Domains Formed within the N-terminal Region of the Quorum-sensing Activator TraR Are Required for Transcriptional Activation and Direct Interaction with RpoA from Agrobacterium*

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TraR, a quorum-sensing activator, induces transcription from its binding site, the tra-box, located upstream of Ti plasmid target promoters. TraR activated expression of a lacZ reporter in Escherichia coli only when RpoA_{\text{At}} from Agrobacterium tumefaciens was co-expressed. As assessed by gel retardation assays RpoA_{\text{At}} but not RpoA_{\text{Ec}} formed a ternary complex with TraR and a tra-box probe in vitro. TraR formed similar ternary complexes with αCTD_{\text{At}} but not with αNTD_{\text{At}}, the C- and N-terminal segments of RpoA_{\text{At}}. As measured by surface plasmon resonance reflectometry, TraR interacted directly with RpoA_{\text{At}} with an affinity about five times greater than that observed with its interaction with RpoA_{\text{Ec}}. The activator interacted with αCTD_{\text{At}} with kinetics and affinities similar to those of the full-sized α-subunit. Positive control (PC) mutations at Asp-10 and Gly-123 of TraR did not affect DNA binding but greatly decreased the TraR-RpoA_{\text{At}} interaction. These two residues combine to form two patches on the activator, one of which may be involved in interaction with RpoA. When co-expressed, mutants of TraR with substitutions at Asp-10 complementing mutants with substitutions at Gly-123 for gene activation in an allele-specific manner. Co-expression studies with TraR and its PC mutants, and also with complementary PC alleles of TraR, coupled with three-dimensional structure are consistent with a hypothesis that both Asp-10/Gly-123 patches are required for activator function.

TraR activates expression of the three operons of the tra regulon on the Ti plasmids of Agrobacterium tumefaciens (1–3) in response to the population density of the donors (3, 4). The activator, a member of the LuxR family of quorum-sensing regulators, binds an 18-bp inverted repeat, the tra-box, located upstream of its target promoters (3, 5). pTiC58 contains three such boxes, one located between two divergently oriented operons, traAFB and traCDG (6), one in the promoter region of the trb operon (2) and one located upstream of the repABC operon (7, 8). TraR activates expression of each of these four gene sets, thereby controlling conjugation and plasmid copy number (4, 8, 9).

Most members of the LuxR family are activators and require as ligands acyl-homoserine lactone (acyl-HSL) signals (reviewed in Ref. 10). The acyl-HSLs are produced by the bacteria themselves, and the cells gauge their population size by sensing the accumulation of these signals in the environment in which they are growing (11). Ligand binding is required for TraR, LuxR, and LasR to form homodimers (12–14), a process required for binding DNA (5, 15, 16). However, other members of the family, including CarR and EsaR, multimerize and bind DNA even in the absence of their acyl-HSL signals (17, 18).

Although much is known about the regulatory circuits controlled by quorum sensing, less is known about the mechanisms by which these activators initiate transcription. Purified homodimers of TraR and LuxR bind target promoters containing appropriate binding sites and, with RNA polymerase (RNAP), activate transcription in vitro (16, 19). According to the crystal structure of TraR, each protomer of the dimeric activator is composed of two domains joined by a 12-residue flexible linker (20, 21). The N-terminal 162 residue segment contains the signal binding site and the primary dimerization domain, whereas the C-terminal 60-residue segment contains secondary dimerization regions and a helix-turn-helix domain, one helix of which makes contacts with specific nucleotides in a tra-box half-site (14, 20, 21).

Genetic, physiological, and biochemical data suggest that LuxR is an ambidextrous activator and, as such, interacts with the α-subunit (22–24) and σ\(^{\text{70}}\) (25) of Escherichia coli RNAP holoenzyme. Given its dimeric structure and the location of the tra-box recognition site within target promoters, TraR also likely makes contacts with one or more components of RNAP. Consistent with this hypothesis, alterations at two residues of TraR, Asp-10 and Gly-123, yield a strong positive control (PC) phenotype; both mutant proteins retain tra-box binding activity as measured by a genetic assay but fail to activate transcription (5). Asp-10 and Gly-123 both are located N-terminally to the linker region of the protein. Moreover, because of the asymmetric alignment and the folding patterns of the N-terminal regions of the two protomers, Asp-10 of one subunit combines with Gly-123 of the other to form two composite surface patches (Fig. 1). One such patch is close to the bound DNA, whereas the other is located at the opposite extremity of the

