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Conserved *cis*-Acting Promoter Elements Are Required for Density-Dependent Transcription of *Agrobacterium tumefaciens* Conjugal Transfer Genes

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Ti plasmids of *Agrobacterium tumefaciens*, in addition to transferring oncogenic DNA to the nuclei of infected plant cells, can conjugally transfer between agrobacteria. Conjugation of wide-host-range octopine-type Ti plasmids requires a tumor-released arginine derivative called octopine. Octopine stimulates expression of the *traR* gene, whose product directly activates other *tra* genes in the presence of an acylated homoserine lactone called *Agrobacterium* autoinducer (AAI). We have localized the transcription starts of three *tra* promoters and find conserved elements (*tra* boxes) at virtually identical positions upstream of each promoter. Disruption of these *tra* boxes abolished induction of each promoter. Deletion analysis of the *traI* promoter indicates that *tra* boxes are the only upstream elements required for transcriptional activation. Since Ti plasmid donor cells both produce and respond to AAI, we tested whether expression of *tra* promoters was enhanced by high concentrations of bacteria. Both *tra* gene expression and conjugation itself were strongly stimulated either by high donor densities or by exogenous AAI.

Many gram-negative bacteria of diverse genera release *N*-acyl homoserine lactones, collectively called autoinducers (AIs), which act as diffusible intercellular pheromones (15, 38). Several AIs are known to activate target genes via cognate regulatory proteins (11, 14, 17, 29, 31, 32). These AI-responsive transcriptional regulators have sequence homology with LuxR, the transcriptional activator of bioluminescence (*lux*) genes in *Vibrio fischeri* (15). Production of autoinducers generally requires a protein homologous to LuxI of *V. fischeri* (15, 38). It has been proposed that LuxR-LuxI-type regulators activate expression of target genes preferentially at high cell densities and thus provide bacteria with a mechanism for sensing their population density.

Agrobacterium tumefaciens, the causative agent of crown gall tumors, uses a LuxR-LuxI-type regulatory system to control the conjugal transfer of its Ti (tumor-inducing) plasmid (14, 22, 32). Transfer has long been known to require compounds called opines that are released from crown gall tumors (12, 18). For plasmids such as pTiR10, octopine acts through the OccR activator (20, 41) to activate *traR*, which is homologous to *luxR* (14). In turn, TraR activates the expression of Ti plasmid conjugal transfer (*tra*) genes in the presence of the *Agrobacterium* AI [*N*-3-(oxo)octanoyl]homoserine lactone (43), also called AAI. Synthesis of AAI requires the Ti plasmid *traI* gene, which is homologous to *luxI* and which is itself activated by TraR (14, 22). A third regulatory protein, TraM, somehow antagonizes TraR activity (13, 21). TraR controls the expression of at least five promoters: two divergent promoters adjacent to the *oriT* site that express genes probably required for conjugal DNA processing, an unlinked promoter that expresses *traI* and genes required to synthesize the putative conjugal pore, a promoter that expresses *traM*, and a promoter that expresses *traR* itself (13, 14, 22).

In previous studies, a transcriptional regulatory cascade involving OccR, TraR, TraI, and target *tra* operons was described genetically. However, the promoters for the *tra* structural genes were not localized. Dyad symmetrical sequences called *tra* boxes upstream of several TraR-activated operons that are similar to each other and to binding sites for LuxR were observed (8, 14, 19, 22). However, the hypothesis that these sites were required for induction remained untested. It was also hypothesized, by analogy to *V. fischeri lux* gene regulation, that expression of these *tra* promoters might be stimulated by high numbers of Ti plasmid conjugal donors. We describe here the structures of three different TraR-regulated promoters. We also demonstrate that both *tra* structural gene expression and conjugation itself are strongly stimulated either by high densities of donor bacteria or by exogenous AAI. This finding provides direct evidence for cell density-dependent gene regulation by LuxR-type regulators in an organism other than *V. fischeri*.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *A. tumefaciens* strains were grown at 30°C in either Luria-Bertani (LB) medium, AT (40) minimal salts media with 15 mM $(NH_4)_2SO_4$ and 0.5% glucose (ATGN), or AT salts with 2 mg of octopine per ml (ATO). AAI was provided in crude form as the sterile culture filtrate of strain R10(pCF218), which strongly overproduces AAI (14). Plasmid pCF336 is a derivative of the broad-host-range cloning vector pSW213 (4) containing *Bam*HI-20a of pTiR10, which contains *tra* box I. pCF278 is a derivative of pSW213 containing *Eco*RI-6 of pTiA6, which contains *tra* boxes II and III. Plasmids pCF338 and pCF291 are identical to pCF336 and pCF278, respectively, except for fill-in mutations at the *Bgl*II sites of *tra* box I or *tra* box II. Fill-in mutations were carried out by sequential treatment with *Bgl*II, T7 DNA polymerase, and T4 DNA ligase and checked by DNA sequencing.

