*$^-$, Figure 2) were derived by direct substitution into this region of pCT415 from pCAS157 and pCAS158, described above.

These plasmids were tested as above for incompatibility towards pRK248 and it was found that only pCT417, in which *korB* is transcribed from the *trpE* promoter, shows strong incompatibility (Table I). This effect was partially repressed by high exogenous tryptophan concentrations. These pCT407 derivatives all have a copy number which is much lower than that of the pMB1 replicon-based plasmids and which, as estimated from the yield of plasmid DNA in alkaline/SDS plasmid DNA extracts, is lower even than that of pRK248. The relative strengths of incompatibility expressed by these sets of high and low copy number plasmid derivatives suggests that *korB* may only express incompatibility either when its expression is boosted by a foreign promoter or its copy number rises above that of mini RK2 plasmids. This property is consistent with *korB* being a potential copy number control element.

From the control circuits described in the Introduction it seemed most likely that the *korB* expression of incompatibility is due to reduction of *trfA* gene expression. If this is the case then the incompatibility might be reduced if the *trfA* level is boosted. Plasmid pCT88 (Thomas, 1981b) carries a Tc^r *trfA*$^+$ fragment joined to pDS3, a Cm^r P15A-derived vector. While this plasmid is *trfA*$^+$ it lacks the normal *trfA* promoter and putative operator sequences so that expression of *trfA* should not respond to the *trfB* and *korB* transcriptional regulation. The key *korB* incompatibility experiments were repeated in both a *recA*$^+$ (MV10) and a *recA*$^-$ (MV12) host in the presence of either pDS3 or pCT88. While pDS3 did not affect the results, pCT88 largely abolished the incompatibility (data not shown), strongly suggesting that it is providing some function which complements a deficiency caused by the presence of *korB*. Therefore we think that *korB* limiting *trfA* expression by transcriptional regulation is the simplest explanation for the observed incompatibility.

The incompatibility effect of korB requires the presence of trfB

Since *trfB* and *korB* both reduced transcription of the *trfA* operon and since this may be the basis of the incompatibility phenotype described for *korB* in the previous section, it was of interest to investigate this incompatibility effect further. Therefore, we examined the incompatibility of the IncQ replicon plasmids described above (pCT407, pCT415 and derivatives) towards plasmid pCT201, a mini RK2 plasmid which lacks the *trfB* region, Figure 3. The *trfA* region of pCT201 comes from plasmid pCT87 (Thomas, 1981b) and contains a point mutation in the putative *trfA* operon promoter which reduces its homology to the *E. coli* consensus promoter and which we propose effectively inactivates *kilD* thus removing the requirement for *trfB*(*korD*) (Smith *et al.*, 1984). Thus a

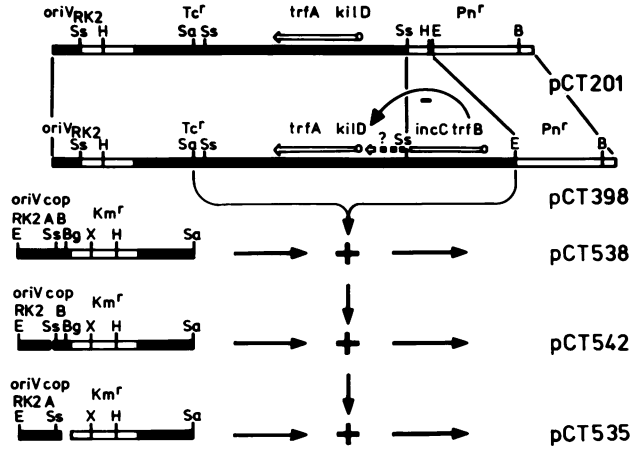

Fig. 3. Structure of pCT201 and pCT398 showing the *trfA* and *trfB* operon transcripts (⟸), the speculative part of the latter being shown as a discontinuous arrow. Only the regulatory circuit of *trfB* acting on the *trfA* operon is shown. Restriction endonuclease cleavage sites are shown as in Figure 2 with the addition of Bg for *Bgl*II. Plasmids pCT538, pCT542 and pCT535 were constructed by joining the *Sal*I to *Eco*RI fragment of pCT398 shown to the *Eco*RI to *Sal*I fragments shown of pCT539, pCT543 and pCT460, respectively.