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The abbreviations used are: HSL, homoserine lactone; RNAP, RNA polymerase; SPRR, surface plasmon resonance refractometry; PC, positive control; αCTD, C-terminal half of the α-subunit; αNTD, N-terminal half of the α-subunit; RpoA_{\text{At}}, enteric form of RpoA; RpoA_{\text{Ec}}, RpoA from A. tumefaciens; 3-oxo-C8-HSL, N-(3-oxo-octanoyl)-L-homoserine lactone; IPTG, isopropyl-1-thio-β-D-galactopyranoside; cfu, colony-forming units; CAP, catabolite activator protein; AAI, Agrobacterium autoducer.
TraR-RpoA Interactions

1. Locations of the Asp-10/Gly-123 patches on the surface of a TraR dimer. The three-dimensional structure of a TraR dimer bound to its DNA binding site (magenta) is shown in ribbon form (A and C) and in surface contour form (B) using atomic coordinates from Zhang et al. (21). A and B show the complex in a side view, and C shows the complex oriented down the axis of the DNA helix. One protomer (residues labeled A) is shown in green and the other (residues labeled B) in blue; the two Asp-10/Gly-123 patches are labeled thus.

dimer. Either or both of these patches could make contact with one or more components of RNAP.

Although TraR binds to the tra-box in E. coli, it does not activate transcription in the enteric host. VirG, another Ti plasmid-encoded activator, shows a similar defect but will activate a VirG-dependent promoter in E. coli when co-expressed with rpoA from A. tumefaciens (26, 27). This observation suggests that VirG does not properly interact with RNAP containing the enteric form of RpoA (RpoAt). In this report we show that when co-expressed with RpoAt, TraR will activate expression from a tra-box promoter in E. coli. We also show that TraR interacts with RpoAt in vitro through the C-terminal domain of the α-subunit. The Asp-10 or Gly-123 mutants bind DNA in vitro but do not interact with the α-subunit. In vivo activation was restored by co-expressing certain alleles of TraR containing different substitutions at the Asp-10 and Gly-123 positions in the same cell. However, activation could not be rescued by co-expressing wild-type TraR with any of these mutants or by expressing an allele of TraR containing otherwise complementing substitutions at both locations.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—A. tumefaciens strain NTL4 (28), a Ti plasmid-cured derivative of the nanopine/agrocinopine-type strain C58, was used in all studies. E. coli strain D1530 was used as the host in all cloning experiments, as well as in assays of gene expression, and E. coli strains BL21(DE3)(pLysS) and SG13009 (26) were used for expressing proteins prior to purification. E. coli strains were grown in LB liquid medium, and A. tumefaciens were grown in LB or in M9/L (29) liquid medium. For studies involving defined media, A. tumefaciens was grown in AB minimal medium containing mannitol as sole source of carbon (ABM; Ref. 30) and E. coli was grown in LB medium containing glucose as sole carbon source (5). Cultures of A. tumefaciens were grown at 28 °C, and E. coli was grown at 28 or at 37 °C. All cultures in liquid medium were grown with shaking to ensure adequate aeration. Antibiotics, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and IPTG were included in medium at concentrations described previously (6). N-(3-Oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL; AAL, synthesized as described previously (51), was added to cultures at the concentrations indicated in the text.

Plasmid Isolation and Genetic Methods—Plasmids that express RpoAAt and RpoAEc remained soluble in cell extracts and were purified by Nickel affinity chromatography. Overexpression and purification of proteins was achieved by cloning HindIII fragment 4 of pTiC58 containing the TraR-dependent trc: lacZ reporter fusion from pPH41 (32) into the HindIII site of pKK223-3. The repression reporter pPBL1 was used to genetically monitor the DNA binding properties of TraR and its mutants as described previously (5). Plasmid pQKK was constructed by replacing the HindIII-Clal region of the polylinker of pKK223-3 (34) with the HindIII-EcoRI polylinker fragment from pUC18. This substitution removed the trc promoter from pKK223-3 and yielded a vector that lacks a promoter upstream of the polylinker. Wild-type traR or its single amino acid substitution mutants were amplified by PCR from pZLQ derivatives containing the appropriate genes. The amplicons, which included the upstream trc promoter of pZLQ, were cloned as Smal fragments into the pQKK polylinker. The traG: lacZ reporter was transferred from pH41 (as a HindIII fragment) into the pQKK-traR derivatives yielding plasmids that express traR or its mutants and also a TraR-dependent reporter fusion.

