Regulation of transfer genes of promiscuous IncP α plasmid RK2: repression of Tra1 region transcription both by relaxosome proteins and by the Tra2 regulator TrbA

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The Tra1 region of broad host range IncP α plasmid RK2 encodes proteins essential for its promiscuous conjugative transfer and includes *oriT*, the site at which nicking occurs to initiate transfer replication. Unregulated expression of the Tra1 region genes would be likely to place a major burden on the host. To investigate the control of these genes the three transcriptional promoters from this region were cloned by PCR and inserted into *xyIE* promoter probe vectors. The strength of *traJp* and *traKp* was estimated to be six to eightfold less than the strong *trfA* promoter which is required for expression of genes for vegetative replication of RK2. The *traG* promoter was about one-tenth the strength of the other two. These promoters are not repressed by products of the central control operon of RK2. However, *traJp* and *traKp*, which are arranged as back to back divergent promoters in the *oriT* region, are repressed by TraK which constitutes part of the relaxosome necessary for nicking at *oriT*. A second relaxosome protein, TraJ, represses *traJp*. *traGp* is not repressed by any relaxosome proteins. All three promoters are repressed by TrbA, which is encoded at the start of the *trb* operon containing the rest of the transfer genes (the Tra2 region). These circuits provide: (i) an autoregulatory way of ensuring production of enough relaxosome proteins without overburdening the host; and (ii) a means of coordinating expression of both blocks of transfer genes.

Keywords: plasmid transfer, transcription, regulation, plasmid RK2, TrbA repressor

INTRODUCTION

Broad host range IncP plasmids are capable of transfer between and replication in most Gram-negative bacterial species (for reviews see Thomas & Helinski, 1989; Guiney & Lanka, 1989). In addition, their conjugative apparatus can promote transfer of DNA from Gram-negative bacteria to Gram-positive bacteria (Trieu-Cuot *et al.*, 1987; Williams *et al.*, 1990) and also to yeast (Heinemann & Sprague, 1989; Sikorski *et al.*, 1990). The best studied

IncP plasmids are the group of indistinguishable IncP α plasmids, RP1, RP4, RK2, R18 and R68 (Holloway & Richmond, 1973; Thomas & Smith, 1987) whose complete sequence has recently been compiled (Pansegrau *et al.*, 1994). The specific plasmids used in this work are RK2 and RP4.

The genes for the conjugative apparatus of these plasmids are organized into two blocks designated Tra1, encoding *tra* genes, and Tra2, encoding *trb* genes. Analysis of the Tra1 region indicated that transfer-associated genes span a 13 kb region from kb coordinate 39.9 to 53.0 (reviewed by Guiney & Lanka, 1989). The limits of the Tra1 region essential for intraspecific *Escherichia coli* matings were determined by deletion analysis (Lessl *et al.*, 1993). This *tra* core encompasses 5.8 kb including the genes *traF*, *-G*, *-H*, *-I*, *-J* and *-K* together with the *oriT* region. TraI, TraJ and TraK bind in the *oriT* region to form the relaxosome

Abbreviation: tsp, transcription start point.

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Fig. 1. Genetic organization of the Tra1 relaxosome region of RK2/RP4. (a) Map of RK2 showing the main features of the plasmid genome, including the antibiotic resistance markers, the replication functions *oriV* and *trfA*, the two key regulators *korA* and *korB* in the central control operon and *trbA* in the *trb* operon, and the two blocks of transfer functions Tra1 and Tra2. (b) Expanded map of the Tra1 region encoding *traH*–*traL* and *oriT* (the site where nicking occurs to initiate transfer replication) as well as parts of *traG* and *traM*. This segment is present in pMS2260. (c) Detailed location of the promoter regions cloned after PCR amplification with primers MZ1–MZ4, and showing the location of the three promoters studied in this paper.

which induces the specific nick at *oriT*. TraF and TraG are thought to form a bridge to the mating pair apparatus. The other Tra1 region genes, which include *traC* encoding a primase (Lanka & Barth, 1981), are not essential for mating between *E. coli* strains although the presence of *traM* significantly increases transfer efficiency (Lessl *et al.*, 1993). Within the core region Cole *et al.* (1993) showed that a mutation that alters the start of *traH* has no effect on intraspecific *E. coli* transfer, but reduces transfer up to sevenfold for mating into some Gram-negative bacterial species.

