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Transcriptional and Translational Control of the Genes for the Mating Pair Formation Apparatus of Promiscuous IncP Plasmids

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The *trb* operon of broad-host-range plasmid RK2 encodes most of the genes required for formation of mating-pair apparatus and is thus essential for the promiscuous spread of this plasmid. Only two promoters, lying upstream of *trbA* and *trbB*, have been identified for this operon. *trbB* encodes a protein belonging to a large family of proteins which function in the assembly of apparatuses associated with the cell surface. *trbA* encodes a repressor protein, one of whose targets is the *trbB* promoter. *trbAp* is arranged as a face-to-face divergent promoter with *trfAp*, the strongest of the three promoters in this region. *trfAp* completely inhibits *trbAp* unless it is repressed by the KorA protein, a key regulator encoded in the plasmid's central control operon. We show that when *trfAp* is firing constitutively, it also appears to interfere with *trbBp*, but that *trbBp* activity increases when *trfAp* activity is decreased by repression or mutation. A second global regulator encoded in the central control operon, KorB, represses *trbBp*, *trfAp*, and *trbAp*. The results presented here show that both KorB and TrbA are necessary for full repression of *trbBp*. The region between *trbA* and *trbB* encodes a large inverted repeat which has been proposed to modulate translation of *trbB* on transcripts which are initiated at *trbAp* but not *trbBp*. Using translational fusions to *lacZ*, we show that translation of *trbB* is completely blocked when transcripts incorporate the inverted repeat upstream of *trbB* but proceeds with reasonable efficiency when deletions remove the sequences predicted to sequester the ribosome binding site. Results from both transcriptional fusion and direct measurement of transcript size and intensity by Northern blot analysis show that most *trbA* transcripts are monocistronic and serve to express only *trbA*, although some transcription continues into *trbB*. The monocistronic *trbA* transcript appears to be the result of transcription termination downstream of *trbA*. Thus, *trbAp* and *trbA* appear to form an operon distinct from the *trbB-trbP* operon. Consequently, *trbA* and the switch that controls its expression help to provide the sequential steps which allow efficient expression of transfer genes during plasmid establishment but tight repression once the plasmid is established.

IncP plasmids have been studied widely because of their ability to transfer between and stably maintain themselves in almost all gram-negative bacterial species (7, 30). They can also promote conjugative transfer of DNA from gram-negative bacteria to gram-positive bacteria as well as to yeast (10, 28, 33, 35). The majority of the genes needed for the surface apparatus responsible for promoting formation of the mating bridges, across which DNA is transferred, are encoded in a single operon (*trb* operon) (8, 17, 18). The genes in this operon are clearly derived from a common block of genes whose different descendants are found in the transfer genes of other plasmids as well as in the virulence functions of the Ti plasmid, responsible for bacterium-plant DNA transfer (24). They also bear a close relationship with less functionally analogous systems such as the *Bordetella pertussis* toxin export system (15, 34). The product of a key gene in this operon, *trbB*, belongs to an even wider family of proteins which are essential to allow assembly of bacterial surface/membrane-associated structures such as pili and transport systems (22). It is conceivable that TrbB acts as some sort of chaperone in this process.

Expression of the genes of the *trb* operon is important for RK2 transfer proficiency, but uncontrolled expression would be a metabolic burden which could limit plasmid survival. We are currently trying to establish how IncP plasmids, which are extremely transfer proficient, strike a balance which achieves transfer proficiency with minimum cost to the host. It has previously been established that there are two promoters upstream of *trbB* (12, 18, 27, 32). One of these (*trbBp*) lies immediately upstream of *trbB*, while the other precedes *trbA*, the first open reading frame in the operon (Fig. 1 and 2). A third promoter in this region, *trfAp*, fires in the opposite direction (25, 27, 29), is very strong, and normally silences *trbAp* when *trfAp* is firing at full strength (13). Thus, *trbAp* is active only when *trfAp* is repressed by KorA. The *trbBp* is thought to be strong but is subject to repression by both KorB (27, 32) and TrbA (12). Preceding *trbB* is a large inverted repeat which if present in the mRNA might regulate translation. mRNA from *trbAp* would include this structure, while mRNA from *trbBp* should not (22). In this report, we focus on the balance between transcription from *trbAp* and *trbBp*, the possible translational control on *trbB* transcripts from *trbAp*, and the repression of *trbBp* by KorB and TrbA.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* K-12 strains used were C600K (*thr-1 leu-6 thi-1 lacY1 supE44 tonA21 galK*) (20) and TG1 [(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ*Δ*M15*]. Plasmids used are listed in Table 1. Bacteria were grown in L broth (16). Solid medium was obtained by addition of 1.5% (wt/vol) agar. Cultures were grown with shaking at 37°C. Antibiotic resistance was selected by addition of penicillin G (sodium salt at 100 μg/ml in liquid

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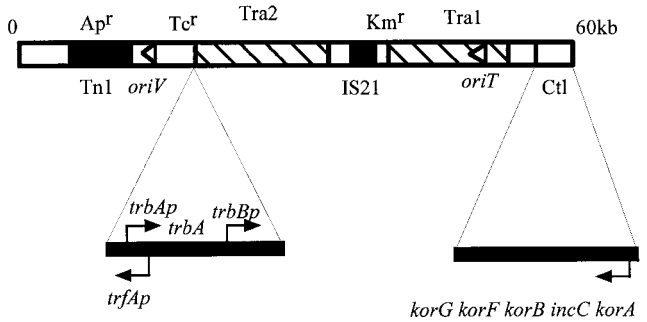

FIG. 1. Linear map of plasmid RK2 showing the main features of its 60-kb genome: transposable elements, Tn*1* and IS*21*; antibiotic resistance markers as indicated in Materials and Methods; origin of vegetative replication, *oriV*; and two blocks of transfer genes, Tra1 and Tra2, including origin of transfer *oriT*. Enlarged are the *trfAp-trbAp-trbBp* region, the *trbA* gene, and the central control operon, Ctl. Arrows by *trfAp*, *trbAp*, and *trbBp* indicate transcription start points and direction of transcription.

medium and 300 μg/ml in solid medium), kanamycin sulfate (50 μg/ml), and streptomycin sulfate (30 μg/ml). For selection for *lacZ* translational fusions and *galK* transcriptional fusions, MacConkey agar supplemented with lactose and galactose, respectively, was used. To propagate M13, TG1 cells were grown in 2TY medium (26) and plated in top agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-1-thiogalactopyranoside (IPTG) at standard concentrations.

Isolation of DNA, genetic manipulation, and sequencing. Plasmid DNA was prepared on both small and large scales by the method of Birnboim and Doly (2) and if necessary by CsCl-ethidium bromide equilibrium density gradient centrifugation. Some of the plasmid samples were prepared according to the Promega Wizard Minipreps protocol. DNA manipulations were carried out by standard techniques (26), using enzymes according to the manufacturers' instructions. Sequencing was performed automatically (by AltaBioscience, School of Biochemistry, University of Birmingham) on an Applied Biosystems 373A DNA sequencer, using a dye terminator kit supplied by the manufacturer.