normal level of repression by the *trfB* product provided in *trans* might be expected to reduce *trfA* expression from pCT201 to a level insufficient to support efficient replication and might lead to plasmid loss. This was observed to be the case (Table II). Interestingly pCT409 (*trfB*$^+$ *incC*$^-$, Figure 2) shows less incompatibility than pCT407 (*trfB*$^+$ *incC*$^+$, Figure 2) suggesting either that *trfB* is not expressed well from pCT409 or that *incC* is involved in the incompatibility observed with pCT407. Because the effect on pCT201 of the *trfB* region in *trans* was very strong it was impossible to be sure whether or not *korB* has any additional effect in its presence. When the key incompatibility tests (pCT407, *trfB*$^+$ *incC*$^+$ and pCT415, *trfB*$^+$ *incC*$^+$ *korB*$^+$. Figure 2) were repeated in the presence of pCT88 (*trfA*$^+$) incompatibility was largely abolished while the presence of pDS3 (*trfA*°) had no effect (data not shown). Since a number of attempts to introduce pCT201 into MV12 (*recA*$^-$) were unsuccessful, these tests could be carried out only in MV10 (*recA*$^+$) in which we cannot rule out the possibility of inter-plasmid recombination to form co-integrates as the basis of the suppression of incompatibility. However, these results are at least consistent with the proposal that the incompatibility towards pCT201 is due to an effect on *trfA* operon expression. It is interesting, therefore, to note that *korB* alone (pCT417 *trfB*° *incC*$^-$ *korB*$^+$) had no significant effect on the rate of loss of pCT201 suggesting that *korB* on its own does not have as much effect on *trfA* operon expression as *trfB* alone. Since addition of *korB* to *trfB* further decreases *trfA* operon expression by a factor similar to the initial effect of *trfB* alone (Shingler and Thomas, 1984b), this suggests that *trfB* may be the primary control with which *korB* interacts as a secondary control element for the *trfA* operon.

Table II. Elimination of pCT201 by plasmids carrying various segments of the *trfB* and *korB* region

Incoming IncQ based plasmid	Relevant RK2 genotypes	Proportion of transformants retaining pCT201[a]	
		+tryptophan	−tryptophan
R300B	−	43/50	45/50
pCT407	*trfB*$^+$ *incC*$^+$ *korB*$^-$	5/50	0/50
pCT409	*trfB*$^+$ *incC*$^-$ *korB*^{0}	30/50	10/50
pCT415	*trfB*$^+$ *incC*$^+$ *korB*$^+$	0/50	0/50
pCT417	*trfB*^{0} *incC*$^-$ *korB*$^+$	43/50	39/50
pCT418	*trfB*^{0} *incC*^{0} *korB*$^-$	42/50	41/50

[a]C600 (pCT201) was transformed with DNA of the plasmids shown and streptomycin-resistant transformants selected on M9.CAA medium with and without tryptophan (200 μg/ml). Transformants were replica plated to test for retention of the tetracycline resistance marker of pCT201.

Efficient replication of mini RK2 replicons depends on the level of transcription from the trfA operon promoter

Since the *trfB* region in *trans*, attached to a vector with a copy number similar to or even lower than that of RK2, inhibits pCT201 replication, it is perhaps surprising that pCT398 (Thomas *et al.*, 1982, Figure 3) in which the *trfB* region has been re-introduced in *cis* appears to be maintained quite efficiently. The most reasonable explanation for this seems to be that since *trfB* region transcription runs towards the *trfA* gene (Figures 1 and 3) then in these mini replicons transcription originating at the *trfB* promoter may contribute significantly to *trfA* expression, at least partly counteracting the effect of repressing transcription from the *trfA* operon promoter. If this is correct then it raises the question as to whether in such mini replicons alterations in the rate of transcription from the *trfA* promoter will significantly alter the level of *trfA* gene expression. This question is of considerable interest here in considering whether or not control of *trfA*