Nucleotides encoding the N-terminal (residues 1–166) and C-terminal (residues 167–336) segments of RpoAt were produced as fragments generated by PCR using Fhu polymerase and pZLQRpoA as template. The N-terminal end of each fragment was constructed to contain an NdeI site. The coding regions were cloned as NdeI-EcoRI fragments into pZLQ and pET14b to generate pZLQ-RpoAAt (αNTD), pH41-RpoAAt (αCTD), pET14-RpoAAt (Hissg-αCTD), and pET14-RpoAAt (Hisgg-αNTD). Clonings at the pZLQ NdeI site generated an ATG initiation codon that is properly spaced with respect to the vector transcriptional and translational signals to ensure high levels of expression of the protein.

Mutagenesis—Site-specific mutations were introduced into the traR gene using the QuikChange kit (Stratagene), and the resulting DNA samples were transformed into E. coli. Plasmid DNA samples from randomly chosen isolates were subjected to DNA sequence analysis, a representative containing the desired substitution mutation was retained, and the fragment bearing the mutant allele was cloned into the appropriate expression vector. The complete double-stranded sequence was determined for all mutant clones using automated methods by the Keck Center for Biotechnology at the University of Illinois at Urbana-Champaign.

Western Analyses—The stability and expression levels of mutant TraR proteins in vivo were determined by Western analysis using anti-TraR antisera as described previously (12).

Overexpression and Purification of Proteins—His-tagged RpoAAt as well as His-tagged forms of αCTDAt and αNTDAt were over-expressed in E. coli strain BL21(DE3)(pLysS) as described previously (12). N-terminal His-tagged RpoAAt from E. coli strain SG13009(pREP4) as described by Lohrke et al. (26). The His-tagged forms of RpoAt and RpoAet remained soluble in cell extracts and were purified under nondenaturing conditions. The two subfragments of RpoAAt were insoluble and were solubilized by treatment with 6 M urea. In both cases the proteins were purified by Nickel affinity chromatography.
TraR-RpoA Interactions

TraR-mediated activation of transcription in E. coli requires RpoA from A. tumefaciens

All constructs were expressed in E. coli strain DH5α grown in A medium containing glucose and IPTG as described under “Experimental Procedures.”

| Activator* | Co-expressed α-subunit| β-Galactosidase activity | -Fold induction* |
|------------|----------------------|--------------------------|------------------|
| None       | None                 | -AAI                      | <1               |
| None       | RpoA<sub>At</sub>    | 2                         | 3               |
| TraR       | None                 | -AAI                      | <1               |
| TraR       | RpoA<sub>At</sub>    | 3                         | 21              |
| TraR       | αNTD<sub>At</sub>    | 2                         | 3               |
| TraR       | αCTD<sub>At</sub>    | 2                         | 3               |

* traR was expressed from pKKTR2-141, which also codes for the
traG:<sub>G</sub>lacZ reporter.

<sup>1</sup> rpoA from A. tumefaciens and from E. coli, as well as the NTD and CTD fragments of RpoA<sub>At</sub>, were expressed from derivatives of pZLQ constructed as described under “Experimental Procedures.”

<sup>2</sup> Expressed as units of β-galactosidase activity/10<sup>9</sup> cfu as described under “Experimental Procedures.” The strains were assayed three times, and results from a representative experiment are shown.