The core *trb* region is located between kb coordinates 18.03 and 29.26, encompassing 11.23 kb. The genes of this region are primarily required for mating pair formation. Nucleotide sequence revealed 12 ORFs designated *trbA*–*L* (Lessl *et al.*, 1992). A series of defined *trb* mutants showed that at least *trbB*, *-C*, *-E*, *-G* and *-L* are essential

for RK2/RP4 transfer between *E. coli* strains. When the *trbA* ORF and the entire *trbB* regulatory region was replaced by *tacp*, the resulting derivative exhibited a transfer-proficient phenotype (Lessl *et al.*, 1993). However, since TraB has been shown to be a regulatory protein it may still have a key regulatory role when the *trb* operon is intact (Jagura-Burdzy *et al.*, 1992).

In contrast to most plasmids of the F-type, the transfer frequency from bacteria which have carried IncP plasmids for a number of generations can be as high as one per donor in one hour, that is, that every bacterium can act as an efficient donor (Winans & Walker, 1985). This has been interpreted as meaning that the IncP transfer genes are expressed constitutively. However, unregulated expression of so many genes, if they have strong promoters, would place a high metabolic burden on the bacterial host while constitutive expression from weak promoters might not give the high degree of transfer proficiency observed. Indeed, we discovered that transcription of the Tra2 region genes (which constitute the *trb* operon) from the *trbB* promoter is repressed both by the KorB protein encoded in the central control operon (Shingler & Thomas 1984a; Motallebi-Veshareh *et al.*, 1992; Thomson *et al.*, 1992) and by the TraB protein encoded by the ORF preceding *trbB* (Jagura-Burdzy *et al.*, 1992).

The work described in this paper focuses on the transfer genes encoded in the Tra1 region. DNA sequence and related genetic analysis has suggested that these genes are organized as two divergent transcriptional units: *traJ*–*traA* running from *oriT* anticlockwise on the genome and *traK*–*traM* running clockwise from *oriT* (Fig. 1). Promoters are proposed to be located before *traG*, *traJ* and *traK* (Ziegelin *et al.*, 1991). The existence of *traJp* and *traGp* has been confirmed by cloning into an expression vector and mapping the transcription start point (Greener *et al.*, 1992). In this paper we present the cloning of all three promoters by PCR, determination of the *traKp* transcription start point and analysis of their regulation by the products of a number of regions.

METHODS

Bacterial strains and plasmids. *Escherichia coli* K12 strains used were C600K (*thr-1 leu-6 thi-1 lacY1 supE44 tonA2 galK3*; McKenney *et al.*, 1981) and RU2537 (F⁺ *pro-22 met-63 recA56 nalA::Tn1723*; Shingler & Thomas, 1984b; from R. Schmitt, Regensburg, Germany). Plasmids used are listed in Table 1. IncP plasmid nomenclature is as recommended by Pansegrau *et al.* (1994). Bacteria were grown in L broth (Kahn *et al.*, 1979). Solid medium was obtained by addition of agar (1.5% w/v). Cultures were grown with shaking at 37 °C. Antibiotic resistance was selected by addition of penicillin G (Pn^R, sodium salt at 100 µg ml⁻¹ in liquid medium and 300 µg ml⁻¹ in solid medium), kanamycin (Km^R, 50 µg ml⁻¹), neomycin (Nm^R, 100 µg ml⁻¹), chloramphenicol (Cm^R, 25 µg ml⁻¹) or streptomycin (Sm^R, 30 µg ml⁻¹).

Isolation of DNA, genetic manipulation and sequencing. Plasmid DNA was prepared on both small and large scales by the method of Birnboim & Doly (1979) and if necessary by CsCl/EtBr equilibrium density gradient centrifugation. DNA manipulations were carried out by standard techniques