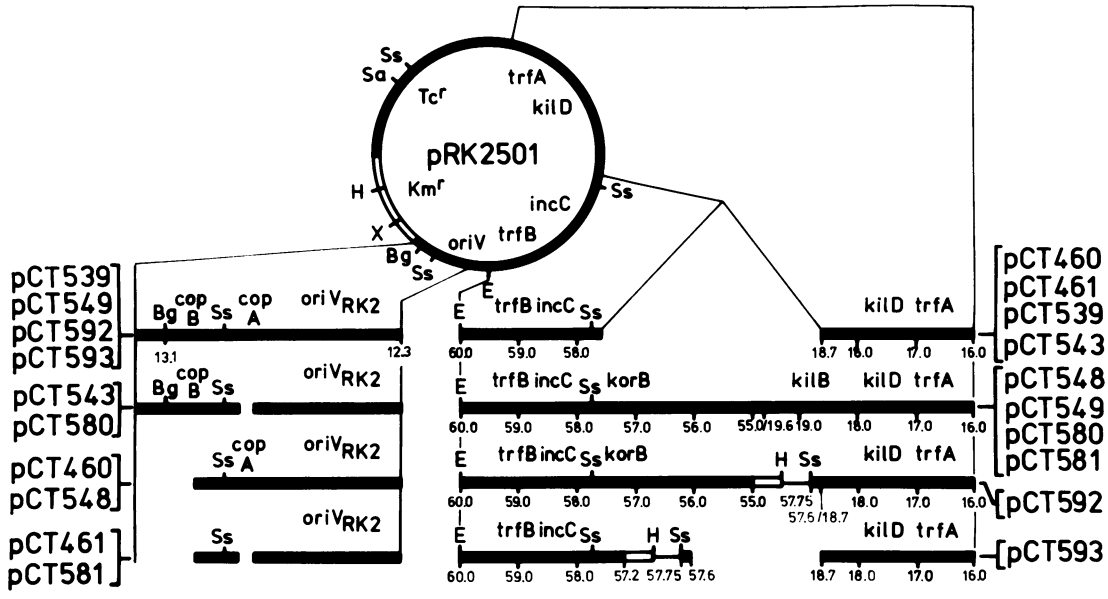

Fig. 4. Structure of plasmids whose copy number was determined to analyse the effect of *korB* on copy number. Plasmid designations on each side indicate which plasmids have the segment shown for either the *oriV*$_{RK2}$ region or *trfA* and *trfB* region. Of the plasmids not previously described pCT549, pCT548, pCT580 and pCT581 were constructed by joining the *Eco*RI to *Sal*I, *trfB*$^+$ *incC*$^+$ *korB*$^+$ *kilB*$^+$ *kilD*$^+$ *trfA*$^+$ fragment of pRK259 (originally from pRK229, Thomas *et al.*, 1980) to the *Eco*RI to *Sal*I *oriV*$_{RK2}$ Km^r fragment present in pCT539, pCT460, pCT543 and pCT461, respectively. Plasmids pCT592 and pCT593 were constructed by first inserting a wild-type Tc^r *trfA*$^+$ *kilD*$^+$ *Hind*III fragment from pCAS132 (Smith and Thomas, 1983) into the *Hind*III site of pRK259.2 (*trfB*$^+$ *incC*$^+$ *korB*$^+$) and pRK259.2 (*trfB*$^+$ *incC*$^+$ *korB*$^-$) (Smith and Thomas, 1983). For each construction, one orientation of the two *Hind*III fragments (in plasmids pCT588 and pCT590, respectively) gives a *Sal*I to *Eco*RI fragment carrying the *trfA* and *trfB* regions juxtaposed as in the mini replicons. This fragment from both pCT588 and pCT590 was joined to the wild-type *oriV*$_{RK2}$ Km^r *Eco*RI to *Sal*I fragment of pCT536 (Thomas *et al.*, 1984) giving plasmids pCT592 and pCT593, respectively. Restriction endonuclease cleavage sites are indicated as in Figures 2 and 3. Linearised segments of RK2 DNA (▬) are shown with RK2 kb coordinates.