Primer extension analysis. Primer extension experiments were performed by using avian myeloblastosis virus reverse transcriptase (26) as described previously (41). Oligonucleotides used to detect transcripts were 5′-TTGCGCCCTT TGGAGCCATTCC-3′ (for *traA* transcripts), 5′-CAGCTGGTCCTGCAATCG GGC-3′ (for *traC* transcripts), 5′-GTCAGAATCAGCATGTTGATC-3′ (for *traI* transcripts), and 5′-CGATCAGGGGTATGTTGGGT-3′ (for *tetA* transcripts), which served as an internal control.

Resection of the *traI* promoter. Resections of the *traI* promoter were constructed by PCR amplification, using oligonucleotides complementary to the upstream region of *traI* in combination with an oligonucleotide designed to fuse the *traI* coding sequence to the *lacZ* gene of pMC1403 (3). Compatible restriction sites were engineered into each oligonucleotide. The resulting PCR products

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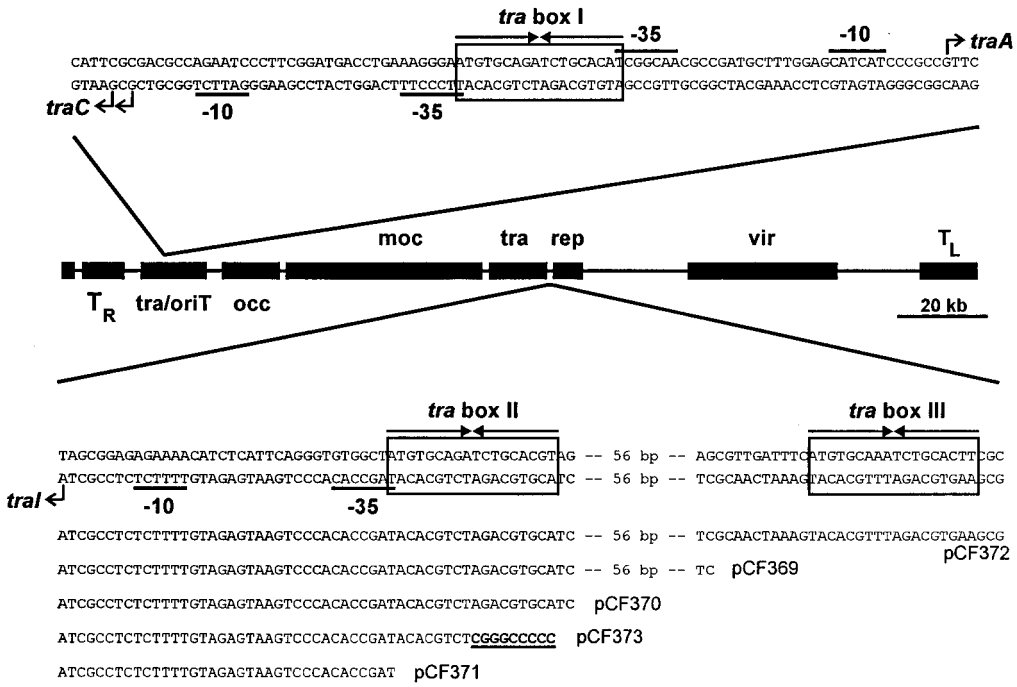

FIG. 1. Sequences, organization, and locations of TraR operator sites. The locations of the divergent *traC*, *traA*, and *traI* promoters were determined by primer extension analyses as described in the text. Short arrows denote transcriptional start sites, predicted −10 and −35 sequences are underlined, and *tra* boxes are boxed and marked with the convergent arrows. Sequences of resected *traI* promoters are shown at the bottom. Underlined sequence in pCF373 is derived from the vector.

