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Bacterial Two-Hybrid Analysis of Interactions between Region 4 of the σ^{70} Subunit of RNA Polymerase and the Transcriptional Regulators Rsd from *Escherichia coli* and AlgQ from *Pseudomonas aeruginosa*

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A number of transcriptional regulators mediate their effects through direct contact with the σ^{70} subunit of *Escherichia coli* RNA polymerase (RNAP). In particular, several regulators have been shown to contact a C-terminal portion of σ^{70} that harbors conserved region 4. This region of σ contains a putative helix-turn-helix DNA-binding motif that contacts the −35 element of σ^{70}-dependent promoters directly. Here we report the use of a recently developed bacterial two-hybrid system to study the interaction between the putative anti-σ factor Rsd and the σ^{70} subunit of *E. coli* RNAP. Using this system, we found that Rsd can interact with an 86-amino-acid C-terminal fragment of σ^{70} and also that amino acid substitution R596H, within region 4 of σ^{70}, weakens this interaction. We demonstrated the specificity of this effect by showing that substitution R596H does not weaken the interaction between σ and two other regulators shown previously to contact region 4 of σ^{70}. We also demonstrated that AlgQ, a homolog of Rsd that positively regulates virulence gene expression in *Pseudomonas aeruginosa*, can contact the C-terminal region of the σ^{70} subunit of RNAP from this organism. We found that amino acid substitution R600H in σ^{70} from *P. aeruginosa*, corresponding to the R596H substitution in *E. coli* σ^{70}, specifically weakens the interaction between AlgQ and σ^{70}. Taken together, our findings suggest that Rsd and AlgQ contact similar surfaces of RNAP present in region 4 of σ^{70} and probably regulate gene expression through this contact.

Sigma factors are subunits of bacterial RNA polymerase (RNAP) that direct the holoenzymes that contain them to promoters of a specific class (20). In *Escherichia coli* there are seven different species of σ factors, and σ^{70} is the principal σ factor (27). The presence of different types of σ factors within a cell with distinct DNA sequence binding specificities provides a mechanism for coordinate regulation of genes that are controlled by promoters of the same class. Competition between different σ factors for the available RNAP core enzyme in part determines which genes are transcribed within a cell at any given time (27). This competition can be influenced by anti-σ factors, which are regulatory proteins that bind σ factors and often prevent their association with the RNAP core enzyme (19, 26). Anti-σ factors ultimately inhibit transcription from the class of promoters recognized by the σ factors that they sequester.

The σ^{70} subunit of RNAP participates in a number of protein-protein interactions, including interactions with other subunits of the polymerase complex (43, 52) and interactions with transcriptional regulators (18, 23). The regulators that interact with σ^{70} often contact a region of σ that contains a putative helix-turn-helix DNA-binding motif responsible for contacting the −35 elements of σ^{70}-dependent promoters (18, 23, 37). This DNA-binding region of σ is conserved in members of the σ^{70} family of proteins and is called region 4 (36).

Recently, Jishage and Ishihama identified a protein in *E. coli* that was preferentially made by cells during the stationary phase of growth and was associated with the σ^{70} subunit of RNAP in stationary-phase extracts (28). The protein was named Rsd (which stands for regulator of sigma D) since it was found to associate specifically with σ^{70} (but not with several alternative σ factors) and was shown to be capable of inhibiting σ^{70}-dependent transcription from certain promoters in vitro (28). The binding site for Rsd on σ^{70} was mapped to a C-terminal tryptic fragment encompassing conserved region 4 (28). On the basis of these observations and because the synthesis of Rsd coincides with the general shutdown in σ^{70}-dependent transcription that occurs as cells enter the stationary phase of growth, Jishage and Ishihama suggested that Rsd might be an anti-σ factor (28). Subsequent work has shown that consistent with this idea, Rsd may facilitate the replacement of σ^{70} by the stationary-phase-specific σ factor σ^{38} in functional RNAP holoenzyme complexes as cells go from the exponential phase to the stationary phase of growth (29).

The sequence of putative anti-σ factor Rsd is similar to the sequence of a regulator of alginate production in *Pseudomonas aeruginosa* called AlgQ (or AlgR2) (28). Alginate is an important virulence factor that imparts the characteristic mucoid phenotype to *P. aeruginosa* isolated from the lungs of cystic fibrosis patients (17). *P. aeruginosa* isolates from other sources are typically nonmucoid and do not exhibit activated expression of genes involved in alginate production (10). However, the production of alginate is believed to promote survival of *P. aeruginosa* in the special environment of the lungs of cystic fibrosis patients, contributing to resistance to both immune

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responses and antibiotics (10). AlgQ was originally identified as a positive transcriptional regulator of the key alginate biosynthetic gene *algD* (8, 32), which is expressed at high levels in mucoid cells. The regulation of the *algD* gene is complex and involves two different σ factors; transcription initiates from two superimposed promoters, one of which is recognized by RNAP containing σ^E (AlgU/AlgT) and the other of which is recognized by RNAP containing σ^{54} (RpoN) (3, 9, 11, 21, 38, 49, 59). Furthermore, at least one DNA-binding protein, AlgR (AlgR1), is known to bind to specific sites upstream of the *algD* promoter and activate transcription (31, 41, 42). The mechanism by which AlgQ positively regulates transcription of the *algD* gene is not known.