Table III. Comparison of the copy number of mini RK2 plasmids

$oriV_{RK2}$ region *cop* loci present	Plasmid:*trfB*$^+$*korB*$^-$ wt[b] *trfA* region	Estimated[a] copy no.	Plasmid:*trfB*$^+$*korB*$^-$ mt[b] *trfA* region	Estimated[a] copy no.	Plasmid:*trfB*$^+$*korB*$^+$ wt[b] *trfA* region	Estimated[a] copy no.
copA$^+$*copB*$^+$	pRK2501	10.1 ± 1.2				
copA$^+$*copB*$^+$	pCT539	11.3 ± 1.5	pCT538	7.35 ± 0.8	pCT549(*kilB*$^+$)	6.0 ± 0.4
copA$^+$*copB*$^+$	pCT593	12.2 ± 2.2			pCT592(*kilB*$^-$)	7.5 ± 0.6
copA$^-$*copB*$^+$	pCT543	17.1 ± 2.2	pCT542	10.4 ± 1.2	pCT580(*kilB*$^+$)	7.5 ± 0.7
copA$^+$*copB*$^-$	pCT460	18.0 ± 2.1	pCT535	12.0 ± 2.6	pCT548(*kilB*$^+$)	6.6 ± 0.7
copA$^-$*copB*$^-$	pCT461	38.7 ± 7.2			pCT581(*kilB*$^+$)	8.6 ± 0.9

[a]Copy number was estimated by comparison of the level of [Me-^{3}H]thymine incorporation into pBR322 and the test plasmid as previously described (Thomas *et al.*, 1984). Averages are based on estimates for a minimum of two separate strains performed in duplicate and the variances are shown.
[b]wt = wild-type; mt = mutant.

operon expression in these replicons can control copy number. To determine whether in such mini replicons the rate of transcription from the *trfA* promoter has any effect on copy number, we tested to see whether or not the *trfA* operon promoter mutation (present in pCT201 and pCT398, referred to above; Figure 3) influences copy number when introduced into the standard mini replicons previously used for copy number determinations. We joined the *trfB*, *trfA* and Tc^r region from pCT398 to the $oriV_{RK2}$ and Km^r region of pCT539 and pCT543 and pCT460 whose copy numbers were previously determined, (Figure 3). Comparison of copy number of these plasmids with those containing the *trfA* operon promoter mutation indicated that this mutation causes a consistent drop in plasmid copy number of ~40% (Table III). Thus, while transcription from the *trfB* promoter may contribute to *trfA* expression in these plasmids, this indicates that when a wild-type *trfA* region is present the majority of the expression is due to transcription from the *trfA* promoter so that a down-mutation in the *trfA* promoter results in a lower copy number. The three pairs of plasmids tested differ in their $oriV_{RK2}$ regions, pCT543/pCT542 and pCT460/pCT535 having deletions which cause an increase in copy number (Thomas *et al.*, 1984). In view of the results obtained below it is interesting to note that the relative effects of these $oriV_{RK2}$ deletions on copy number is independent of whether or not the *trfA* region is wild-type or mutant. These results strongly suggest that the copy number of mini RK2 replicons can be reduced if transcription from the *trfA* operon promoter is reduced.

Inclusion of korB in cis in mini RK2 replicons reduces the copy number

To test what effect *korB* in *cis* has on mini RK2 plasmid copy number we took advantage of the fact that pRK229 contains *korB* and *kilB*, Figure 1 (Smith and Thomas, 1983). Plasmids pCT549 and pCT539 have the wild-type $oriV_{RK2}$ region but differ in the *trfA* and *trfB* region in that pCT549 has the *trfB*$^+$ *incC*$^+$ *korB*$^+$ *kilB*$^+$ *kilD*$^+$ *trfA*$^+$ segment of pRK229 while pCT539 has only *trfB*$^+$ *incC*$^+$ *kilD*$^+$ *trfA*$^+$ (from pRK248, Figure 4). Comparison of copy numbers indicated that pCT549 has a copy number approximately half that of pCT539 (Table III). Thus the copy number of pCT549 lies within the range of 5–7 copies/chromosome equivalent estimated for parental RK2 (Figurski *et al.*, 1979).