Table 1. Plasmids used in this study

Plasmid	Size (kb)	Replicon	Selective marker	Other properties	Reference
pBR322	4.36	pMB1	Pn ^R Tc ^R	No RK2 sequence	Bolivar <i>et al.</i> (1977)
pGBT30	6.3	pMB1	Pn ^R	<i>lacI</i> ^a <i>tacp</i>	Jagura-Burdzy <i>et al.</i> (1991)
pGBT43	10.1	pSC101	Km ^R	<i>xylE</i> pp* <i>trfAp-xylE</i> <i>trbBp-xylE</i> <i>tacp-trbA</i>	Jagura-Burdzy <i>et al.</i> (1992)
pGBT58	10.35	pSC101	Km ^R		
pGBT63	10.60	pSC101	Km ^R		
pGBT80	4.33	pMB1	Pn ^R		
pGBT100	6.0	pMB1	Pn ^R	<i>xylE-galK</i> pp*	Jagura-Burdzy & Thomas (1994)
pMS2260	8.9	pMB1	Pn ^R Cm ^R	RP4 co†:47.5–52.9	Pansegrau <i>et al.</i> (1990)
pJF166uΔ5	8.2	pMB1	Pn ^R	<i>tacp-traJ traH</i> <i>tacp-traK traL</i> ‡ <i>tacp-traJ</i>	Fürste <i>et al.</i> (1989)
pJF161nΔ1	6.3	pMB1	Pn ^R		
pJF161nΔ5	6.3	pMB1	Pn ^R		
pCAS50	36	ColE1	Km ^R	RK2 co†:28.8–60.1 0–0.8	Smith & Thomas (1983)
pCT132	22	RK2/R6K	Tc ^R Km ^R TrpE ⁺	RK2 <i>oriV</i> <i>trfA</i> <i>korA</i>	C. M. Thomas (unpublished)
pCT132Δ1	14.9	R6K	Km ^R TrpE ⁺	RK2 <i>oriV</i> pCT132Δ <i>Bam</i> HI– <i>Sal</i> I	This paper
pCT153.1	58	R6K	Km ^R	RK2 co†:12.0–60.0	Thomas (1981)
pCT415	17	IncQ	Sm ^R	RK2 co†:55.0–60.0	Thomas & Hussain (1984)
R300B	8.9	IncQ	Sm ^R Su ^R	No RK2 sequence	Barth (1979)
pMZT1	6.4	pMB1	Pn ^R	<i>traJp-xylE</i> <i>traKp-xylE</i> <i>traGp-xylE</i> <i>traJp-xylE</i> <i>traKp-xylE</i> <i>traGp-xylE</i> Tn insertion in pMS2260	This paper
pMZT2	6.4	pMB1	Pn ^R		
pMZT3	6.4	pMB1	Pn ^R		
pMZT4	10.5	pSC101	Km ^R		
pMZT5	10.5	pSC101	Km ^R		
pMZT6	10.5	pSC101	Km ^R		
pMZT2260	19.5	pMB1	Pn ^R Cm ^R Km ^R		
pMZT2261	19.5	pMB1	Pn ^R Cm ^R Km ^R		
pMZT2262	19.4	pMB1	Pn ^R Cm ^R Km ^R		
pMZT2263	19.5	pMB1	Pn ^R Cm ^R Km ^R		
pMZT2260.1	9.5	pMB1	Pn ^R Cm ^R		
pMZT2261.1	9.5	pMB1	Pn ^R Cm ^R		
pMZT2262.1	9.4	pMB1	Pn ^R Cm ^R		
pMZT2263.1	9.5	pMB1	Pn ^R Cm ^R		

* Promoter probe plasmid.

† The figures shown indicate RK2/RP4 kb coordinates.

‡ This plasmid contains a truncated *traL*.

(Sambrook *et al.*, 1989) using enzymes according to the supplier's instructions. Sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using a Sequenase version 2 kit (USB) or automatically on an Applied Biosystems 373A DNA sequencer using a dye terminator kit supplied by the manufacturers.

PCR cloning of promoter fragments. Reactions were performed by standard procedures (Mullis *et al.*, 1986) using pCAS50 as a template (Smith & Thomas, 1983), with parameters as follows: 5 min denaturation at 94 °C followed by 25 rounds

of temperature cycling (94 °C for 15 s, 54 °C for 30 s, 71 °C for 90 s) and a final 5 min step at 71 °C. Routinely 10 pmol of each primer was used with 50 ng template and 1 unit *Taq* polymerase in 50 µl reaction buffer as recommended by the suppliers (Northumbria Biologicals Ltd). The primers were designed to place *Bam*HI sites at each end of the product to facilitate insertion into promoter probe vectors containing *xylE* as the reporter gene. Amplified fragments were purified by electroelution, digested with *Bam*HI, precipitated and ligated with *Bam*HI-treated pGBT100 or pGBT43 promoter probe vectors. An *Eco*RI site located in one of each pair of PCR primers

Fig. 2. Sequences of promoter regions. (a) Sequence of the *oriT/traJp/traKp* region showing primers M21 and M22 used for PCR, promoter locations, *oriT* nick site, start of *traJ* and *traK* and binding sites for TraJ and TraK. For TraK the solid line shows the core binding sites which occur at low concentration, while the dashed lines show the extended binding which occurs at higher concentration. +TraK indicates a possible alternative tsp which is only seen in the presence of TraK. (b) Sequence of the *traI/traG* junction region containing *traGp*. Confirmation/determination of the tps is shown in Fig. 3.

allowed the orientation of inserted fragment to be determined easily. The identity of the inserted fragment was confirmed by DNA sequencing.

Measurement of *xylE* activity. The level of *xylE* expression was determined by enzymic assay of catechol 2,3-oxygenase activity using the method of Zukowski *et al.* (1983). One unit is defined as the amount of enzyme necessary to convert 1 mmol substrate to product in 1 min under standard conditions. Protein concentration was determined by the biuret method (Gornall *et al.*, 1949). All the assays presented in this paper were done on stationary phase cultures since the repression was more clearly observed than in exponentially growing cultures. We found significant variation in absolute activities for the same promoter-reporter gene plasmids in different experiments but the relative *xylE* activities between different promoters and with different combinations of repressors were reproducible within experiments. Cultures of bacteria with repressor genes under *tacp* control were grown in the absence of IPTG because induction was found to result in poor growth and plasmid loss.