We were interested in testing the idea that like Rsd, AlgQ interacts with region 4 of the σ^{70} subunit of RNAP. In this study we tested this idea explicitly by using a bacterial two-hybrid system. We found that both Rsd and AlgQ can interact with a σ^{70} moiety encompassing region 4 in vivo, and we identified an amino acid substitution in region 4 that specifically weakens the interaction of Rsd and AlgQ with the σ moiety.

MATERIALS AND METHODS

Plasmids and strains. *E. coli* XL1-blue (Stratagene) was used as the recipient strain for all plasmid constructions. *E. coli* KS1 harbors on its chromosome the *lac* promoter derivative p*lac*O_R2-62 driving expression of a linked *lacZ* reporter gene and has been described previously (14). *E. coli* SF1 harbors an F′ episome containing the *lac* promoter derivative p*lac* O_R2-55/Cons −35 driving expression of a linked *lacZ* reporter gene and has also been described previously (13).

Plasmids pACλcI, pACΔcI, pBRα, pBRα-σ^{38}, pBRα-σ^{70}, and pBRα-σ^{70} (R596H) have all been described previously (13, 14). Plasmid pACλcI-Rsd encodes λcI (residues 1 to 236) fused to Rsd (residues 1 to 158) via three alanine residues. pACλcI-Rsd was made by cloning the appropriate *Not*I-*Bam*HI-digested PCR product into *Not*I-*Bst*YI-digested pACλcI32 (24); expression of the *cI-rsd* fusion gene was therefore under the control of the *lac*UV5 promoter. Plasmid pACλcI-AlgQ encodes λcI (residues 1 to 236) fused to AlgQ (residues 1 to 160) via three alanine residues, and it was made in a manner similar to the manner in which pACλcI-Rsd was made. Expression of the *cI-algQ* fusion gene on pACλcI-AlgQ is also under the control of the *lac*UV5 promoter. Plasmid pACλcI-AsiA encodes λcI (residues 1 to 236) fused to AsiA from bacteriophage T4 (residues 1 to 90) via three alanine residues, and it was made in a manner similar to the manner in which pACλcI-Rsd was made. Plasmid pACΔ-35λcI-AsiA contains the *cI-asiA* fusion gene from plasmid pACλcI-AsiA under the control of a *lac*UV5 promoter variant in which the −35 element of the promoter has been deleted. Plasmid pACΔ-35λcI-AsiA, therefore, expresses less of the λcI-AsiA fusion protein than plasmid pACλcI-AsiA expresses under identical conditions. pACΔ-35λcI-AsiA was made by cloning the appropriate *Hin*dIII-*Bst*YI fragment from pACλcI-AsiA into plasmid pA3B2 (57) cut with both *Hin*dIII and *Bst*YI. Plasmid pBRα-σ^{70}PA encodes residues 1 to 248 of the α subunit of *E. coli* RNAP fused to residues 532 to 617 of the σ^{70} subunit of *P. aeruginosa* RNAP. The hybrid α-σ^{70}*PA* gene was made by performing PCR and was cloned into *Hin*dIII-*Bam*HI-digested pBRα. Expression of the chimeric gene on pBRα-σ^{70}PA is, therefore, under the control of tandem *lpp* and *lac*UV5 promoters. pBRα-σ^{70}PA (R600H) is a derivative of pBRα-σ^{70}PA in which the R600H substitution in the σ moiety of the chimera was introduced by PCR.

The *lac* promoter derivative p*lac*COP-93+O_L2-62 was made by the PCR and contains two λ operators; a near-consensus λ operator (TACCACCGGCGGTGATA) and O_L2 (CAACACCGCCAGAGATA) are centered 93 and 62 bp, respectively, upstream of the transcriptional start site of the *lac* core promoter. Plasmid pFW11-COP-93+O_L2-62 was constructed by cloning an *Eco*RI-*Hin*dIII-cut PCR product containing p*lac*COP-93+O_L2-62 into pFW11 (56) cut with *Eco*RI and *Hin*dIII. Plasmid pFW11-COP-93+O_L2-62 was then transformed into strain CSH100, and the promoter-*lacZ* fusion was recombined onto an F′ episome and mated into strain FW102 (56) to create reporter strain F′93+62. The PCR-amplified regions of all plasmids were sequenced to confirm that no errors had been introduced as a result of the PCR process.

Experimental procedures. Cells were grown in LB supplemented with kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), carbenicillin (50 μg/ml), and isopropyl-β-D-thiogalactoside (IPTG) at the concentration indicated. Cells were permeabilized with sodium dodecyl sulfate-$CHCl_3$ and assayed for β-galactosidase activity essentially as described previously (39). Assays were performed at least three times in duplicate on separate occasions, and representative data sets are shown below. The values are averages based on one experiment; duplicate measurements differed by less than 10%.