It is possible that there is a mutational difference between the *trfA* regions of pCT549 and pCT539 which results in reduced *trfA* expression in and hence reduced copy number of pCT549. Therefore, two plasmids were constructed which are isogenic for $oriV_{RK2}$, Km^r, Tc^r and *trfA*, *kilD* regions but which differ in the *trfB*, *korB* region such that pCT592 is *korB*$^+$ while pCT593 is *korB*$^-$ (Figure 4). The *korB*$^+$ plasmid was found to exist at ~7.5 copies/chromosome equivalent, while the *korB*$^-$ plasmid was found at ~12.2 copies/chromosome equivalent (Table III). Since these two plasmids do not have *kilB* but do have a *trfA kilD* region identical to previously studied mini RK2 replicons, this difference in copy number seems likely to be due to the presence or absence of *korB*.

It was also of interest to determine how deletions in the $oriV_{RK2}$ region, which increase copy number when *korB* is absent (Thomas *et al.*, 1984), affect copy number when *korB* is present. The plasmids pCT548, pCT580 and pCT581 (Figure 4), were constructed as pCT549 but with the $oriV_{RK2}$ Km^r region from pCT460 (*copB*$^-$), pCT543 (*copA*$^-$) and pCT461 (*copA*$^-$ *copB*$^-$), respectively. The results (Table III) show that the presence of *korB* substantially reduces the relative effect that the $oriV_{RK2}$ region deletions have on mini RK2 plasmid copy number. Thus, whereas each $oriV_{RK2}$ deletion increases copy number ~60–100% in the absence of *korB*, the increase is only 10–25% in the presence of *korB*. In this respect the effect of *korB* appears to differ from that of the *trfA* operon promoter mutation described above and is such that *korB* does seem to be a major copy number control element in RK2 being able largely to override other mutations which could potentially affect plasmid copy number.

Discussion

The results reported in this paper suggest strongly that *korB* is an important copy number control element in RK2 and may represent the missing element previously postulated (Thomas *et al.*, 1984). Firstly, *korB* expresses incompatibility towards mini RK2 replicons either when it is attached to a vector with a copy number well above the normal RK2 copy number or when *korB* is expressed from a foreign, strong promoter but not when *korB* is at low copy number and expressed from its natural promoter(s). This incompatibility is largely abolished by placing in *trans* a *trfA* gene expressed from a promoter not regulated by *korB*. Secondly, when *korB* is in *cis* to mini RK2 replicons the copy number is reduced to a level similar to that expected for RK2 itself. The fact that these mini replicons have copy numbers near the top of the range reported for RK2 (5–7 copies/chromosome equivalent, Figurski *et al.*, 1979) may be due to their smaller size and their lack of relaxation complex (Guiney and Helinski, 1979), both factors being likely to increase yield of plasmid DNA by standard isolation methods. Thus, through its inhibition of *trfA* gene expression, *korB* may act to prevent replication of RK2-derived replicons as their copy number rises above a level similar to that expected for RK2 itself.