Isolation of RNA and primer extension. Total RNA was isolated as described by Aiba *et al.* (1981). Standard primer extension reactions were performed with AMV reverse transcriptase (Life Sciences Inc.) using 50 µg RNA and M21, M22 and M23 oligonucleotides (Figs 1 and 2) labelled at the 5' end with [γ - 32 P]ATP and polynucleotide kinase.

Transposon mutagenesis. Transposon mutants of pMS2260 with Tn1723 (Km^R) were isolated by transformation of RU2537 followed by selection for elevated neomycin resistance after incubation of transformant colonies at room temperature for 3 d. The insertions were mapped by restriction analysis and DNA sequencing. For each of the insertions (pM2T2260–pM2T2263) the transposon was deleted by *EcoRI* digestion and religation, creating plasmids pM2T2260.1 to pM2T2263.1. This procedure leaves a 35 bp insertion at the site of mutation that will cause a phase change in any ORF it disrupts.

RESULTS

Cloning and mapping of Tra1 region promoters

On the basis of DNA sequence analysis and mapping of RNA polymerase binding sites (Lanka *et al.*, 1984, Ziegelin *et al.*, 1991) it was predicted that the Tra1 region contains three promoters. Two of these are located very close together as back-to-back divergent promoters in the *oriT* region and have been designated *traJp* and *traKp*. A third putative promoter was located downstream of *traJp* and was designated *traGp*. Cloning of random fragments into a promoter probe vector confirmed the existence of *traJp* and *traGp* and established the transcription start points in *E. coli* and a number of other species (Greener *et al.*, 1992). To extend these studies we used PCR to generate fragments containing *traJp* and *traKp* together and *traGp* on its own. The location and sequences of the primers used are shown in Figs 1 and 2. The promoter fragments were joined to the *xylE* reporter gene in both a high copy number vector (pGBT100, based on the pMB1 replicon) and a low copy number vector (pGBT43, based on the pSC101 replicon) to enable a wide range of second, compatible plasmids to be introduced into the same cell.

DNA sequencing confirmed the identity of the cloned fragments and showed that no mutations had been

Fig. 3. Levels of mRNA initiated at *traJp*, *traKp* and *traGp* in the absence and presence of repressors. The abundance of 5' segments of mRNA was estimated indirectly from the cDNA level synthesized by primer extension on mRNA templates isolated from different strains in which either *traJp*, *traKp* or *traGp* were present with pGBT30 (no RK2 genes), pJF161nΔ1 (*traK*), pJF166uΔ5 (*traJ*) or pGBT80 (*trbA*) *in trans*. Radioactive images were analysed with the phosphorimager. Tracks are numbered as shown on each histogram.

introduced during amplification. Plate tests to detect *xylE* expression in colonies of bacteria containing the transcriptional fusions confirmed the existence of all three promoters. Quantitative assays with the low copy number derivatives showed that *traJp* (pMZT4, 0.62 XylE units) and *traKp* (pMZT5, 0.54 XylE units) are of similar strength and that they are one-seventh the strength of *trfAp* (the promoter for the vegetative replication operon; pGBT58, 4.00 XylE units) and one-fifth the strength of *trbBp* (the promoter for the *trb* operon encoding genes largely required for mating pair formation during transfer, pGBT63, 3.22 XylE units). *traGp* (pMZT6, 0.06 XylE units) was about one-tenth the

Table 2. Activities of *TraI* region promoters

Plasmid/promoter	XylE activity in the presence of the following plasmids <i>in trans</i> *		Relative activity†
	pCT132Δ1 (no RK2 sequence)	pCT153.1 (RK2 DNA from 12 to 60 kb; see Fig. 1)	
pMZT1/ <i>traJp</i>	5.9 ± 0.5	0.51 ± 0.13	0.09
pMZT2/ <i>traKp</i>	2.7 ± 0.05	0.30 ± 0.15	0.11
pMZT3/ <i>traGp</i>	1.12 ± 0.12	0.86 ± 0.26	0.77

* Data from four experiments ± SD. XylE activity is expressed in units as described in Methods.

† The relative activity was calculated using the activity in the presence of pCT132Δ1 as 1.00.

strength of *traJp* and *traKp*. Thus in this context *traJp* and *traKp* are medium strength promoters while *traGp* is weak.