were cloned into pBluescriptSK+ (Stratagene, La Jolla, Calif.) and sequenced. These sequences were excised as *Eco*RI-*Bam*HI fragments and inserted into pMC1403, to isolate *traI-lacZ* translational fusions. The resulting fusions were excised as *Pst*I-*Sal*I fragments and inserted into the broad-host-range plasmid pUCD2 (5) digested with the same enzymes. These pUCD2 derivatives were pCF372 (containing nucleotides −143 to +359 with respect to the *traI* transcription start site), pCF369 (containing nucleotides −112 to +359), pCF370 (containing nucleotides −54 to +359), and pCF371 (containing nucleotides −36 to +359). An additional resection was generated by deleting the smaller of two *Bgl*II-*Pst*I fragments from pCF372. The resulting plasmid, pCF373, contained an insert spanning nucleotides −45 to +359. These plasmids were introduced by electroporation into strain A136(pCF218), which lacks the Ti plasmid but which overproduces the plasmid-encoded TraR protein (14).

Ti plasmid conjugal transfer assays. Wild-type *A. tumefaciens* R10 harboring pCF240.113, a plasmid carrying a *traA-lacZ* fusion (14), and the Ti plasmidless, rifampin-resistant, streptomycin-resistant conjugal recipient C58C1RS were cultured to stationary phase in ATGN and concentrated 20-fold. The R10 (pCF240.113) cell suspension was serially diluted, mixed with approximately 10^9 cells of C58C1RS, spotted onto 0.2-μm-pore-size nitrocellulose filters that were placed in a 5-cm-diameter petri dish containing 5 ml of ATO agar, and incubated at 30°C for 15 h. Cells were resuspended and titers were determined for (i) Occ^+ transconjugants (by plating on ATO agar supplemented with rifampin [50 μg/ml] and streptomycin [500 μg/ml]) and (ii) conjugal donors (by plating on ATO agar and subtracting the number of transconjugants). Conjugation efficiencies were calculated as the ratio of transconjugants per recovered donor. *traA* expression in donor cells was measured during this experiment by using the *traA-lacZ* fusion on pCF240.113 (14) and expressed as specific activity per recovered donor.

RESULTS

Localization of the *traA*, *traC*, and *traI* promoters. *traA* and *traC* are the first genes of two divergent *tra* operons (Fig. 1), and the expression of both operons requires TraR and AAI (14). The predicted translation start sites for *traA* and *traC* are separated by 253 bp, and it was hypothesized that this intergenic region might contain two divergent promoters. To localize the *traA* and *traC* transcriptional start sites, RNA was isolated from a single culture of strain R10(pCF240.134), which contains a *traC-lacZ* fusion (14) after growth in ATGN and in ATGN supplemented with octopine and AAI, and annealed in separate reactions with oligonucleotide primers complementary to the *traA* or *traC* transcripts. We detected a *traA* reverse transcript 29 nucleotides long (Fig. 2A) and two *traC* reverse transcripts 96 and 97 nucleotides long (Fig. 2B). The *traA* reverse transcript was observed only from induced cultures (Fig. 2A, lane 4). The *traC* reverse transcript was at least 10-fold more abundant in induced cultures than in uninduced