Table IV. Plasmids used in this study

Plasmid	Size (kb)	Selective[a] markers	Other relevant properties	Source or reference
pBR322	4.36	Pn^r Tc^r	pMB1 replicon	Bolivar *et al.*, 1977
pKO4	3.9	Pn^r	pMB1 replicon	McKenney *et al.*, 1981
pCAS107	7.1	Pn^r Km^r	pMB1 replicon	Smith and Thomas, 1983
pCAS155	6.8	Pn^r	pMB1 replicon	Smith and Thomas, 1984b
pCAS156	5.8	Pn^r	pMB1 replicon	Smith and Thomas, 1984b
pCAS157	10.6	Pn^r	pMB1 replicon	Smith and Thomas, 1984b
pCAS158	9.2	Pn^r	pMB1 replicon	Smith and Thomas, 1984b
pDS3	2.5	Cm^r	P15A replicon	D.Stalker, unpublished
pCT88	9.6	Cm^r Tc^r	P15A replicon	Thomas, 1981b
R300B	8.9	Su^r Sm^r	IncQ replicon	Barth, 1979
pCT407	12.2	Sm^r	IncQ replicon	Thomas *et al.*, 1982
pCT409	9.6	Sm^r	IncQ replicon	Shingler and Thomas, 1984b
pCT415	17.0	Sm^r Cm^r [b]	IncQ replicon	Shingler and Thomas, 1984b
pCT417	22.2	Sm^r Cm^r [b]	IncQ replicon	This study, Table I,II
pCT418	20.8	Sm^r Cm^r [b]	IncQ replicon	This study, Table I,II
pRK248	9.6	Tc^r	RK2 replicon	Thomas *et al.*, 1980
pRK2501	11.1	Tc^r Km^r	RK2 replicon	Kahn *et al.*, 1979
pCT201	10.5	Tc^r Pn^r	RK2 replicon	Thomas *et al.*, 1982
pCT460	10.8	Tc^r Km^r	RK2 replicon	Thomas, 1983
pCT461	10.75	Tc^r Km^r	RK2 replicon	Thomas, 1983
pCT535	10.8	Tc^r Km^r	RK2 replicon	This study, Table III
pCT538	11.1	Tc^r Km^r	RK2 replicon	This study, Table III
pCT539	11.1	Tc^r Km^r	RK2 replicon	Thomas *et al.*, 1984
pCT542	11.05	Tc^r Km^r	RK2 replicon	This study, Table III
pCT543	11.05	Tc^r Km^r	RK2 replicon	Thomas *et al.*, 1984
pCT548	14.4	Tc^r Km^r	RK2 replicon	This study, Table III
pCT549	14.7	Tc^r Km^r	RK2 replicon	This study, Table III
pCT580	14.65	Tc^r Km^r	RK2 replicon	This study, Table III
pCT581	14.35	Tc^r Km^r	RK2 replicon	This study, Table III
pCT592	14.6	Tc^r Km^r	RK2 replicon	This study, Table III
pCT593	12.4	Tc^r Km^r	RK2 replicon	This study, Table III

[a]Selection was carried at the following concentrations: Chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; penicillin, 300 μg/ml in agar, 150 μg/ml in liquid; streptomycin, 30 μg/ml; tetracycline, 25 μg/ml.
[b]This chloramphenicol resistance gene is from *S. aureus* and was not used for selection although it can be screened for on L agar with chloramphenicol at 10 μg/ml.

While it seems that this study may have identified all the segments of RK2 necessary to maintain the relatively low copy number of parental RK2 it remains to be determined exactly how the copy number control works. At least two major questions remain. First, it is not certain whether the combination of *trfB* and *korB* is sufficient to reduce copy number to that of RK2 or whether they simply reduce *trfA* expression to a level low enough for other potential regulatory elements to become important. A candidate for such an additional element may be *incC* which is expressed as part of the *trfB korB* operon (Figure 1). The results in Table II are consistent with *incC* contributing to the incompatibility of pCT407 towards pCT201. In addition, recent studies (C.M. Thomas, unpublished) indicate that pCT549 (*korB*$^+$, Figure 4) is susceptible to *incC* while pCT539 (*korB*$^-$, Figure 4) is not. Possible roles for the *incC* product in incompatibility and copy number control are to reduce *trfA* protein concentration by interfering with transcription, translation or protein stability, or to interact directly with some *oriV*$_{RK2}$ function. Further analysis of this question is clearly needed and is currently underway in this laboratory.

A second major question is how does *korB* largely override the effect of *oriV*$_{RK2}$ region deletions which, in the absence of *korB*, have a large effect on plasmid copy number? It cannot simply be due to a general lowering of *trfA* operon expression because where this is achieved by means of a point mutation in the *trfA* operon promoter as in pCT538, pCT542 and pCT535 (Table III, Figure 3) the result is a generally lower copy number, with *oriV*$_{RK2}$ region deletions still having the same proportional effect as with wild-type *trfA* region. These observations suggest that the effect of *korB* on *trfA* operon expression, and thus replication rate, responds rapidly to increases in plasmid copy number above the normal level for RK2 so that with the *oriV*$_{RK2}$ deletions a new steady-state is achieved only slightly higher than before. Our recent observations suggest that the *trfB korB* operon is autoregulated (Smith *et al.*, 1984) and if this were the only transcriptional unit giving *korB* expression it is hard to see how *korB* product levels would respond to small copy number increases sufficiently to shut off *trfA* expression and so block further replication. However, a second promoter in the *trfB* region directs *korB* transcription, Figure 1 (Smith and Thomas, 1984b) and lies in a region where we have been unable to identify any sequences which we propose to be associated with *trfB* and *korB* transcriptional regulation (Smith *et al.*, 1984). This promoter may thus be a major source of *korB* expression at normal copy numbers. From the data in Table II, we have inferred that *korB* alone does not have as much effect on *trfA* operon expression as *trfB* alone. Thus *korB* seems to act as a secondary *trfA* operon regulation, possibly to achieve a fine tuning of expression which could be achieved through a linear response of *korB* expression from its secondary promoter to copy number changes. Once again further analysis is necessary to be sure exactly how the observed circuits work to regulate copy number.