Cloning of promoter fragments. The fragment cloned in pGBT61 was amplified by PCR on the template pMMV115 with primers p2 and p10 (Fig. 2; RK2 coordinates 18007 to 18915) and cloned into the *Bam*HI site of the promoter probe plasmid pGBT43, creating a *trfAp-xylE* transcriptional fusion. This plasmid was used as a source of RK2 DNA from this region. Plasmid pGBT73 was prepared by cloning a PCR fragment, amplified between primers p2 and p10 (RK2 coordinates 18007 to 18915 [Fig. 2]), on the template pVI124, which carries the *trfAp-1* mutation (29), and cloned into the *Bam*HI site of pGBT43 in the opposite orientation, so that *trbAp* and *trbBp* are firing into *xylE*.

pMZT11 was obtained by subcloning the *Bam*HI fragment from pGBT61 into pDM3 (a promoter probe plasmid derived from and almost identical to pGBT43) in the orientation such that *trbAp* and *trbBp* are firing into *xylE*. PCR amplification of the fragment containing *trfAp trbAp-1* and *trbBp*, later cloned as pMZT13, was carried out with primers p1 and p10 on the template pGBT61 (Fig. 2). This fragment was cloned as a *Bam*HI fragment in pDM3 in the same orientation as pMZT11. The fragment cloned in pMZT14 was amplified between p4 and p10 (RK2 coordinates 18396 to 18915) on pMZT16 template and cloned into the *Bam*HI site, creating a *trbBp-1-xylE* fusion.

PCR. Reactions were performed by standard procedures (23), with parameters as follows: 5 min of denaturation at 96°C followed by 25 rounds of temperature cycling (96°C for 15 s, 55°C for 30 s, and 72°C for 90 s) and a final 5-min step at 72°C. Routinely 10 pmol of each primer was used with 50 ng of template and 1 U of *Taq* polymerase in 50 μl of reaction buffer as recommended by the manufacturer (Northumbria Biologicals Ltd.). Amplified fragments were purified by electroelution, digested with appropriate restriction enzyme, and cloned as described above. The identity of the inserted fragment was confirmed by DNA sequencing. Most of the primers used are shown in Fig. 2.

Site-directed mutagenesis. Site-directed mutagenesis was performed according to the Sculptor in vitro mutagenesis system (Amersham Life Sciences) protocol. The 0.9-kb *Bam*HI fragment was subcloned from plasmid pGBT61 into M13 mp8 RF, creating plasmid pMZT15. The orientation of the insert was established by *Eco*RI digestion, and the insert was sequenced with a universal primer. Single-stranded DNA preparation of recombinant pMZT15, as well as all M13 handling, was done as specified by Sambrook et al. (26). Mutated oligonucleotide p9 was designed as a 20-mer carrying two changes in the *trbBp* −10 sequence. This mutation introduced a *Kpn*I site at the same time. Two modifications were introduced to the Amersham protocol: ligase was added after the extension reaction, and the ligation time was prolonged to overnight at 4°C; and the amount of T5 exonuclease was reduced from 2,000 to 800 U (19a). The mutated clone obtained, pMZT16, was sequenced before being used for further constructions.

Construction of the vector for translational fusions. The vector for cloning translational fusions was derived from pMC1403, a prototype β-galactosidase hybrid protein gene fusion vector (3). As we wanted to be able to clone fragments containing promoters firing in both directions, we prepared the vector with a strong transcriptional terminator inserted between the cloning site and plasmid replication origin. We first inserted a *Hin*dIII linker into the *Eco*RI site in pMC1403 (an *Eco*RI site was regenerated at both sides of the *Hin*dIII). The *Hin*dIII-to-*Sal*I fragment from pMC1403 carrying *Hin*dIII, *Eco*RI, *Sma*I, and *Bam*HI was ligated into the *Hin*dIII-*Sal*I fragment of pAPG3Δ7 (5) carrying the dual rRNA, strong transcriptional terminators rrnBT1 and -T2, penicillin resistance cassette, and *ori* of pBR322. This plasmid was called pMZT9. To allow the study of promoterless fragments, we inserted *tacp* on a *Hin*dIII-*Eco*RI fragment from pGBT30 (11) and a *Hin*dIII fragment carrying *lacI*^{q} of the same origin, creating pMZT10 (Fig. 3).

Construction of translational fusions. Fragments cloned as translational fusions pMZT17, pMZT18, and pMZT19, containing the whole or part of the inverted repeat and *trbBp-1*, were amplified between primers p6 and p10, p7 and p10, and p8 and p10, respectively, on the template pMZT12. These fragments were cloned between the *Eco*RI and *Bam*HI sites of pMZT10.

To bring the *trbB* start codon in frame with *lacZ*, we cut the plasmids at the single *Bam*HI site, filled it with deoxynucleoside triphosphates and DNA polymerase Klenow fragment, and religated. We initially screened for red colonies on MacConkey medium to obtain in-frame fusions, but many were false clones, carrying insertions or deletions. We therefore also screened them for the presence of a *Cla*I site which should appear only if the fusion was connected properly. The *Cla*I site overlaps a GATC sequence which is a substrate for the Dam methylase and thus blocks the *Cla*I site in the host strain, TG1, used for cloning. Therefore, we used PCR to amplify the junction region between LACZ2 (a primer complementary to *lacZ* sequence located about 100 nucleotides away from junction) and an adjacent primer (p6, p7, or p8) which was then tested for cutting by *Cla*I. Positive clones were sequenced.

Construction of a plasmid overexpressing TrbA. TrbA open reading frame was amplified by PCR with primers p3 and p5 on pGBT61 as a template. The fragment was purified and cloned into *Eco*RI/*Sal*I sites on pGBT30 (11). TrbA was expressed from *tacp* induced with IPTG. TrbA overexpression was checked on overnight cultures by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a Phast system (Pharmacia).

Construction of plasmids for Northern blot analysis. To detect transcripts from *trbAp* and *trbBp*, the *Eco*RI-*Bam*HI fragment with these promoters was subcloned from pMZT11 into pMZT9. Linker carrying the sequence of the *trp* transcriptional terminator was then inserted between *Bam*HI and *Cla*I sites. The linker had an additional *Hin*dIII site to facilitate screening for the right clones. The sequences of oligonucleotides for linker construction were 5′GATCCAAG CTTAGCCCGCCTAATGAGCGGGCTTTTTTTTAT3′ and 5′CGATAAAAA AAAGCCCGCTCATTAGGCGGGCTAAGCTTG3′.

To detect transcripts from *trbAp* and *trbBp-1* promoters, the *Eco*RI-*Bam*HI fragment was subcloned from pMZT12 onto pMZT9 and the *trp* transcriptional terminator was inserted between *Cla*I and *Sal*I sites. The linker also carried additional *Hin*dIII site to facilitate screening. The sequences of oligonucleotides were 5′TCGACAAAAAAAAGCCCGCTCATTAGGCGGGCTAAGCTTA T3′ and 5′CGATAAGCTTAGCCCGCCTAATGAGCGGGCTTTTTTTTG3′.

Probes for Northern blotting. The *Eco*RI-*Sal*I fragment from pMZT24 carrying the *trbA* open reading frame was used as a probe specific for detection of transcript from *trbAp*. The *Eco*RI-*Bam*HI fragment from pMZT17 carrying the inverted repeat and beginning of *trbB* was used to detect transcripts reaching *trbB* region. The probes were labeled by the random priming method with [α-^{32}P]dCTP (4).