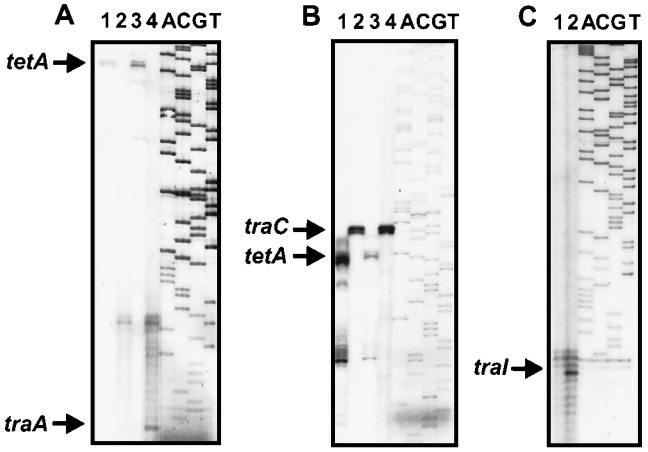

FIG. 2. Localization of TraR-regulated promoters. Radiolabeled oligonucleotides complementary to three *tra* transcripts were used for primer extension reactions using avian myeloblastosis virus reverse transcriptase. Primer extension reaction mixtures were size fractionated adjacent to dideoxy sequencing ladders generated by using the same primers. (A) A *traA*-specific primer (lanes 2 and 4) and a *tet*-specific primer (lanes 1 and 3) were hybridized to total RNA isolated from strain R10(pCF240.143) cultured in ATGN (lanes 1 and 2) or ATGN supplemented with 800 μg of octopine per ml and 0.4 μM crude AAI (lanes 3 and 4). (B) Conditions identical to those described for panel A except that a *traC*-specific primer (lanes 2 and 4) was used. (C) A *traI*-specific primer was used to direct reverse transcription by using RNA isolated from strains A136(pCF251)(pVK211) (lane 2) and A136(pDH99)(pVK211) (lane 1).

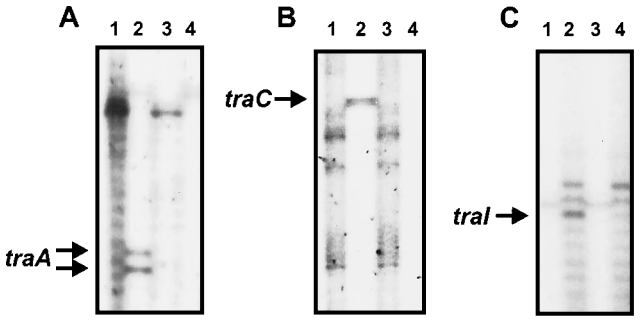

FIG. 3. *tra* box disruptions abolish TraR-dependent transcription. Primer extension reactions were as described for Fig. 2. (A) *traA* reverse transcripts detected by using RNA isolated from A136(pCF251)(pCF336) (lane 2) and A136 (pCF251)(pCF338) (lane 4) cultured in ATGN supplemented with 0.4 μM AAI. (B) *traC* reverse transcripts detected by using the same RNA as used for panel A. (C) *traI* reverse transcripts detected by using RNA prepared from A136(pCF251)(pCF278) (lane 2) and A136(pCF251)(pCF291) (lane 4). Lanes 1 and 3 in all panels are *tetA*-specific reverse transcription reactions, although in some cases, full-length *tetA* transcripts are not shown.

cultures after normalization against the control mRNA (Fig. 2B, lane 4).

Sequences showing some similarity to *Escherichia coli* σ^{70} promoters were identified upstream of the *traA* and *traC* transcriptional start sites (Fig. 1). Adjacent to the possible −35 site of each promoter was a perfect 18-bp inverted repeat, designated *tra* box I. This sequence is similar to the *lux* operator identified in *V. fischeri*, which provides a binding site for LuxR (8, 37). The position of this motif with respect to the *traA* and *traC* promoters (−43 and −43/−45, respectively) is highly similar to the position of the *V. fischeri lux* box with respect to the *luxI* promoter (−44 [11]).

A third, unlinked *tra* promoter is required for expression of an operon containing *traI* and 11 genes believed to direct synthesis of the conjugal mating pore (1, 14, 22) (Fig. 1). We localized this promoter by primer extension analysis using RNA isolated from A136(pVK211)(pCF251) and A136 (pVK211)(pDH99). pVK211 is a cosmid containing *traI*, while pCF251 is a derivative of pDH99 that overexpresses TraR (14, 27). A 39-nucleotide reverse transcript was synthesized by using RNA from the former strain (Fig. 2C, lane 2) but was not detectable from the latter strain (Fig. 2C, lane 1). The apparent *traI* transcription start site is directly downstream from sequences resembling a σ^{70} promoter (Fig. 1). Like the *traC* and *traA* promoters, the putative −35 sequence for *traI* overlaps by one base an 18-bp inverted repeat centered at position −43 (Fig. 1). This sequence is identical to *tra* box I at all positions except one, and we designate this element *tra* box II.