It is intriguing to note the parallels between regulation of RK2 copy number by limitation of *trfA* gene expression and regulation of IncFII plasmid replication by limitations of *repA* gene expression both at the levels of transcription and translation (Molin *et al.*, 1981; Stougard *et al.*, 1981; Light and Molin, 1983; Brady *et al.*, 1983). However, in the case of RK2, this control seems to be superimposed on an *oriV*$_{RK2}$ region that is also involved in replication control and incompatibility whereas such elements have not yet been associated with the replication origin of IncFII plasmids. It is interesting to note that given the complex circuits that are present on RK2 it is perhaps not surprising that it has proved difficult to isolate copy number mutants directly from RK2. If *korB* is a major control element which can override other potential copy mutant effects, then *korB*$^-$ mutants would have to be a first step in obtaining copy mutants and this would not be possible until a *kilB*$^-$ mutant was obtained. In addition, defects in each copy number control element so far identified only have a relatively small effect on copy number, so that possibly only a doubling of copy number can be expected at each stage. Clearly such a system may have advantages for a stable extrachromosomal element.

Materials and methods

Bacterial strains and plasmids

E. coli K12 strains C600, C600△*trp*E5 (MV10), C600*thy* (CR34) and C600△*trp*E5 *recA*56 (MV12) were used. Plasmids used for novel constructions are described and referenced in the text. Plasmids used to generate the results described are listed in Table IV. For plasmid genotypes: +, signifies a functional gene; −, signifies an inactive gene; ±, signifies reduced expression of a functional sequence; °, signifies the complete absence of the coding sequence for a gene.

Method

Media, growth conditions, bacterial transformation, isolation and analysis of plasmid DNA, restriction endonucleases and *in vitro* recombination, and plasmid copy number determination were as described previously (Thomas *et al.*, 1984). Plasmid incompatibility tests were carried out as described in the footnotes to appropriate Tables.

Acknowledgements

We are grateful to K.Ellis and P.Barth for communication of unpublished results, to C.A.Smith and M.A.Cross for critical reading of the manuscript; to C.A.Smith and V.Shingler for useful discussion during the course of research; to H.Howell for technical help; to E.Badger and B.S.Price for help in preparation of the manuscript. This research was financed in part by MRC Project Grant No. G80/0374/9CB.