Transcription start points (tsps) were determined by primer extension on mRNA extracted from bacteria carrying these recombinant plasmids. The results (Fig. 3) confirmed the transcription start point for *traJp* and *traGp* as determined by Greener *et al.* (1992), but showed clearly a single initiation point for *traJp* rather than initiation at a pair of successive bases. In the case of *traKp* we have mapped the multiple tsps at successive GAA residues downstream of the -10 sequence predicted by Ziegelin *et al.* (1991).

For *traGp* we observed shorter extended species in addition to the band which corresponds to the tsp previously mapped by Greener *et al.* (1992). These are probably the result of non-specific primer annealing since they are not repressed by TrbA (see below). Since TrbA reduces transcription from the cloned *traGp* fragment by more than 90%, the sum of the bands which originate from promoters in this fragment should be repressed by this amount. The shorter species are not affected and it seems very unlikely therefore that they are related to the promoter activity being measured with the promoter-probe assays.

traJp and *traKp* are repressed by proteins of the relaxosome region

To determine whether or not transcription from the *tra* region promoters is subject to repression by the products of the RK2 genome we constructed strains in which a second plasmid carrying all or part of the RK2 genome was placed *in trans* to the promoter probe fusion plasmids monitoring each of the three promoters. The presence of pCT153.1 carrying a segment of the RK2 genome from coordinate 12 to 60 kb, showed clearly that *traJp* and *traKp* are subject to repression but that *traGp* is repressed only a little, if at all, when it is on a high copy number

Table 3. Activity of Tra1 region promoters in the presence of central control operon genes

Plasmid/promoter	Plasmid <i>in trans</i>	RK2 genes <i>in trans</i>	XylE activity*
pMZT1/ <i>traJp</i>	R300B	None	2.75 ± 0.05
	pCT415	<i>korB incC korA</i>	2.60 ± 0.14
pMZT2/ <i>traKp</i>	R300B	None	1.45 ± 0.22
	pCT415	<i>korB incC korA</i>	2.20 ± 0.14
pMZT3/ <i>traGp</i>	R300B	None	0.25 ± 0.06
	pCT415	<i>korB incC korA</i>	0.30 ± 0.01

* Data from three experiments ± SD. XylE activity is expressed in units as described in Methods.

plasmid (Table 2). The possibility that the repression of *traJp* and *traKp* is due to the products of the central control operon was tested with plasmids R300B (no RK2 sequence) and pCT415 (*korA incC korB korF korG*). This showed that the 55–60 kb region on the RK2 genome has no effect (Table 3). By contrast, when pMS2260, carrying the Tra1 core region, was placed *in trans* we observed strong repression of *traJp* and *traKp* but no repression of *traGp* (Table 4). We concluded therefore that *traJp* and *traKp* are repressed by relaxosome proteins but that *traGp* is not.

Identification of the relaxosome region repressors by mutagenesis and subcloning of *traH* to *traK*

Since the region encoding *oriT* and the relaxosome proteins caused strong repression of *traJp* and *traKp*, it was important to define which genes were required for this effect. We first used transposon mutagenesis of

pMS2260 to inactivate individual genes. The locations of insertions were established by restriction mapping and sequence analysis. The insertion in pMZT2260.1 is located in the vector part of pMS2260 (at bp 2385 of the pBR329 sequence as presented by Covarrubias & Bolivar, 1982). This plasmid was used as a control and gave similar levels of repression to pMS2260 itself. The insertion in pMZT2261.1 was mapped at the penultimate codon (leucine) of *traK*. The 5 bp duplicated by the insertion corresponds to coordinates 5371–5376 according to the sequence GB_BA: ECRP4 present in GenBank under the accession numbers X54459 and S87583 (Ziegelin *et al.*, 1991). The insertion in pMZT2263.1 occurred near the 5' end of *traH* after the 14th amino acid codon, duplicating nucleotides 2609–2613. This insertion also disrupts *traI* since *traH* lies entirely within *traI*. In both pMZT2261.1 and pMZT2263.1 no deletions were generated by the transposon insertion. However, in the case of pMZT2262.1 the transposon caused an internal *traK* deletion located in its 5' terminal part (after the 19th codon). This deletion removed 125 bp between coordinates 5031 and 5156. The results (Table 4) demonstrated that inactivation of *traK*, while all the other relaxosome genes remained intact (pMZR2262.1), relieved completely the repression on *traKp* and gave an almost 10-fold derepression of *traJp*. The insertion in the very end of *traK* (pMZT2263.1) was found not to affect *traK* repression activity. The *traH/I* mutation (in pMZT2263.1) did not decrease repression of *traKp* and gave at most twofold derepression of *traJp*. Thus *traK* seems to be the most important gene for repression of the *oriT* region promoters.