Interaction experiments were initiated by injecting a preparation of purified native TraR or one of its substitution mutants in EB at concentrations as noted under “Results” into the buffer stream of both flow cells 1 and 2. Buffer flow was continued, and sensograms were recorded for a period up to 15 min, allowing analysis of association and dissociation reactions. The reference sensor signal from flow cell 1 was used to correct by subtraction the experimental binding data observed in the reaction occurring in flow cell 2. Following completion, chips were regenerated by injecting the following solutions in succession into the buffer stream: two 10-μl volumes of 0.3 M imidazole, two 10-μl volumes of RB, and a single 10-μl volume of 0.3% SDS. Both flow cells of the chip were then saturated with NB in preparation for the next run.

Values for kinetic parameters including the association rate constant, k<sub>a</sub>, and the dissociation rate constant, k<sub>d</sub>, were determined using the BIAevaluation 3.1 program (Biacore). The data exhibited best fit using the simple 1:1 Langmuir binding model available in the program. Deviations between experimental and fitted data normally were less than ± 2 resonance units.

β-Galactosidase Assays—β-Galactosidase activity, expressed as units/10<sup>9</sup> colony-forming units (cfu), was quantified as described previously (6). Assays were performed in triplicate, and each experiment was repeated at least twice.

**RESULTS**

**TraR Requires Agrobacterium RpoA to Initiate Transcription in E. coli**—In E. coli TraR binds to the tra-box sequence but does not initiate transcription from a native TraR-dependent promoter (5). However, TraR strongly activates transcription of the same promoter in A. tumefaciens. Moreover, purified TraR, when combined with RNAP from A. tumefaciens, activates transcription from tra-box promoters in vitro (19). These observations suggest that TraR cannot form an activation complex with enteric RNAP. Given that some activators interact with the α-subunit of RNAP (reviewed in Ref. 35), we tested whether expression of rpoA<sub>At</sub> would restore the activator function to TraR in an E. coli host. As expected, TraR failed to activate the traG:<sub>G</sub>lacZ reporter in E. coli DH5α (Table I). However, when rpoA<sub>At</sub> was co-expressed with traR, the reporter was induced 6-fold in the enteric host (Table I), and activation was depend-
TraR Interacts Directly with RpoA from Agrobacterium tumefaciens—We used surface plasmon resonance refractometry (SPRR) to assess whether TraR can bind RpoA in the absence of DNA. The sensograms clearly indicated that TraR in the mobile phase interacted with His$_6$-RpoA$_{At}$ subunits from both A. tumefaciens and E. coli (Fig. 3, A and B). However, the association rate constant, $k_a$, for the interaction between TraR and His$_6$-RpoA$_{At}$ was almost 4-fold greater than that calculated for the interaction between TraR and His$_6$-RpoA$_{Ec}$ (Table II). Following completion of the binding reaction, the sensograms indicated that both TraR-RpoA complexes dissociated at a slow but measurable rate (Fig. 3, A and B). Moreover, the dissociation rate constants, $k_d$, for the two TraR-RpoA complexes were not significantly different (Table II).

TraR Interacts with the C-Terminal but Not the N-Terminal Domain of RpoA$_{At}$—Several well studied activators interact with the C-terminal half of the $\alpha$-subunit ($\alpha$CTD) (35). Using PCR, we constructed His-tagged expression clones of the $\alpha$CTD fragment of RpoA$_{At}$. Strains of E. coli co-expressing either of these two constructs along with TraR failed to activate the reporter (Table I). When tested by gel retardation and SPRR, the His$_{10}$-CTD fragment did not interact with TraR in either assay (Fig. 4A and data not shown). However, the His$_{10}$-CTD fragment altered the mobility of the TraR-DNA complex in the gel retardation assay (Fig. 4B) and, as judged by SPRR, interacted directly with TraR (Fig. 4B). The rate constants of the binding reactions with His$_{10}$-CTD approximated those calculated for the interaction between TraR and full-sized RpoA$_{At}$ (Table II).