A third *tra* box-like sequence was found upstream from *tra* box II (designated *tra* box III; Fig. 1). The possibility that this sequence has a role in *traI* expression is examined below. It also seemed possible that this site could have some role in expression of the nearby *rep* operon, which is expressed divergently from *traI* and is required for vegetative plasmid replication (14, 39). However, primer extension analysis did not reveal a transcript in this region divergent from *traI* (data not shown).

Disruptions of *tra* boxes I and II abolish TraR-AAI-dependent transcriptional activation. To determine whether *tra* boxes are required for induction of *traA*, *traC*, and *traI*, we mutagenized *tra* boxes I and II of the broad-host-range plasmids pCF336 and pCF278, respectively (see Materials and Methods). pCF336 contains the *Bam*HI-20a fragment of pTiA6, which contains the *traC* and *traA* promoters, while pCF278 contains the *Eco*RI-6 fragment, which contains the *traI* promoter. Both plasmids have unique *Bgl*II sites at the middle of *tra* box I and *tra* box II, respectively. They were treated with *Bgl*II, DNA polymerase, and ligase to add four nucleotides at these sites. The resulting plasmids (pCF338 and pCF291) and their wild-type parents were introduced into the Ti plasmidless strain A136(pCF251) and assayed for production of *tra* transcripts in the presence of AAI. These mutations abolished detectable production of all three transcripts (Fig. 3, compare lanes 2 and 4 of each panel). The effect of the *tra* box II mutation was also tested by comparing A136(pCF251)(pCF278) and A136(pCF251)(pCF291) for the production of AAI. A culture supernatant of the former strain contained approximately 30 μM AAI activity, while a supernatant from the latter strain contained less than 0.3 μM AAI activity. The residual expression of *traI* from the mutant plasmid did not require TraR. This finding suggests strongly that *tra* box II is essential for TraR-dependent production of AAI.

Deletion analysis of the *traI* upstream region. The experiments described above did not rule out the possibility that a site upstream of these *tra* boxes was required for regulation and that the four-nucleotide insertions altered the helical phase of such a site with respect to its target promoter. To address this issue and to assess the relative roles of the two *tra* boxes in *traI* expression, we used PCR amplification to construct five resections of the *traI* promoter (Fig. 1, bottom) and used these resected promoters to express a *traI-lacZ* translational fusion. Three of the resulting plasmids (pCF372, pCF369, and pCF370) contained *tra* box II, and all three expressed similar levels of β-galactosidase in the presence of AAI (Table 1), indicating that the presence or absence of *tra* box III did not significantly affect *traI* expression. Plasmid pCF373, which lacks a portion of *tra* box II, expressed approximately 1% as much β-galactosidase as the three larger plasmids, although this low-level expression required TraR and AAI. Plasmid pCF371, which is missing virtually all of *tra* box II but retains an intact −35 element, did not express detectable β-galactosidase (Table 1). Note that plasmids pCF370, pCF373, and pCF371 span a region of only 12 nucleotides, thereby providing precise localization of this site. We conclude that *tra* box II is the sole *cis*-acting site essential for *traI* expression.

Ti plasmid conjugation efficiency is strongly stimulated by high numbers of conjugal donors. We sought to test directly the model that the promoters described above were regulated in a cell density-dependent manner. Strain R10(pCF240.113) contains wild-type Ti plasmid pTiR10 and a multicopy plasmid bearing a *traA-lacZ* fusion (14). We performed a series of filter matings in which different number of donors (from 10^4 to 10^8 donors per filter) were mixed with a large excess of conjugal recipients (see Materials and Methods). In the absence of

TABLE 1. Effects of upstream deletions on *traI-lacZ* expression

Plasmid[a]	Deletion end point	β-Galactosidase sp act (Miller units) in the presence of AAI[b]
pCF372	−136	831
pCF369	−103	580
pCF370	−50	833
pCF373	−40	5
pCF371	−33	<1

[a] Upstream deletion construct tested in *A. tumefaciens* A136 harboring pCF218.

[b] Crude AAI added to approximately 0.4 μM (1%). In all cases, specific activity in the absence of AAI was <1 Miller unit.