To determine whether *traK* can act alone as a repressor of the *oriT* region promoters we used a plasmid (pJF161nΔ1) containing the *traK* ORF under control of the *tac*

Table 4. The effect of mutations in relaxosome genes on *traJp*, *traKp* and *traGp* expression

Plasmid/promoter	Plasmid <i>in trans</i>	RK2 genes <i>in trans</i>	XylE activity*	Relative activity
pMZT4/ <i>traJp</i>	pBR322	None	0.73 ± 0.23	1
	pMS2260	<i>tra H⁺ I⁺ J⁺ K⁺ L⁺</i>	0.023 ± 0.02	0.03
	pMZT2260.1	<i>tra H⁺ I⁺ J⁺ K⁺ L⁺</i>	0.016 ± 0.01	0.02
	pMZT2261.1	<i>tra H⁺ I⁺ J⁺ K[±] L⁺</i>	0.013 ± 0.02	0.02
	pMZT2262.1	<i>tra H⁺ I⁺ J⁺ K⁻ L⁺</i>	0.16 ± 0.07	0.22
	pMZT2263.1	<i>tra H⁻ I⁺ J⁺ K⁺ L⁺</i>	0.036 ± 0.02	0.05
pMZT5/ <i>traKp</i>	pBR322	None	1.08 ± 0.54	1
	pMS2260	<i>tra H⁺ I⁺ J⁺ K⁺ L⁺</i>	0.12 ± 0.05	0.11
	pMZT2260.1	<i>tra H⁺ I⁺ J⁺ K⁺ L⁺</i>	0.086 ± 0.08	0.08
	pMZT2261.1	<i>tra H⁺ I⁺ J⁺ K[±] L⁺</i>	0.086 ± 0.07	0.08
	pMZT2262.1	<i>tra H⁺ I⁺ J⁺ K⁻ L⁺</i>	1.46 ± 0.75	1.35
	pMZT2263.1	<i>tra H⁻ I⁺ J⁺ K⁺ L⁺</i>	0.045 ± 0.09	0.04
pMZT6/ <i>traGp</i>	pBR322	None	0.11 ± 0.07	1
	pMS2260	<i>tra H⁺ I⁺ J⁺ K⁺ L⁺</i>	0.20 ± 0.12	1.8

* Data from three experiments ± SD. XylE activity is expressed in units as described in Methods.

Table 5. Activity of *traJp* and *traKp* in the presence of plasmids overproducing TraJ and TraK

Plasmid/ promoter	Plasmid <i>in trans</i>	RK2 genes <i>in trans</i>	XylE activity*	Relative activity
pMZT4/ <i>traJp</i>	pGBT30	None	9.80 ± 1.5	1
	pJF166uΔ5	<i>traJ</i>	2.30 ± 0.3	0.23
	pJF161nΔ1	<i>traK</i>	0.32 ± 0.02	0.03
pMZT5/ <i>traKp</i>	pGBT30	None	6.66 ± 1.01	1
	pJF166uΔ5	<i>traJ</i>	ND	ND
	pJF161nΔ1	<i>traK</i>	0.24 ± 0.05	0.04

ND, not done because of plasmid instability.

* Data from three experiments ± SD. Assays were done in the absence of IPTG. XylE activity is expressed in units as described in Methods.

promoter. The results showed that TraK on its own is able to repress both *traJp* and *traKp* about 30-fold (Table 5). Similar experiments with *ptac-traJ* (pJF161nΔ5) showed that TraJ can repress *traJp* fourfold. We were unable to determine the effect of *traJ* on *traKp* because the combination of pJF161nΔ5 and pMZT5 was unstable. A plasmid overproducing TraI is not available due to its lethal effect on the bacterial host (E. Lanka, personal communication). Even attempts to use a plasmid which has a 3'-truncated version of *traI* under the control of the *tac* promoter (pJF166nΔ5) caused problems of instability when introduced into the same cell with promoter probe fusions.

Since *traK* can act alone to repress *traKp* and inactivation of *traK* in pMS2260 causes complete derepression of *traKp*, we concluded that TraK is probably the only relaxosome protein which regulates this promoter. For *traJp* the situation appears to be more complicated since inactivation of *traK* in pMS2260 only causes partial derepression. Since *traJ* under *tacp* control also causes repression, we concluded that TraJ may be responsible for some of the residual repression by pMS2260 when *traK* is inactivated, while *traI* and/or *traH* may also play a role. Nevertheless TraK once again seems to be the primary repressor protein.