![Figure 3](image-url)  
**Fig. 3.** RpoA from A. tumefaciens but not RpoA from E. coli interacts directly with TraR. Interactions of fluid-phase TraR with His$_6$-RpoA$_{At}$ (A) and His$_6$-RpoA$_{Ec}$ (B) immobilized on the nitrotriacetic acid sensor chip were assayed by SPRR as described under “Experimental Procedures.” 30-μl samples of TraR at concentrations of 350 nM (trace 1), 250 nM (trace 2), 200 nM (trace 3), 125 nM (trace 4), and 62.5 nM (trace 5) were injected into the eluent buffer stream, and association and dissociation reactions were monitored as described under “Experimental Procedures.”
The acyl-HSL signal bound the 251-bp tra-mixture of wild-type TraR and His6-RpoAAt formed a ternary binding properties of TraR and its PC mutants (5). Although a based genetic screen that we developed to assess the DNA-Interaction with the promoter probe validates the repression-

phenotype mutations at Asp-10 and Gly-123 to determine whether the PC phenotype of the original mutants is specific to the substituted residues. As assessed by Western analysis, each mutant protein is as stable in vivo as wild-type TraR (data not shown). Each mutant was tested in vivo for DNA binding using the repressor assay in E. coli and for transcriptional activation of the TraR-dependent reporter fusion in A. tumefaciens, all in cells grown with or without 3-oxo-C8-HSL. Wild-type TraR activated the traG::lacZ reporter and repressed the chimeric tra-box::lacZ reporter, whereas the original D10N and G123R PC mutants (5) failed to activate transcription but retained strong repressor activity (Table III), all in a quorumone-dependent manner (data not shown). The D10A and G123A mutants also exhibited PC properties; both strongly repressed the traG::lacZ reporter but did not significantly activate the traG::lacZ reporter (Table III). When co-expressed with TraR, like the original PC mutants, TraRD10A and TraRG123A exhibited dominant negativity; both strongly inhibited activation of the reporter by the wild-type activator (Table IV).

When we co-expressed the Asp-10 and Gly-123 Positive Control Mutants Complement Each Other—Because Asp-10 of one pro-
tomer and Gly-123 of the other combine to form two surface patches on the TraR dimer (Fig. 1; Refs. 20 and 21), we tested whether combinations of the two PC mutants could complement one another when co-expressed in the same cell. Strains expressing either allele of traR alone did not signifi-
cantly activate the reporter fusion (Table V). However, when co-expressed TraRD10A and TraRG123A cooperated to strongly activate transcription of the reporter (Table V). Similarly, a combination of TraRD10A and TraRG123R strongly activated transcription, whereas co-expressing TraRD10N with TraRG123A yielded only weak activity. When co-expressed, TraRD10N and TraRG123R failed to activate the reporter. In all cases activation was dependent on growth of the cultures with the acyl-HSL signal.

Because certain alleles of the two PC mutants complement another, we constructed and tested a derivative of TraR with complementing substitution mutations at both positions. The double mutant TraRD10A/G123R failed to activate the traG::lacZ reporter (Table III). However, the double mutant retained repressor activity, and repression required the acyl-

HSL (Table III). When co-expressed with wild-type TraR, the double mutant exerted strong dominant negativity (Table IV).

![Fig. 4.](image1.png)

**Fig. 4.** The C-terminal but not the N-terminal fragment of RpoAAt interacts with TraR and its promoter. A, gel retardation analysis. 20-μl samples containing probe DNA only (lane 1, 20 ng), probe DNA and TraR (lane 2, 250 nM), probe DNA with His<sub>6</sub>-αNTD (lane 3, 1 μM; lane 4, 2 μM; lane 5, 5 μM) or with His<sub>6</sub>-αCTD (lane 6, 1 μM; lane 7, 2 μM; lane 8, 4 μM), or probe DNA and TraR (250 nM) with His<sub>6</sub>-αNTD (lane 9, 1 μM; lane 10, 2 μM; lane 11, 4 μM) or with His<sub>6</sub>-αCTD (lane 12, 1 μM; lane 13, 2 μM; lane 14, 4 μM) or His<sub>6</sub>-RpoAAt at any concentration tested (Fig. 5).