References

Barth,P.T. (1979) in Timmis,K.N. and Pühler,A. (eds.), *Plasmids of Medical, Environmental and Commercial Importance,* Elsevier/North Holland, Amsterdam, pp. 399-410.
Barth,P.T. and Grinter,N.J. (1977) *J. Mol. Biol.,* **113**, 455-474.
Barth,P.T., Grinter,N.J. and Bradley,D.E. (1978) *J. Bacteriol.,* **133**, 43-52.
Bechhofer,D.H. and Figurski,D.H. (1983) *Nucleic Acids Res.,* **11**, 7453-7469.
Bolivar,R., Rodriguez,R.L., Green,P.J., Betlach,M.C., Heynecker,H.C., Boyer,H.W., Crosa,J.H. and Falkow,S. (1977) *Gene,* **2**, 95-113.
Brady,G., Frey,J., Danbara,H. and Timmis,K.N. (1983) *J. Bacteriol.,* **154**, 429-436.
Burkhardt,H.-J., Riess,G. and Pühler,A. (1979) *J. Gen. Microbiol.,* **114**, 341-348.
Figurski,D.H. and Helinski,D.R. (1979) *Proc. Natl. Acad. Sci. USA.,* **76**, 1648-1652.
Figurski,D., Meyer,R. and Helinski,D.R. (1979) *J. Mol. Biol.,* **133**, 295-318.
Figurski,D.H., Pohlman,R.F., Bechhofer,D.H., Prince,A.S. and Kelton, C.A. (1982) *Proc. Natl. Acad. Sci. USA,* **79**, 1935-1939.
Guiney,D.G. (1982) *J. Mol. Biol.,,* **162**, 699-703.
Guiney,D.G. and Helinski,D.R. (1979) *Mol. Gen. Genet.,* **176**, 183-189.
Guiney,D.G. and Yakobsen,E. (1983) *Proc. Natl. Acad. Sci. USA,* **80**, 3593-3598.
Kahn,M., Kolter,R., Thomas,C., Figurski,D., Meyer,R., Ramaut,E. and Helinski,D.R. (1979) *Methods Enzymol.,* **68**, 268-280.
Kolter,R. and Helinski,D.R. (1978) *Plasmid,* **1**, 571-580.
Lanka,E. and Barth,P.T. (1981) *J. Bacteriol.,* **148**, 769-781.
Lanka,E., Lurz,R. and Furste,J.P. (1983) *Plasmid,* **10**, 303-307.
Light,J. and Molin,S. (1983) *EMBO J.,* **2**, 93-98.
McKenney,K., Shimatake,H., Court,D., Schmeissner,U., Brady,C. and Rosenberg,M. (1981) in Chirikjian,J.C. and Papas,T.S. (eds.), *Gene Amplification and Analysis, Vol. II: Analysis of Nucleic Acids by Enzymatic Methods,* Elsevier/North Holland, Amsterdam, pp. 383-415.
Meyer,R.J. (1979) *Mol. Gen. Genet.,* **77**, 155-161.
Meyer,R. and Helinski,D.R. (1977) *Biochim. Biophys. Acta,* **487**, 109-113.
Meyer,R. and Hinds,M. (1982) *J. Bacteriol.,* **152**, 1078-1090.
Molin,S., Stougard,P., Light,J., Nordstrom,M. and Nordstrom,K. (1981) *Mol. Gen. Genet.,* **181**, 123-130.
Pohlman,R.F. and Figurski,D.H. (1983a) *Plasmid,* **10**, 82-95.
Pohlman,R.F. and Figurski,D.H. (1983b) *J. Bacteriol.,* **156**, 584-591.
Shingler,V. and Thomas,C.M. (1984a) *J. Mol. Biol.,* in press.
Shingler,V. and Thomas,C.M. (1984b) *Mol. Gen. Genet.,* in press.
Smith,C.A. and Thomas,C.M. (1983) *Mol. Gen. Genet.,* **190**, 245-254.
Smith,C.A. and Thomas,C.M. (1984a) *J. Mol. Biol.,* in press.
Smith,C.A. and Thomas,C.M. (1984b) *J. Gen. Microbiol.,* in press.
Smith,C.A., Shingler,V. and Thomas,C.M. (1984) *Nucleic Acids Res.,* **12**, 3619-3630.
Stalker,D.M., Thomas,C.M. and Helinski,D.R. (1981) *Mol. Gen. Genet.,* **181**, 8-12.
Stokes,H.W., Moore,R.J. and Krishnapillai,V. (1981) *Plasmid,* **5**, 202-212.
Stougard,P., Molin,S. and Nordstrom,K. (1981) *Proc. Natl. Acad. Sci. USA,* **78**, 6008-6012.
Thomas,C.M. (1981a) *Plasmid,* **5**, 10-19.
Thomas,C.M. (1981b) *Plasmid,* **5**, 277-291.
Thomas,C.M. (1983) *Plasmid,* **10**, 184-195.
Thomas,C.M., Meyer,R. and Helinski,D.R. (1980) *J. Bacteriol.,* **141**, 213-222.
Thomas,C.M., Stalker,D. and Helinski,D.R. (1981) *Mol. Gen. Genet.,* **181**, 1-7.
Thomas,C.M., Hussain,A.A.K. and Smith,C.A. (1982) *Nature,* **298**, 674-676.
Thomas,C.M., Cross,M.A., Hussain,A.A.K. and Smith,C.A. (1984) *EMBO J.,* **3**, 57-63.

Received on 5 April 1984; revised on 25 April 1984