RNA isolation. RNA was isolated from logarithmic-phase cultures of *E. coli* TG1. Pellets from 10-ml cultures were suspended in 200 μl of TKM buffer (10 mM Tris [pH 7.4], 10 mM KCl, 5 mM $MgCl_2$) and quickly frozen in liquid nitrogen until all of the samples were ready. Then 14 μl of 10% (wt/vol) SDS, 7 μl of 3 M sodium acetate (pH 5.0), and 250 μl of acidic phenol were added to each sample, and the samples were vortexed. The samples were incubated for 10 min at 65°C and spun down. Phenol extraction was repeated once more and followed by chloroform extraction. RNA was precipitated with 2.5 volumes of ethanol after addition of 0.1 volume of 3 M sodium acetate.

Northern blot analysis of RNA. RNA was separated on a 1.5% formaldehyde agarose gel (26). The same amount of RNA was loaded in each lane. Boehringer Mannheim RNA molecular weight markers (0.3 to 7.4 kb) were used to establish the sizes of the RNA bands. Transfer to filter, hybridization, and washing were done as described elsewhere (9). To correct the results of Northern blotting for differences in plasmid copy number, plasmid DNA was prepared from each culture used for RNA isolation. After appropriate restriction digestion, DNA was separated on an agarose gel, and band intensities were measured densitometrically.

Insertion of *trbA-trbB* intergenic region between *tacp* and the *galK* reporter gene. Plasmid pMZT29 was constructed by inserting a *Bam*HI fragment from pMZT14 (*trbBp-1*) upstream of *galK* in pKO4. Then *tacp* on a *Hin*dIII fragment was subcloned from pGBT30 upstream of the intergenic *Bam*HI fragment, creating pMZT30. pMZT31 has *tacp* inserted directly upstream of *galK* of pKO4. Plasmids pMZT22 and pMZT23 were constructed by inserting upstream of *galK* in pKO4 *Hin*dIII-*Bam*HI fragments carrying *tacp* and relevant intergenic regions

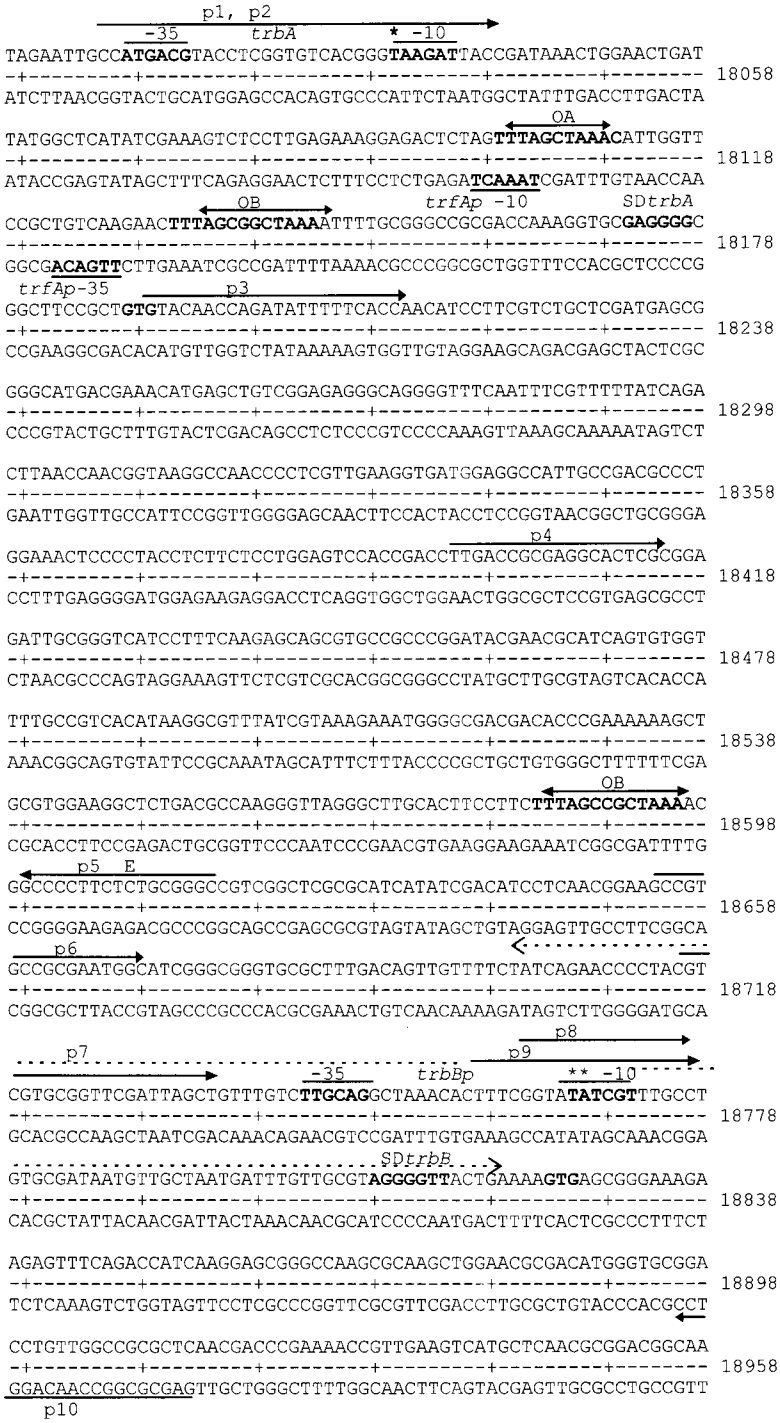

FIG. 2. Sequence of the *trfAp-trbAp-trbBp* region. Coordinates refer to the complete sequence of RK2, which is available as GenBank accession no. LZ7758 (24). −35 and −10 regions of relevant promoters are indicated. OA, KorA operator; OB, KorB operator; SD, Shine-Dalgarno sequence; E, end of the *trbA* gene. Primers p1 and p9 were used to introduce mutations into −10 regions of *trbAp* (T→C) and *trbBp* (TA→CC); asterisks mark the sites of mutations. Primers p2, p3, p4, p5, p6, p7, p8, and p10 were used for PCR amplification of the relevant fragments. Primers p1 and p2 have the same sequence except that p2 does not introduce a mutation. The dashed line shows the inverted repeat, which we propose forms a stem-loop that inhibits translation of *trbB* on transcripts from *trbAp*.

equivalent to those present in pMZT17 and pMZT19. The *Hin*dIII-*Bam*HI fragments were removed from precursors of pMZT17 and pMZT19 before the *Bam*HI site was filled in with Klenow enzyme to bring the translational fusions in frame.

Measurement of reporter gene activity. The assays described below were performed on exponentially growing bacteria. Plasmid DNA was extracted from a normalized density of bacteria to check the identities and relative copy numbers of plasmids. Any significant variations in copy number were compensated for in determining gene activity. Protein concentration was determined by the biuret method (6).