![Fig. 5.](image2.png)

**Fig. 5.** The Asp-10 and Gly-123 PC mutants of TraR bind the tra-box but do not form ternary complexes with RpoAAt. Samples (20 μl) containing the 251-bp DNA probe (20 ng) alone (lane 1) or mixed with wild-type TraR only (lane 2), TraRD10N only (lane 3), or TraRG123R only (lane 4), each at a final concentration of 100 nM, or with TraRD10N (100 nM) and His<sub>6</sub>-RpoAAt, at 140 nM (lane 5), 280 nM (lane 6), or 560 nM (lane 7), or with TraRG123R (100 nM) and His<sub>6</sub>-RpoAAt at 140 nM (lane 8), 280 nM (lane 9), or 560 nM (lane 10), or with wild-type TraR (100 nM) and His<sub>6</sub>-RpoAAt at 140 nM (lane 11), 280 nM (lane 12), or 560 nM (lane 13) were subjected to electrophoresis; protein-DNA complexes were detected as described under “Experimental Procedures.” I is free probe; II is the TraR-probe complex; III is the TraR-probe-αCTD complex.
studies have shown that this transcription factor requires a direct contact with a component of the RNAP. We have used purified proteins to show that a quorum-sensing reporter, traRD10A-G123R, which is a dominant-negative mutant, represses transcription of three times, and the results shown are from a representative experiment.

Strains of A. tumefaciens NTL4 harboring the appropriate plasmids were grown in AB minimal medium containing manitol, IPTG, and 3-oxo-C8-HSL at a final concentration of 25 nM.

- Fold activation is calculated as $\frac{\text{rpoA}_{\text{mutant}}}{\text{rpoA}_{\text{wildtype}}}$.
- Fold repression is calculated as $\frac{\text{rpoA}_{\text{wildtype}}}{\text{rpoA}_{\text{mutant}}}$.
- Fold inhibition is calculated as $\frac{\text{rpoA}_{\text{wildtype}} + \text{rpoA}_{\text{mutant}}}{\text{rpoA}_{\text{mutant}}}$.

### Table III

| Allele of TraR | β-Galactosidase activity | Fold activation | β-Galactosidase activity | Fold repression |
|---------------|--------------------------|----------------|--------------------------|----------------|
| None          | 2                        | 198            | 2                        | 12             |
| Wild type     | 425                      | 212            | 17                       | 12             |
| D10N          | 2                        | <1             | 18                       | 11             |
| D10A          | 2                        | 2              | 23                       | 9              |
| G123R         | 3                        | <1             | 19                       | 10             |
| G123A         | 10                       | 4              | 17                       | 12             |
| D10A-G123R    | 3                        | <1             | 18                       | 11             |

- traR and its mutant alleles were derived from derivatives of pZLQ constructed as described under “Experimental Procedures.”
- Activation by TraR and its mutant alleles was assessed in A. tumefaciens NTL4 harboring the reporter plasmid pH4I41 and the pZLQ derivative expressing the traR gene grown in AB minimal medium containing manitol, IPTG, and 3-oxo-C8-HSL at a final concentration of 25 nM.
- Fold activation is calculated as $\frac{\text{rpoA}_{\text{mutant}}}{\text{rpoA}_{\text{wildtype}}}$.
- Fold repression is calculated as $\frac{\text{rpoA}_{\text{wildtype}}}{\text{rpoA}_{\text{mutant}}}$.

### Table IV

Dominant-negative properties of TraR substitution mutants

All assays were conducted in A. tumefaciens NTL4 harboring pH4I41, which codes for wild-type traR and the TraR-dependent lacZ reporter fusion. Strains were grown in AB minimal medium containing manitol, IPTG, and 3-oxo-C8-HSL at a final concentration of 25 nM.

| Extra traR gene | β-Galactosidase activity | Fold activation |
|-----------------|--------------------------|----------------|
| None            | 5                        | 125            |
| Wild type       | 15                       | 245            |
| traRD10N        | 4                        | 4              |
| traRD10A        | 4                        | 4              |
| TrgG123R        | 4                        | 5              |
| traRG123A       | 4                        | 4              |
| TraRD10A-G123R  | 4                        | 4              |

- The second copy of traR or one of its mutant alleles as indicated was derived from derivatives of pZLQ constructed as described under “Experimental Procedures.”
- $\text{AAI}$ was added to a final concentration of 25 nM at the beginning of growth.
- $\text{AAI}$ was added to cultures to final concentrations of 25 nM at the beginning of growth.
- NA, not applicable.