TrbA coordinates expression of both *tra* and *trb* operons

We recently reported that *trfAp* and *trbBp* are under control of TrbA, encoded by the first ORF in the *trb* operon and that TrbA may have some special association with control of genes required for conjugative transfer (Jagura-Burdzy *et al.*, 1992). It was therefore of interest to determine whether TrbA also influences transcription from *tra* promoters. We introduced a plasmid carrying *tacp-trbA* (pGBT80) *in trans* to promoter probe vectors carrying promoters *traJp*, *traKp* or *traGp* and determined its effects on expression of the *xylE* reporter gene. The results (Table 6) showed that indeed TrbA does give

Table 6. Activity of Tra1 region promoters in the presence of a plasmid overproducing TrbA

Plasmid/ promoter	Plasmid <i>in trans</i>	RK2 genes <i>in trans</i>	XylE activity*	Relative activity
pMZT4/ <i>traJp</i>	pGBT30	None	10.3 ± 1.10	1
	pGBT80	<i>trbA</i>	1.40 ± 0.60	0.13
pMZT5/ <i>traKp</i>	pGBT30	None	9.40 ± 3.90	1
	pGBT80	<i>trbA</i>	0.94 ± 0.18	0.10
pMZT6/ <i>traGp</i>	pGBT30	None	0.40 ± 0.03	1
	pGBT80	<i>trbA</i>	0.06 ± 0.05	0.15

* Data from four experiments ± SD. Assays were done in the absence of IPTG. XylE activity is expressed in units as described in Methods.

7–10-fold repression of transcription from all the three promoters.

Although these effects of pGBT80 were observed in the absence of IPTG induction and therefore should correspond to only a low level of TrbA expression, the translation of *trbA* has been dramatically improved by the addition of the T7 gene 10 translational signals. To determine whether the unmodified *trbA* gene can also have this effect we repeated the assays with TrbA supplied from pVI109.2 (Shingler & Thomas, 1984a). As a control we used pVI107.2 (Shingler & Thomas, 1984b) in which the beginning of *trbA* is deleted. The results showed that *traGp* is repressed fivefold, *traJp* is repressed fourfold and *traKp* is repressed two- to threefold. This confirms that an effect of TrbA on Tra1 region promoters is likely to occur in cells carrying the unmodified *trbA* gene alone.

Repressors of Tra1 region promoter activities decrease mRNA levels

To confirm that the repression observed using *xylE* as a reporter gene reflected decreased mRNA concentration we performed primer extension on mRNA extracted from bacteria carrying each promoter in the presence of different regulators. The results (Fig. 3) confirmed that for each of the repression activities demonstrated in Tables 5 and 6, the level of detectable mRNA was decreased proportionately. We noted two anomalous results. First, with *traJp* the presence of TraJ not only reduces the intensity of the primary transcript but also causes the appearance of a new longer species. The location of the TraJ operator (Fig. 2a), would be consistent with a displacement of RNAP to an upstream position. Second, although TraK repression of *traKp* resulted in almost complete disappearance of the normal mRNA 5' end, a new shorter transcript could be detected corresponding to transcription start points shown in Fig. 2(a) (this additional *traKp* tsp is not shown in Fig. 3). This raises the possibility that TraK represses one promoter but activates a second. However, since reporter gene assays do not detect this putative activation event in the presence of

Fig. 4. Summary of control circuits controlling expression of conjugative transfer genes of IncP α plasmids. The key genes are *korA*, whose product derepresses *trbAp* activity (Jagura-Burdzy & Thomas, 1994); *korB*, whose product represses *trbBp* (Shingler & Thomas, 1984a; Motallebi-Veshareh *et al.*, 1992; Thomson *et al.*, 1993) as well as binding to additional operators in the *tra* and *trb* regions (Balzer *et al.*, 1992; Williams *et al.*, 1993); and *trbA*, whose product represses *trbBp*, *traGp*, *traJp* and *traKp*. *KorA* represses transcription at all the other places at which it is shown to bind. *KorB* only represses transcription at the known promoter regions where it binds; many of its binding sites are not near promoters and its effect at these sites is not known.

TraK, we do not wish to draw any conclusions based on this observation until further experiments have been carried out.

DISCUSSION

In this paper we have confirmed the existence of the three promoters *traJp*, *traKp* and *traGp* in the Tra1 region. We confirmed the transcription start point for *traJp* and *traGp* as described by Greener *et al.* (1992), but for *traJp* we found that there was only a single initiation point. In the case of *traKp* we mapped transcription initiation at the GAA residues downstream of the promoter region predicted by Ziegelin *et al.* (1991).

We have demonstrated that the Tra1 region promoters are not expressed constitutively. *traJp* and *traKp* can be autogenously repressed by their own products: *traJp* can be repressed about fourfold by TraJ, while *traKp* can be repressed about 30-fold by TraK. TraK can also repress *traJp* about 30-fold (Table 5). The assays in which we used mutants (Table 4) suggest that while *traK* is the only repressor of *traKp*, full repression of *traJp* requires additional genes since we still observed fourfold repression after inactivation of *traK*. The small amount of derepression observed with the pMS2260 mutant with an insertion in *traH/I* suggests that these gene(s) may contribute to this residual activity. However, the results obtained with plasmids expressing individual genes suggest that it is TraJ which is the second repressor of *traJp*. None of the relaxosome proteins affects *traGp*.