The level of *xylE* expression was determined by enzymatic assay of catechol 2,3-oxygenase activity in exponentially growing bacteria (37). One unit is defined

TABLE 1. Plasmids used in this study

Plasmid	Size (kb)	Replicon	Selective marker(s)	Description[a]	References
R300B	8.9	IncQ	Sm, Su	No RK2 sequence	1
pAPG3Δ7	4.5	pMB1	Pn	Source of transcriptional terminator	5
pCT409	9.6	IncQ	Sm	RK2 coordinates 58.7–59.6 *korA*	31
pCT415	17.0	IncQ	Sm	RK2 coordinates 55.0–60.0 *korA korB*	31
pDM1.1	11.5	IncQ	Sm	Expression vector	19
pDM1.2	14.0	IncQ	Sm, Tet	Expression vector	19
pDM3	10.1	pSC101	Km	*xylE*pp, derivative of pGBT43	19
pGBT30	6.3	pMB1	Pn	Expression vector	11
pGBT43	10.1	pSC101	Km	*xylE*pp	12
pGBT61	11.0	pSC101	Km	*trfAp-xylE*	This study
pGBT63	10.6	pSC101	Km	*trbBp-xylE*	12
pGBT73	11.0	pSC101	Km	*trfAp-1 trbAp trbBp-xylE*[b] (18007–18915)	This study
pK04	4.0	pMB1	Pn	*galK*pp	20
pMC1403	9.9	pMB1	Pn	β-Galactosidase gene fusion vector	3
pVI124	10.1	p15A	Cm, Tet	*trfA-trbB*	27
pMMV115	18.4	pMB1	Pn	*trfA-trbB*	12
pMZT2	6.4	pMB1	Pn	*traKp-xylE*	36
pMZT7	9.9	pMB1	Pn	pMC1403 with *Hin*dIII inserted into *Eco*RI	This study
pMZT9	10.1	pMB1	Pn	Vector for gene fusions	This study
pMZT10	11.5	pMB1	Pn	Vector for gene fusions	This study
pMZT11	11.0	pSC101	Km	*trfAp trbAp trbBp-xylE*[b] (18007–18915)	This study
pMZT12	11.0	pSC101	Km	*trfAp trbAp trbBp1-xylE*[b]	This study
pMZT13	11.0	pSC101	Km	*trfAp trbAp-1 trbBp-xylE*[b]	This study
pMZT14	10.6	pSC101	Km	*trbBp-1-xylE*	This study
pMZT15	8.0	M13 mp8 RF		Substrate for site-directed mutagenesis	This study
pMZT16	8.0	M13 mp8 RF		Product of site-directed mutagenesis	This study
pMZT17	11.7	pMB1	Pn	*tacp* (18653–18915) *trbB 'lacZ*	This study
pMZT18	11.7	pMB1	Pn	*tacp* (18715–18915) *trbB 'lacZ*	This study
pMZT19	11.6	pMB1	Pn	*tacp* (18761–18915) *trbB 'lacZ*	This study
pMZT20	13.8	pMB1	Pn	*xylE* inserted into *Eco*RI of pMZT17	This study
pMZT21	11.0	pMB1	Pn	*trbAp trbBp* (18007–18915) *trbB' lacZ*	This study
pMZT22	4.2	pMB1	Pn	*tacp* (18653–18915)-*galK*	This study
pMZT23	4.2	pMB1	Pn	*tacp* (18761–18915)-*galK*	This study
pMZT24	6.5	pMB1	Pn	*trbA* overexpressed from *tacp*	This study
pMZT29	4.5	pMB1	Pn	pKO4 with *trbBp-1* fragment from pMZT14	This study
pMZT30	4.8	pMB1	Pn	pMZT29 and *tacp*	This study
pMZT31	4.3	pMB1	Pn	pKO4 and *tacp*	This study
pMZT35	11.0	pMB1	Pn	pMZT9 and *trfAp*, *trbAp*, *trbBp*, and *trp* terminator	This study
pMZT36	11.0	pMB1	Pn	pMZT9 and *trfAp*, *trbAp*, *trbBp-1*, and *trp* terminator	This study

[a] Numbers in parentheses are coordinates of the inverted repeat upstream of *trbB* as presented in Fig. 2. *xylE*pp and *galK*pp are promoter probes.
[b] Both *trbAp* and *trbBp* can potentially cause transcription across *xylE*.

as the amount of enzyme necessary to convert 1 mmol of substrate to product in 1 min under standard conditions.

GalK assays were done on exponentially growing cells as described by McKenney et al. (20), with the only modification that samples of reaction mixture were not filtered but only placed on DE81 filters. This was followed by three washings in 1 liter of water, and then filters were dried and placed in a phosphorimager cassette for at least one night. The storage phosphor screen was scanned, and the results were quantified with the program IMAGEQUANT. Galactokinase units represent nanomoles of galactose phosphorylated per minute per milliliter of cells.

β-Galactosidase activity was assayed on logarithmically growing bacteria by the method of Miller (21). The cell membranes were disrupted by adding 2 drops of chloroform and 1 drop of 0.1% SDS, instead of toluene, to each 1 ml of reaction mix. Activity was calculated in Miller units.

RESULTS

Reporter plasmids to monitor transcription of *trbB*. To study transcription into the *trbB* gene, we inserted into a *xylE* promoter probe plasmid both *trbBp* alone, giving pGBT63, and *trbBp* in the presence of *trfAp* and *trbAp*, giving pMZT11 (see Materials and Methods). Comparison of XylE activities from the two plasmids (Table 2) showed that in the absence of *korA* and *korB*, transcription into *trbB* is reduced sixfold when all three promoters are present. Since the constitutive activity of *trbBp* is much greater than that of *trbAp*, this finding suggested that something in the *trfAp-trbAp* region was inhibiting *trbBp*. Since the very strong *trfAp* negatively affects *trbAp*, the most obvious possibility was that it also affects *trbBp*. To test this hypothesis, we constructed pGBT73 (see Materials and Methods), which is identical to pMZT11 but has the *trfAp-1* (27) mutation, which reduces *trfAp* strength (29) and thus derepresses *trbAp* (13). As predicted, transcription into *trbB* increased (Table 2) when the wild-type *trfAp* was replaced by the *trfAp-1*.

The other major difference between pGBT63 and pMZT11 that could clearly influence the activity of *trbBp* is the presence of the *trbA* gene, whose product is known to repress *trbBp* (12). It is hard to see how this would cause the increase in *trbBp* activity observed between pMZT11 and pGBT73, because the *trfAp-1* mutation should increase *trbA* transcription and thus repress rather than activate *trbBp*. However, to rule out this possibility, we used PCR amplification with an altered primer to incorporate the *trbAp-1* mutation into the long fragment with *trfAp*, *trbAp*, and *trbBp* to give pMZT13. This mutation abolishes *trbAp* activity by changing its −10 sequence from TAAGAT to CAAGAT (13), and so there should be very little *trbA* transcription, irrespective of the activity of the *trfA* promoter. We then decreased *trfAp* activity in pMZT13 by placing