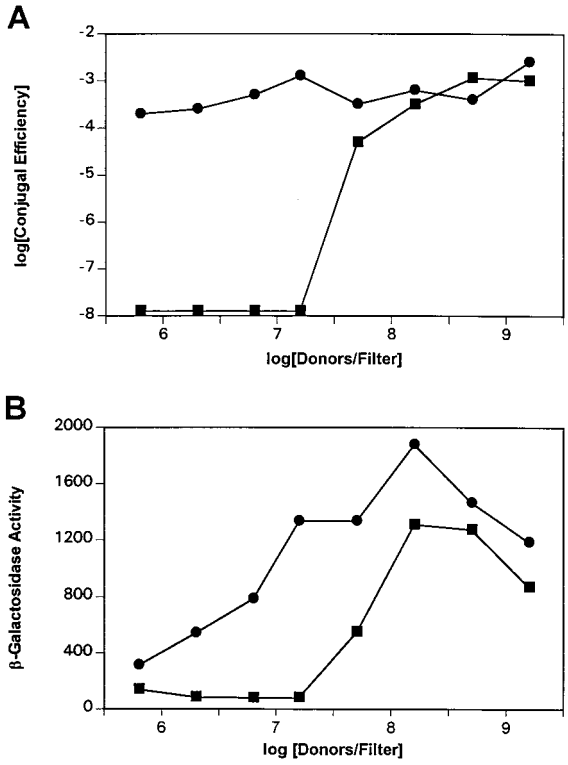

FIG. 4. Donor density dependence of *traI* expression and conjugal transfer. (A) Strain R10(pCF240.113) was cultured in ATGN, serially diluted, mixed with 10^9 cells of strain C58C1RS, deposited on nitrocellulose filters, and incubated for 15 h on ATO agar (■) or ATO supplemented with 0.4 μM AAI (●). Titers of donor bacteria and transconjugants were determined for filters. (B) Donors from the mating experiment in panel A were assayed for *traA-lacZ* expression by measuring total β-galactosidase activity per recovered donor and calculating total activity per 10^9 donor cells.

exogenous AAI, conjugation was undetectable when conjugal donors were inoculated at fewer than 3×10^7 cells per filter and was at least 10,000-fold more efficient at higher donor cell densities (Fig. 4A). In the presence of AAI, donor cells transferred the Ti plasmid with approximately equal efficiencies at all tested cell densities (Fig. 4A).

β-Galactosidase specific activity in donor cells was measured in the same experiment and plotted as a function of inoculated donor cells per filter. In the absence of exogenous AAI, β-galactosidase was weakly expressed when fewer than 3×10^7 donors were applied to the filter (Fig. 4B) and increased more than 25-fold by higher donor densities. Similar patterns of *tra* expression were found in the absence of conjugal recipients, while expression of a *lac* promoter control was not cell density stimulated (data not shown). This induction closely paralleled the conjugation efficiencies shown in Fig. 4A. Addition of AAI strongly stimulated β-galactosidase activity at low donor densities (Fig. 4B). Unexpectedly, however, *traA* expression was moderately stimulated by high donor densities even in the presence of exogenous AAI.

DISCUSSION

We have identified the transcriptional start sites of the *traA*, *traC*, and *traI* genes and found that all three promoters show an architecture strikingly similar to that of the *luxI* promoter of *V. fischeri*. All three *tra* promoters contain sequences similar to *E. coli* σ^{70} promoters and *tra* boxes that overlap rather weak possible −35 elements (8, 15, 19). The *tra* boxes are similar in sequence and position to the LuxR binding site upstream of the *luxI* promoter and to a possible binding site for the *Pseudomonas aeruginosa* LasR protein that is just upstream of the *lasB* promoter (15, 19). TraR and other LuxR homologs have homology in their DNA binding regions with a larger class of transcription factors, the FixJ/NarL superfamily (7, 15). Several of these proteins are known to bind to sites upstream of their target promoters to activate transcription (16, 28, 33, 37). In contrast to the conserved positions of the *cis*-acting sites for TraR, LuxR, and LasR, the locations of binding sites for other members of this superfamily are highly variable.