### Discussion

Based on the SPRR studies we conclude that TraR, like CAP, FNR, and Mor (36), associates directly with RpoA by binding to the C-terminal end of the α-subunit. These constitute the first studies using purified proteins to show that a quorum-sensing transcription factor directly contacts a component of RNAP. LuxR also most likely contacts RpoA; physiological and genetic studies have shown that this transcription factor requires RpoA that is wild type in its C-terminal domain, to activate transcription (23, 24).

Two lines of evidence suggest that TraR and RpoA interact at a Tra-box promoter region and that this interaction is required for RNAS to initiate transcription. First, TraR only activates transcription in E. coli when RpoAαs is co-expressed (Table I). This observation also suggests that whereas TraR interacts with RpoAαs, the activator does not productively interact with RpoAαs, a conclusion that is consistent with the binding kinetics observed between TraR and the RpoA subunits from A. tumefaciens and E. coli (Table II). Second, in the absence of TraR, the RpoA subunits from A. tumefaciens and E. coli do not detectably bind to the probe DNA (Fig. 2B). However, combining RpoAαs with the activator and probe DNA yielded a ternary complex. Significantly, such a complex was not detected when RpoAαs was added to the reaction (Fig. 2B). These results suggest that TraR assists in the establishment or maintenance of an association between RpoA and the promoter.

That TraR exhibits specificity for the α-subunit of A. tumefaciens is intriguing but not unexpected. VirG, the response regulator of the two-component system that controls the Ti plasmid vir regulon, also activates transcription in E. coli only when co-expressed with $\text{rpoA}_{\alpha_1}$ (26, 27). These observations suggest that the α-subunits of A. tumefaciens, an α-proteobacterium, and E. coli, a γ-proteobacterium, although functionally equivalent, differ in their activator recognition properties. Based on studies with activators in E. coli, three domains of RpoA, the 265, the 261, and the 287 determinants, are important for activation of transcription by CAP (reviewed in Ref. 36). The critical residues in the 265 and 261 determinants, which apparently are involved in DNA binding and UP element recognition and interaction with αs (36), respectively, are strongly conserved between the α-subunits from the two bacteria.
ever, the 287 determinants of the two orthologs, which in RpoA_{Ec} mediates interaction with CAP, differ at four of the eight critical residues. Significantly, two of these residues of RpoA_{Ec}, Val-287 and Arg-317 (corresponding to Ala-286 and Glu-316 of RpoA_{At}), are among the six residues of this region that make direct contacts with CAP (37). We speculate that these divergent residues define specificity differences between the α-subunits of A. tumefaciens and coli. Consistent with this interpretation, the eight residues of the 287 determinant of RpoA_{At}, including those four that vary in RpoA_{Ec}, are virtually invariant in the α-subunits of all of the other α-proteobacteria for which DNA sequence information is available.

Clearly Asp-10 and Gly-123 are required for activation by TraR. These two residues are surface-exposed (Fig. 1), and, because of the asymmetric folding pattern and the antiparallel monomer-monomer interactions of the N-terminal domains, the Asp-10 of one protomer is adjacent to the Gly-123 residue of the other. This close contact is also observed in the polymerase (Fig. 1 and Refs. 20 and 21). Thus, two residues combine to form a surface patch, one located close to the DNA-binding domain and the other some distance away from the promoter binding site (Fig. 1). We propose that at least one of these patches interacts with the C-terminal domain of RpoA_{At}. Consistent with this hypothesis, substitution mutations at either residue abolish activation without affecting DNA binding, and neither mutant dimer interacts significantly with RpoA_{At} (Fig. 5 and data not shown).

Neither Asp-10 nor Gly-123 of TraR is absolutely conserved among all of the proteins (data not shown). This lack of conservation suggests that the two Asp-10/Gly-123 patches for activation, it is conceivable that one interacts with the α-subunit whereas the second interacts with another subunit of the polymerase. Consistent with such an interaction, we have recently observed that co-expression of the A. tumefaciens σ^{70} in addition to RpoA_{At} in E. coli allows TraR to activate transcription from a tra-box promoter to levels comparable with that observed in Agrobacterium. Similarly, genetic analyses suggest that LuxR interacts with σ^{70} of RNAP in E. coli (25), an observation consistent with the recent report that purified LuxR stabilizes the interaction of RNAP holoenzyme with the lux promoter in vitro (16).

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