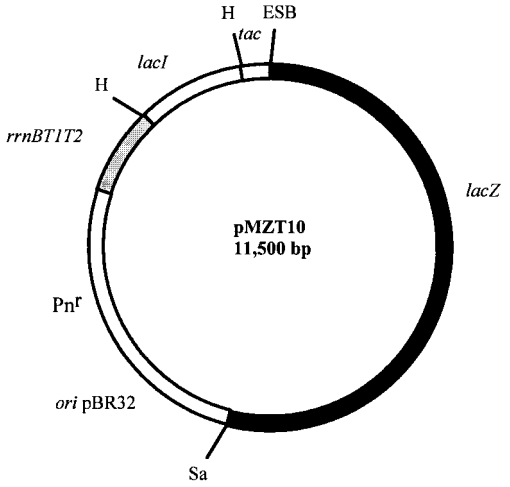

FIG. 3. Vector pMZT10 for cloning translational fusions. Restriction sites: E, *Eco*RI; S, *Sma*I; Sa, *Sal*I; B, *Bam*HI; H, *Hin*dIII. Construction of this plasmid is described in Materials and Methods. Black sections indicate the *lacZ* gene from pMC1403; grey sections indicate rRNA gene terminators from pAPG3Δ7.

in *trans korA* (supplied by pCT409). As a control, we placed in *trans* R300B, which has the same replicon (IncQ) and selectable marker as pCT409, but no *korA*. Comparison of these two strains showed a threefold increase in transcription into *trbB* in the presence of *korA*. Thus, *trbBp* activity was restored to 63% of the level observed for *trbBp* on its own (pGBT63) in the presence of R300B. This increase must come from *trbBp*, because *trbAp* is inactivated, and must be caused by the change in *trfAp* activity, since *korA* does not cause activation of *trbBp* when this promoter is on its own in reporter plasmid pGBT63 (Table 2). This observation suggests that *trfAp* dominates over both of the *trb* promoters and that this dominance is reduced or eliminated when *trfAp* is mutated or repressed by *korA*.

Mutational inactivation of the *trbB* promoter. To assess the contribution of *trbAp* to transcription into *trbB*, it was essential to obtain derivatives in which *trbBp* was inactivated. To do this, we used site-directed mutagenesis to change the −10 region of the putative promoter (32) from TATCGT to CCTCGT (*trbBp-1*). This mutation introduced a *Kpn*I site to facilitate the screening for the mutant genotype. Site-directed mutagenesis was done on a fragment containing *trbAp*, *trfAp*, and *trbBp* which was subcloned from pGBT61 (see Materials and Methods) to M13 mp8 (pMZT15). The clone obtained (pMZT16) with the *trbBp* mutation was checked by sequencing and then used for further constructions. To determine how much the *trbBp-1* mutation decreased *trbBp* activity, a fragment containing only *trbBp-1* was amplified on the pMZT16 template. The primers used were the same as those used to generate the fragment in pGBT63 (Fig. 2 and Materials and Methods). The fragment was inserted into pDM3, a *xylE* promoter probe vector which is a derivative of pGBT43, yielding pMZT14. The activities of pGBT63 and pMZT14 were then compared. This comparison showed that the mutation results in a 33-fold reduction in promoter activity (0.065 XylE units, compared to 2.13 for wild-type [wt] *trbBp* [Table 2]). A second mutation in *trbBp* (*trbBp-2*) which changes the −35 sequence from TTG CAG to TTGCGG arose spontaneously but was found not to alter *trbBp* activity significantly. We chose to use *trbBp-1* in further studies.

TABLE 2. Influence of *trfAp-trbAp* region on activity of *trbBp*

Reporter plasmid (promoter[s] present)	*korA* in R300B/pCT409	XylE activity (U)[a]
pGBT63 (*trbBp*)	−	2.13 ± 0.24
	+	2.47 ± 0.12
pMZT11 (*trbBp trfAp trbAp*)	−	0.34 ± 0.08
pGBT73 (*trbBp trfAp-1 trbAp*)	−	2.31 ± 0.11
pMZT13 (*trbBp trfAp trbAp-1*)	−	0.47 ± 0.04
	+	1.35 ± 0.23
pMZT14 (*trbBp-1*)	−	0.065 ± 0.007

[a] Results from three experiments.

TABLE 3. Effects of *korB* and *trbA* on activity of *trbBp* and *trbBp-1*

Reporter plasmid	Regulatory plasmid 1	Regulatory plasmid 2	XylE activity (U)[a]
pGBT63	R300B (−[b])	pGBT30 (−)	0.71 ± 0.056
(*trbBp-xylE*)	R300B (−)	pMZT24 (*trbA*)	0.073 ± 0.019
	pCT415 (*korA korB*)	pGBT30 (−)	0.26 ± 0.042
	pCT415 (*korA korB*)	pMZT24 (*trbA*)	0.066 ± 0.022
pMZT14	R300B (−)	pGBT30 (−)	0.02 ± 0.0014
(*trbBp-1-xylE*)	R300B (−)	pMZT24 (*trbA*)	0.019 ± 0.0006
	pCT415 (*korA korB*)	pGBT30 (−)	0.007 ± 0.0016
	pCT415 (*korA korB*)	pMZT24 (*trbA*)	0.005 ± 0.0017

[a] Results from three experiments.
[b] −, no repressor.

Both TrbA and KorB are necessary for full repression of the *trbB* promoter. Since *trbBp* in the absence of repressors is a much stronger promoter than *trbAp*, a direct role for transcription from *trbAp* in expression of *trbB* would depend on tight repression of *trbBp*. Table 3 shows that when *korB* and *trbA* are provided by separate expression plasmids in *trans*, *trbA* has a stronger effect on *trbBp* (pGBT63) than *korB* (10-fold versus approximately 3-fold). This may be because the level of TrbA, produced from pMZT24 (*tacp-trbA*), is abnormally high. The *trbBp-1* mutation appeared to alter the regulatory characteristics of *trbBp*: *trbA* had virtually no effect, while *korB* still appeared to cause the normal amount of repression.

When we measured the repression of transcription into *trbB* in the context of the three-promoter fragment by placing *korA* and *korB* in *trans*, *trbA* would be expressed in *cis* (Table 4). The contribution of *trbA* was assessed by comparing the wt fragment (pMZT11), which should give normal expression of TrbA, with the fragment carrying the *trbAp-1* mutation (pMZT13), which should give no expression of *trbA*. When just *korA* was present (on pCT409), inactivation of *trbAp* had very little effect on transcription into *trbB* (1.29 U for pMZT11, compared to 1.35 U for pMZT13—a 5% rise [Table 4]). However, in the presence of *korA* and *korB* (pCT415), the *trbAp-1* mutant had a much bigger effect on the level of repression, causing a 14-fold rise in activity (0.03 U for pMZT11, compared to 0.41 U for pMZT13 [Table 4]). This finding clearly

TABLE 4. Comparison of transcription into *trbB* when *trbAp* or *trbBp* is inactivated

xylE reporter	Genotype	XylE activity (U) in the presence of different repressors[a]		
		R300B (no repressor)	pCT409 (*korA*)	pCT415 (*korA korB*)
pMZT11	*trfAp trbAp trbBp*	0.34 ± 0.08	1.29 ± 0.23	0.03 ± 0.005
pMZT12	*trfAp trbAp trbBp-1*	0.021 ± 0.001	0.073 ± 0.016	0.025 ± 0.002
pMZT13	*trfAp trbAp-1 trbBp*	0.47 ± 0.04	1.35 ± 0.23	0.41 ± 0.045

[a] Results from three experiments.

indicates that when *trbA* is provided in *cis* under the control of its normal promoter, both KorB and TrbA play a role in maintaining the normal low activity of *trbBp*.