The existence of a single *tra* box between *traA* and *traC* suggested that these genes could have the same *cis*-acting element. Although it remains possible that the *tra* box insertion mutations that we made acted by changing the phase of some site further upstream, we favor the interpretation that *tra* box I is the only essential *cis*-acting site required for *traA* and *traC* expression. If so, this is the first example of a LuxR-type protein strongly activating divergent promoters from a single operator. Although this may seem similar to the situation for the divergent *luxR* and *luxI* promoters, both of which can be activated by LuxR (10, 34), the organization of the *traA* and *traC* promoters is quite different. The *luxICDABEG* promoter is adjacent to the *lux* box, such that this site overlaps the −35 element by one nucleotide (8, 11). In contrast, the *luxR* promoter is located 144 bp away from the *lux* box. Furthermore, LuxR protein activates the *luxR* promoter weakly and only under certain conditions (34, 36). In contrast, the *traA* and *traC* promoters have perfect symmetry centered around *tra* box I and are both strongly activated by TraR (Fig. 1). This compact spacing of two promoters and a shared *cis*-acting site is highly unusual (2).

A truncated *V. fischeri* LuxR protein and RNA polymerase (RNAP) bind synergistically to the 20-bp *lux* box (8, 37). Assuming that *tra* boxes provide binding sites for TraR, the dyad symmetry of *tra* boxes suggests that TraR could bind as a dimer, as has been found for many other regulatory proteins (6, 14). The fact that the *traA* and *traC* promoters are positioned symmetrically with respect to *tra* box I leads us to speculate that RNAP bound to *traA* could make contacts to one bound TraR protomer, while RNAP bound to *traC* could make identical contacts to the other bound TraR protomer. RNAP might transcribe these promoters simultaneously, although the close spacing of these promoters might prevent their simultaneous occupancy. The position of *tra* boxes suggests that TraR may be a class II-type transcriptional activator as defined by Ishihama (23). However, detailed understanding of the activation mechanism and putative interactions with RNAP await further studies.

The conserved spacing between *tra* boxes and the *traA*, *traC*, and *traI* promoters suggests that interactions between putatively bound TraR and RNAP may be similar. A plasmid lacking the distal seven nucleotides of *tra* box II (pCF373) expressed *traI* at extremely low but still detectable levels, indicating that TraR can weakly recognize the promoter proximal half of this *tra* box. Similar data have been obtained for *V. fischeri*: 5′ deletions removing part of the *lux* box reduced but did not abolish transcriptional activation via LuxR (8). Another possible TraR binding site, *tra* box III, is centered 132 bp upstream of the *traI* start site (Fig. 2). *tra* box III is the least similar to the consensus sequence, with one divergent nucleotide in each arm of the repeat. There is precedent for the existence of distal, regulatory sequences in *V. fischeri lux* gene regulation (8, 35). However, deletion of *tra* box III did not affect TraR-dependent activation of *traI*. Therefore, the func-

tion of *tra* box III, if any, remains unresolved. The proximity of this element to the *rep* operon suggested that it might have some role in vegetative replication, especially since coregulation of *tra* genes and genes required for vegetative replication has been reported in other plasmids (24). However, we could not detect a *rep* transcript originating near *tra* box III.

LuxR-LuxI-type regulatory systems are often hypothesized to activate target genes at high cell densities (25, 30, 38). Culturing *A. tumefaciens* on a solid medium dramatically increases *tra* gene expression and AAI synthesis (14, 42). We report here that TraR stimulated conjugation only at high densities of donor cells and that this density dependence was overcome by providing AAI exogenously. As expected, *traA* expression in the absence of exogenous AAI was strongly expressed at densities sufficient for efficient conjugation and poorly expressed at lower densities. Similar patterns of *tra* gene expression were observed in the absence of recipient cells, indicating that in contrast to pheromone-regulated conjugation systems (9), Ti plasmid conjugation is not responsive to signals released from recipient cells. Exogenous AAI caused *traA* to be expressed at lower cell densities, although expression was still somewhat stimulated by high donor densities (Fig. 4B). This was somewhat surprising, especially since AAI rendered conjugation by these same cells density independent. Residual cell density dependence of gene expression in the presence of AAI was observed in the absence of recipient cells for the *traA*, *traI*, and *traC* promoters (data not shown). However, this stimulation by high cell densities in the presence of AAI is evidently too mild to result in elevated conjugation efficiencies (Fig. 4A). It should be pointed out that these conjugation assays were quantitated over 4 orders of magnitude, while *traA* expression in the presence of AAI varied by less than 1 order of magnitude.

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