The strongest regulatory effect is caused by the TraK protein which is essential for transfer. Its exact role in transfer is not known, but it increases the yield of relaxed intermediates as a result of relaxosome formation (Fürste *et al.*, 1989). It binds cooperatively to DNA in the *oriT* region covering about 200 bp (Fig. 2), in which protected and unprotected stretches can be found, and dramatically

increases bending of this region. Binding to TraK reduces the apparent length of the *oriT* fragment in the electron microscope, suggesting that DNA is wound around the protein core of several TraK molecules forming a highly ordered nucleoprotein structure. TraK binding to *oriT* protects the divergent *traJ* and *traK* promoter sequences from DNaseI cleavage and hydroxyl radical attack (Ziegelin *et al.*, 1992). Thus, the repression caused by TraK protein is probably the result of exclusion of RNA polymerase from these promoters.

TraJ binding to the right arm of an imperfect 19 bp inverted repeat sequence in the *oriT* region has been proposed to be the first step in relaxosome formation (Ziegelin *et al.*, 1989). The TraJ–DNA complex is recognized by TraI that nicks the strand to be transferred (Pansegrau *et al.*, 1990). However, TraI itself can cleave specifically in short oligonucleotides spanning *oriT* (Pansegrau *et al.*, 1993) so the recognition by TraI is not exclusively mediated by the TraJ–*oriT* complex. Since the binding sites for both TraJ and TraI are located downstream from the transcription start point either protein alone or the complex between the two proteins may prevent RNA polymerase from entering elongation mode after initiation at *traJp*.

The binding sites for both TraJ and TraK are only found in the *oriT* region. It was therefore not surprising that they do not affect the activity of *traGp*, when it is cloned on its own. However, because no transcriptional terminator was found between *traJp* and *traGp* it is conceivable that transcription from both of these promoters normally contributes to expression of *traG* and downstream genes. Ziegelin *et al.* (1991) observed very low levels of proteins encoded by the genes transcribed from *traJp* and *traKp* in cells harbouring these operons in their native configuration. They proposed that this was the result of a very low activity of both promoters. Our data extend this proposal. We showed that not only are

traJp and *traKp* about one-tenth the strength of the *trfA* and *trbB* promoters, but in addition they are autoregulated, explaining the very low level of gene expression observed. Ziegelin *et al.* (1991) also observed that products of the genes downstream of *traGp* are present in much higher amounts. However, our results show that *traGp* is about 10 times weaker than the *oriT* promoters which is not consistent with it alone being responsible for the easily visible levels of protein. Transcription from *traJp* may augment *traGp* transcription while a mechanism which stabilizes the mRNA for *traG* and downstream genes could help to boost protein levels from scarce mRNA. Further studies may resolve this point.

All the three promoters studied in this paper were repressed by TrbA, which we showed previously to repress both *trbBp* and *trfAp* (Jagura-Burdzy *et al.*, 1992). The repression by TrbA protein is not very strong (about 7–10-fold) in comparison to repression caused by TraK (30-fold) (Tables 5 and 6). It would be interesting to determine whether it has any effect on *traJ* and *traK* promoters, when the relaxosome proteins are present.

Our primer extension studies confirmed that the observed repression of our transcriptional reporter genes alters the mRNA level initiated at each promoter. This, together with the presence in the promoter regions of binding sites for the repressors TraK (Ziegelin *et al.*, 1992), TraJ (Pansegrau *et al.*, 1990) and TrbA (M. Zatyka, unpublished) is consistent with the regulation acting at the level of transcription initiation. In Fig. 4 we summarize our current knowledge about regulation of genes in the Tra1 and Tra2 regions in the context of the global regulation by KorA and KorB, the products of the central control operon. KorB was shown to repress *trbBp* (Shingler & Thomas, 1984a; Motallebi-Veshareh *et al.*, 1992; Thomson *et al.*, 1993). KorA derepresses *trbAp* activity (Jagura-Burdzy & Thomas, 1994). The *trbA* product seems to be not only a repressor of *trfAp* and *trpBp* (Jagura-Burdzy *et al.*, 1992) but also of *traJp*, *traKp* and *traGp* in the Tra1 region, and in this way possibly coordinates Tra1 and Tra2 expression. Furthermore, *traJp* and *traKp* are subject to repression by local circuits – TraK represses both *traJp* and *traKp*, while *traJp* seems to be repressed autogenously by its own product.

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