Analysis of the contribution of *trbAp* and *trbBp* to the transcription of *trbB*. To assess the contribution that *trbAp* makes to transcription of *trbB* when *trbBp* is repressed, we used plasmids which would allow us to determine how much transcription still occurs after inactivation of *trbBp*. The wild-type 0.9-kb fragment containing *trfAp*, *trbAp*, and *trbBp* is present in pMZT11, giving transcription into *xylE* from *trbB*. The equivalent construction but with the *trbBp-1* mutation was cloned from pMZT16 into the *xylE* promoter probe plasmid to give pMZT12. By comparison of transcriptional activities into *xylE* in pMZT11 and pMZT12 in the presence and absence of different repressors and activators of the system (provided by pCT415 and pCT409, respectively), we tried to deduce the contribution of *trbAp* and *trbBp* to transcription into *trbB*.

In the absence of central control operon repressors (*korA* and *korB*) and thus in the presence of a low, uninduced level of TrbA, transcription from the mutated fragment is approximately 17-fold less than for pMZT11 with wt *trbBp* (compare results for pMZT12/R300B and pMZT11/R300B in Table 4). The remaining transcriptional activity (approximately 6%) after inactivation of *trbBp* should be due to both the residual activity of *trbBp* and the uninduced level of *trbAp*. Addition of pCT409 (*korA* alone) gave an approximately 3.5-fold increase in transcription for both pMZT11 and pMZT12, consistent with derepression of both *trbAp* and *trbBp*. To induce what we expect to be the normal steady-state level of transcription from *trbAp*, we placed in *trans* pCT415, an IncQ plasmid with a copy number similar to that of RK2, which carries the central control operon of RK2 including *korA* and *korB*. This will not only activate *trbAp* but also result in repression of *trbBp*. Comparison of pMZT11 (wt *trbAp* and wt *trbBp*) with pMZT12 (wt *trbAp* and *trbBp-1*) in the presence of pCT415 showed that the level of transcription into *trbB* under these conditions is reduced only about 17% by knocking out *trbBp*. This observation suggests that the observed transcription depends to only a small extent on *trbBp*, the remaining transcription coming from *trbAp*. However, since the level of XylE activity is very low and the *trbBp-1* mutant alters sensitivity to TrbA, it is difficult to say exactly how much *trbB* transcription originates at *trbAp* under these conditions, although it may be greater than 50%.

Detection of transcripts from *trbAp* and *trbBp* by Northern blotting. To provide an independent method of assessing the contributions of *trbAp* and *trbBp* to the transcription of *trbB*, a Northern blot analysis was performed. To increase the chance of detecting well-defined transcripts of relatively short length, *Eco*RI-*Bam*HI fragments from pMZT11 (*trbAp trbBp*) and pMZT12 (*trbAp trbBp-1*) were subcloned into pMZT9, and the *trp* transcriptional terminator sequence was inserted downstream, creating pMZT35 (*trbAp trbBp trpt*) or pMZT36 (*trbAp trbBp-1 trpt*). RNA from bacteria with pMZT35 and pMZT36 in the presence of R300B (no repressors), pCT409 (*korA*), and pCT415 (*korA korB*) was analyzed with the probe specific for *trbA* or with the probe which detected any transcript reaching the inverted repeat and *trbB* region. The results obtained after measuring the intensity of transcripts with the program IMAGEQUANT and correcting for the differences in the amount of plasmid DNA present are presented in Fig. 4 and Table 5. We observed that transcription from *trbAp* present on pMZT35 was eightfold greater in the presence of pCT409 than when R300B was present in *trans*. Introduction of pCT415 caused slight repression of *trbA*, but transcription was still fivefold higher than in the presence of R300B. The majority of the *trbA* transcript was about 0.5 kb (species I in Fig. 4a). This

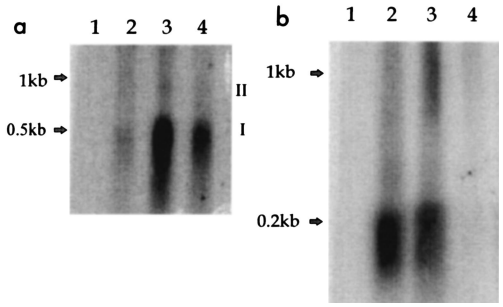

FIG. 4. (a) Northern blot analysis of *trbA* transcript from pMZT35 (*trbAp trbBp*). RNA was extracted and blotted as described in Materials and Methods. The probe was the *Eco*RI-*Sal*I fragment from pMZT24. Lane 1, TG1, no plasmid; lane 2, pMZT35 plus R300B (no repressors); lane 3, pMZT35 plus pCT409 (*korA*); lane 4, pMZT35 plus pCT415 (*korA korB*). Most transcript is about 0.5 kb (species I), which means it either terminates or is processed downstream of the *trbA* gene. A weak band of about 0.9 kb (species II) is visible in lane 3 and was detected in lane 4 (see Results) when *trbAp* was induced, which suggests that it may be a *trbA* transcript which continues through *trbB* and terminates at the *trp* terminator sequence inserted at the end of the cloned fragment. (b) Northern blot analysis of *trbB* transcript from pMZT35 (*trbAp trbBp*). RNA was extracted and blotted as described in Materials and Methods. The probe was the *Eco*RI-*Bam*HI fragment from pMZT17. Lane 1, TG1, no plasmid; lane 2, pMZT35 plus R300B (no repressors); lane 3, pMZT35 plus pCT409 (*korA*); lane 4, pMZT35 plus pCT415 (*korA korB*). The *trbB* transcript is about 0.15 kb.

means that *trbAp* serves primarily to express *trbA*. However, a very weak band of 0.9 kb (species II in Fig. 4a) visible in lane 3 (upon induction by KorA) could be also detected. Although not visible in lane 4, we did detect species II very weakly in the presence of pCT415, using IMAGEQUANT. This finding suggests that a small amount of *trbA* transcript may reach *trbB*. However, this transcript was not detected by the probe specific for the region downstream of *trbA* as clearly as by the *trbA* probe, and so the identity of this transcript remains uncertain. *trbB* transcript detected from pMZT35 is strongly (30-fold) repressed by the presence of pCT415, as expected from promoter probe studies. However, in the presence of pCT409, no increase in transcriptional activity was observed, which was unexpected since in the reporter gene studies, repression of *trfAp* by KorA resulted in derepression of *trbBp* (Table 2). When *trbA* transcript was detected from pMZT36 (*trbA trbBp-1*), it was clearly induced in the presence of pCT409. When we looked at mutated promoter *trbBp-1* in the presence of pCT415, we did not see the strong repression exhibited with the wild type but instead saw only a 2.5-fold decrease.

Analysis of transcriptional termination in the *trbA-trbB* intergenic region. Since the Northern blot analysis indicated that

TABLE 5. Effects of repressors KorA and KorB on levels of transcripts from *trbAp* and *trbBp* (measured from Northern blots with the program IMAGEQUANT)

Plasmid	Repressor(s)	Transcript level[a]	
		trbA	*trbB*
pMZT35 (*trfAp trbAp trbBp*)	None (R300B)	1.0	1.0
	KorA (pCT409)	8.0	0.5
	KorA, KorB (pCT415)	5.0	0.03
pMZT36 (*trfAp trbAp trbBp-1*)	None (R300B)	1.0	1.0
	KorA (pCT409)	8.5	0.6
	KorA, KorB (pCT415)	11.2	0.4

[a] For each plasmid, transcript level in the absence of repressors is set at 1. Levels in the presence of repressors are compared only to levels with no repressors present.

transcripts from *trbAp* stop before they reach *trbB*, we used a reporter system to check how much transcription passes across the region between the *trbA* and *trbB* genes. Starting from reporter plasmid pKO4, we inserted a *Bam*HI fragment from pMZT14 (*trbBp-1*) upstream of *galK* (pMZT29) and then inserted a *Hin*dIII fragment with *tacp* from pGBT30 upstream of this intergenic region so that transcription from *tacp* into *galK* would have to pass across the putative terminator (pMZT30). *tacp* was also inserted directly into pKO4, giving pMZT31. To allow proper regulation of *tacp*, Lac repressor was provided in *trans* from pDM1.21. The results of assaying GalK activity in the presence and absence of IPTG are presented in Table 6. A small amount of transcription comes from the cloned *trbA-trbB* intergenic region, presumably due to residual *trbBp-1* activity (pMZT29). Placement of *tacp* upstream resulted in little change in activity even when IPTG was added (pMZT30). However, when the *trbA-trbB* intergenic region is not present (pMZT31), normal induction of *tacp* was observed. This finding indicates strongly that the region contains a block to transcription. The region consists of two segments: the inverted repeat preceding *trbB* and the region between this and the end of *trbA*. Since transcriptional termination is often associated with inverted repeats, we tested whether this inverted repeat was sufficient for the observed block on transcription coming from *trbA*. We constructed reporter plasmids with just the inverted repeat or half of it between *tacp* and *galK*. For this purpose, we prepared transcriptional fusions pMZT22 and pMZT23. The subcloned fragments consisted of *tacp* and the relevant fragment of the inverted repeat region. Strains were set up as described above, and GalK was assayed in the presence and absence of IPTG. Table 6 shows that *galK* was induced 10- to 20-fold by addition of IPTG with both pMZT22 and pMZT23, indicating that the inverted repeat does not block transcription. The uninduced level of GalK in pMZT22 is approximately twofold higher than in pMZT23. This increase could at least partially be due to the presence of the *trbBp-1* promoter on pMZT22, but as *trbBp-1* is a very weak promoter, we do not think that it would be responsible for the whole difference. It is conceivable that the transcript containing the stem-loop structure (pMZT22) is more stable than the transcript from pMZT23. While this region may influence mRNA stability, it is clearly not sufficient for the transcriptional block that we observe with the whole region. Therefore, the region upstream of the inverted repeat must contain vital information for this activity.

Translation of *trbB* is inhibited on transcripts originating upstream of the inverted repeat preceding *trbB*. Since a small proportion of *trbA* transcript may continue into *trbB*, translational control on such a transcript would determine whether it contributes to the production of TrbB when *trbBp* is repressed by KorB and TrbA. To assess whether the predicted stem-loop around the *trbB* start site inhibits translation of *trbB* on transcripts originating upstream of the inverted repeat region, we created translational fusions to *lacZ* by using pMZT10 as the basic reporter plasmid (Fig. 2 and 3; Materials and Methods). With pMZT12 as a template, since it carries the *trbBp-1* mutation, fragments were amplified by PCR and inserted upstream of *lacZ*. The whole inverted repeat (pMZT17), just a part of the inverted repeat such that the Shine-Dalgarno sequence is unmasked (pMZT18), or only half of the inverted repeat, which gives no possibility of creating any hairpin loop in the transcript (pMZT19), was cloned this way. β-Galactosidase activity was determined in the absence and presence of IPTG to induce *tacp* (Table 7). Our first observation was that after induction of *tacp* by addition of IPTG, we could see only a very small increase in the β-galactosidase activity in plasmid pMZT17, which carries the complete putative stem-loop between *tacp* and *lacZ*. This is consistent with translational control by the stem-loop. However, to exclude the possibility that this effect was caused by mutation of *tacp* in this plasmid, we inserted a *xylE* cassette from the promoter probe plasmid pMZT2 (36) into the *Eco*RI site downstream of *tacp* and upstream of the translational fusion in pMZT17, creating pMZT20. XylE assays in the presence and absence of IPTG showed that *tacp* was induced about 15-fold, from 0.09 to 1.4 U. Therefore, the lack of translation of *lacZ* from pMZT17 was not the result of a loss of *tacp* activity crossing to the region but must have been due to a translational effect of the DNA inserted between *tacp* and *lacZ*. Removal of the sequences complementary to the *trbB* Shine-Dalgarno sequence resulted in ability of *lacZ* to be induced 10-fold (pMZT18). Removal of the half of the inverted repeat, so that no stem-loop structure would be formed (pMZT19), and also the −35 sequence of *trbBp-1* resulted in decrease of uninduced level of β-galactosidase, probably due to inactivation of *trbBp-1*. *lacZ* could now be induced about 100-fold. We think that this effect is due mainly to efficient translation from the unmasked translational signals, but as the level is threefold higher than in pMZT18, it is possible that some effect is due to removal of additional sequences that can modulate efficiency of *trbB* expression in a way that we do not currently understand.

TABLE 6. Transcription into *galK* from *tacp* in the presence and absence of the region between *trbA* and *trbB*

Plasmid	*tacp*	Intergenic region[a]	GalK activity (U)[b]	
			−IPTG	+IPTG
pKO4	−	None	0.3	0.3
pMZT29	−	18396–18914[c]	4.6	4.6
pMZT30	+	18396–18914[c]	3.9	4.5
pMZT31	+	None	0.8	16.5
pMZT22	+	18653–18915[d]	4.9	42.0
pMZT23	+	18761–18915[e]	2.3	47.1

[a] RK2 coordinates as presented in Fig. 2.
[b] Results from three or six repetitions.
[c] The whole region between *trbA* and *trbB*.
[d] The whole inverted repeat.
[e] Half of the inverted repeat.

TABLE 7. Effect of the inverted repeat upstream of *trbB* on expression of *trbB-lacZ* translational fusions from the *tac* promoter with all or part of the inverted repeat between *tacp* and *trbB*

Reporter plasmid (region present[a] [RK2 coordinates])	IPTG	Total β-galactosidase activity (U)[b]
pMZT17 (18653–18915[c])	−	362
	+	444
pMZT18 (18715–18915[d])	−	359
	+	3,631
pMZT19 (18761–18915[e])	−	109
	+	10,455

[a] RK2 coordinates as in reference 24 and Fig. 2.
[b] Results are from a single experiment, but the relative β-galactosidase values were reproducible in all experiments performed.
[c] Complete inverted repeat.
[d] Reduced inverted repeat lacking sequences complementary to *trbB* Shine-Dalgarno sequence.
[e] Removal of half of the inverted repeat so that no hairpin can be formed.

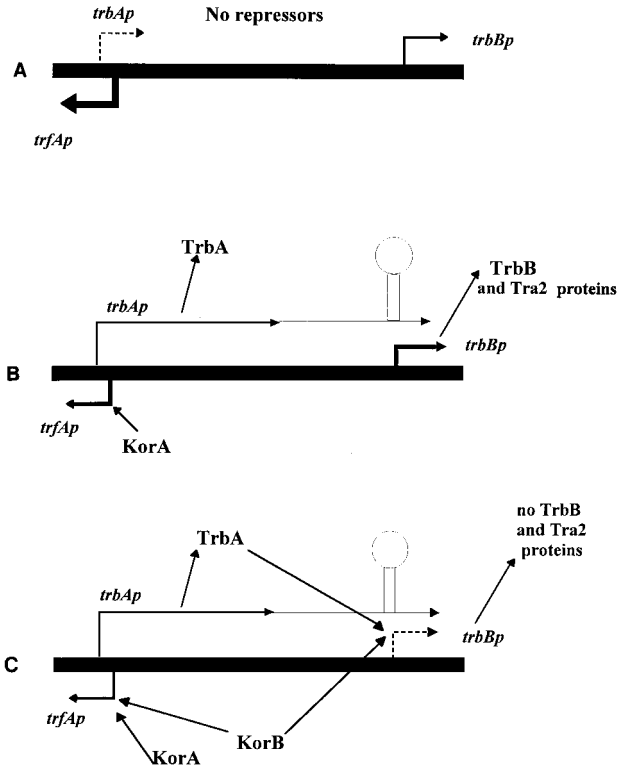

FIG. 5. Summary of expression events that are postulated to happen during plasmid establishment in the cell. (A) After entering the new host cell, while no repressors are produced, transcription from *trfAp* dominates over that of *trbBp* and *trbAp*. Sizes of the arrows indicate approximately the relative strengths of promoters. A dashed line indicates no transcription. (B) When transcription starts and KorA repressor appears, *trfAp* is repressed. This leads to derepression of both *trbAp* and *trbBp*. TrbA repressor is synthesized from transcripts originating at *trbAp*. Transcription from *trbBp* boosts expression of *trb* genes, including *trbB*. (C) As the repressors accumulate, *trbBp* is shut off. Translational control on longer *trbA* transcript prevents any expression of *trbB* from this transcript.

DISCUSSION

The purpose of the work described in this report was to assess the importance of the *trbA* promoter in expression of genes of the *trb* operon of promiscuous plasmid RK2 and to determine how translation of *trb* may be affected by the position from which transcription is initiated. The genes required for mating-pair formation start with *trbB*, and a strong promoter is located upstream of *trbB*, but this promoter is repressed by KorB and TrbA and should be largely switched off when the plasmid is established in a steady state. On the other hand, the *trbA* promoter, which lies upstream of *trbA*, the gene preceding *trbB*, is switched on when the plasmid reaches a steady state and, if transcription continues through into *trbB*, could contribute to the steady-state expression of the *trb* operon. By inhibiting *trbBp* by mutation, we were able to show that some transcription from *trbAp* reaches *trbB*, but the level was much lower than we would expect if all transcripts from *trbAp* reached *trbB*. Our Northern analysis indicated that *trbAp* is responsible primarily for transcription of the *trbA* gene. This transcript appears to terminate downstream of *trbA* in the region between *trbA* and *trbB*. The existence of a transcriptional terminator in this region was confirmed by insertion between *tacp* and *galK* which blocked the ability of *tacp* to increase *galK* expression upon induction with IPTG. It therefore appears likely that *trbA* and *trbB* are separate transcriptional units and that the *trb* operon starts with *trbBp*.

The use of translational fusions clearly showed that the inverted repeat present upstream of *trbB* is able to prevent its translation from transcripts from *trbAp*. This effect is due to sequestration of Shine-Dalgarno sequence of *trbB* in a stem-loop structure, since deletions which unmask this sequence gave a significant increase in reporter gene activity in translational fusions (Table 7). In light of the Northern blot results, we think that the role of this control is to prevent any translation of *trbB* on transcripts from *trbAp*.

As the result of these analyses, we discovered some interesting properties of the repressors and activators of this system. The results of Northern blots confirmed that *trbAp* is activated by KorA and slightly repressed by KorB (pMZT35 [Table 5]), as observed in our recent reporter gene study (14). However, when we detected *trbA* transcript from the fragment which carried both *trbAp* and the mutated *trbBp-1* promoter (pMZT36), we could not see repression of *trbAp* by KorB. Instead, there was a further increase in *trbAp* activity. We cannot explain this observation. Consistent with the results of reporter gene assays, the Northern blot showed that *trbBp* is strongly repressed by the presence of pCT415. Our reporter gene study showed clearly that *trbBp* needs both TrbA and KorB to be fully repressed (pMZT13 [Table 4]). We do not know why this effect is not visible in the three-plasmid system assay (Table 3). It may be due to the fact that there is an excess of TrbA, which is expressed from high-copy-number plasmids. An interesting possibility is that TrbA does not act in the same way in *cis* and in *trans*.

Northern blot analysis showed that *trbBp-1* is repressed only twofold by pCT415, which produces KorA and KorB. pCT415 should repress *trbB* by the direct effect of KorB as well as indirectly through induction of *trbA* expression. Since *trbBp-1* is still repressed by KorB alone but not TrbA alone, it appears likely that the small effect of pCT415 is due to loss of TrbA sensitivity. The TrbA binding site near *trbBp* has still not been defined, and so this mutation may contribute to its identification. This observation may also suggest that the unchanged levels of *xylE* activity in pMZT12 and pCT415 in comparison to pMZT12/R300B are due to the changed susceptibility of the *trbBp-1* promoter to its repressors and not to the fact that the transcription comes under these circumstances from *trbAp*. Reporter gene assays show that *trbBp* is repressed by the presence of active *trfAp*, and repression of *trfAp* by KorA leads to derepression of *trbBp*. However, by Northern blot analysis, we do not see an increase in transcription from *trbBp* in the presence of pCT409 in comparison to R300B. Unfortunately, under this condition we see either no change or a slight decrease in *trbBp* activity. However, since the reporter gene studies give significant differences and are internally consistent with *trfAp-1* mutation and repression by KorA, we are inclined at present to think that results of the reporter gene assays are more likely to be correct.

The results described here clarify a number of key issues about the roles of *trbAp* and *trbBp*. They still leave questions about the mechanism by which these promoters are regulated and the ways in which their interactions result in integration of the changing activities of the various RK2-encoded repressors. Further site-directed point mutations will be necessary to define sequences required for TrbA and KorB action, but the present work provides a basis for rational design of such an investigation.

Finally, it is worth reviewing what we now understand about the expression of the genes in this region during plasmid establishment. After entering the new host cell, while no repres-

sors are produced, transcription from *trfAp* dominates over that of *trbBp* and *trbAp*. Replication protein TrfA is produced. When transcription starts and KorA repressor appears, *trfAp* is repressed. This leads to derepression of both *trbAp* and *trbBp*. TrbA repressor is synthesized from transcripts originating at *trbAp*. Transcription from *trbBp* boosts expression of *trb* genes, including *trbB*. As the repressors accumulate, *trbBp* is shut off. Translational control on the read-through transcript from *trbAp* prevents any expression of *trbB* from this transcript (Fig. 5). Thus, the monocistronic *trbA* operon may be best seen as part of the establishment cascade eventually leading to minimal expression of the *trb* operon and probably contributes little to the steady-state expression of the *trb* genes.

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