RESULTS

λcI-Rsd activates transcription from a test promoter in the presence of a chimeric α-subunit harboring region 4 of *E. coli* σ^{70}. We sought to detect an interaction between Rsd and region 4 of σ^{70} in vivo by using a bacterial two-hybrid system that we had recently developed (12, 14). This bacterial two-hybrid system is based on the finding that any sufficiently strong interaction between a protein bound upstream of a suitable test promoter and a component of RNAP can activate transcription in *E. coli* (12, 14). Thus, two proteins that interact with one another can mediate transcriptional activation in *E. coli* provided that one protein is fused to a DNA-binding protein and the other is fused to a component of RNAP (12, 14).

Our strategy for detecting an interaction between Rsd and region 4 of σ^{70} involved the use of two chimeric proteins, one comprising Rsd fused to the repressor of bacteriophage λ (λcI) and the other comprising a modified form of the α-subunit of RNAP in which the C-terminal domain (CTD) of α has been replaced by a C-terminal fragment of σ^{70}. We reasoned that RNAP containing the resulting α-σ^{70} chimera would display a target for Rsd that could be contacted by a DNA-bound λcI-Rsd dimer (Fig. 1A). Having fused the entire Rsd protein (residues 1 to 158) to the C terminus of λcI, we placed the gene encoding this chimeric protein on a plasmid vector downstream of the IPTG-inducible *lac*UV5 promoter, thus creating plasmid pACλcI-Rsd. We used plasmid pBRα-σ^{70} as a source of the α-σ^{70} chimera. This plasmid encodes a chimera in which residues 528 to 613 of *E. coli* σ^{70} are fused to residues 1 to 248 of the α-subunit of RNAP (13). We introduced plasmids pACλcI-Rsd and pBRα-σ^{70} into *E. coli* KS1 (14), which harbors on its chromosome the *lac* promoter derivative p*lac*O_R2-62 (bearing a single λ operator centered 62 bp upstream of the transcriptional start site) linked to a *lacZ* reporter gene. We then tested the ability of the λcI-Rsd chimera to activate transcription from the p*lac*O_R2-62 test promoter in the presence of the α-σ^{70} chimera. Figure 1B shows that λcI-Rsd activated transcription from the test promoter up to ~24-fold in cells containing the α-σ^{70} chimera compared to control cells containing only wild-type α. An additional control revealed that λcI (lacking the fused Rsd moiety) did not activate transcription from the test promoter in the presence of the α-σ^{70} chimera (Fig. 1B). We also found that λcI-Rsd did not activate transcription from the test promoter in the presence of an α-σ^{38} chimera (comprising residues 1 to 248 of α fused to residues 243 to 330 of σ^{38}) encoded by plasmid pBRα-σ^{38} (13; data not shown).

Substitution R596H in the σ moiety of the α-σ^{70} chimera weakens the interaction between σ and Rsd. Our ability to link the protein-protein interaction between Rsd and its target on σ^{70} to transcriptional activation provided us with a useful genetic tool for dissecting this interaction. We were particularly interested in identifying mutant forms of σ^{70} that were specif-

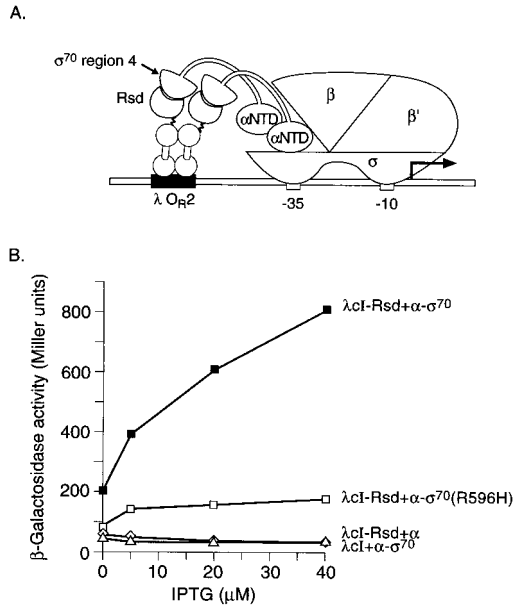

FIG. 1. Transcriptional activation by λcI-Rsd in the presence of the α-σ^{70} chimera. (A) Replacement of the RNAP α-CTD by a C-terminal fragment of *E. coli* σ^{70} (residues 528 to 613) permits interaction with the Rsd moiety of a λcI-Rsd chimera bound to DNA. The diagram depicts the test promoter p*lac*O_R2-62, which bears the λ operator O_R2 centered 62 bp upstream from the transcriptional start site of the *lac* core promoter. In reporter strain KS1 the p*lac*O_R2-62 test promoter is located on the chromosome and drives expression of a linked *lacZ* gene. The N-terminal domain of α is designated αNTD. (B) Effect of λcI-Rsd on transcription in vivo from p*lac*O_R2-62 in the presence of the α-σ^{70} or α-σ^{70}(R596H) chimera. KS1 cells harboring compatible plasmids expressing the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity.

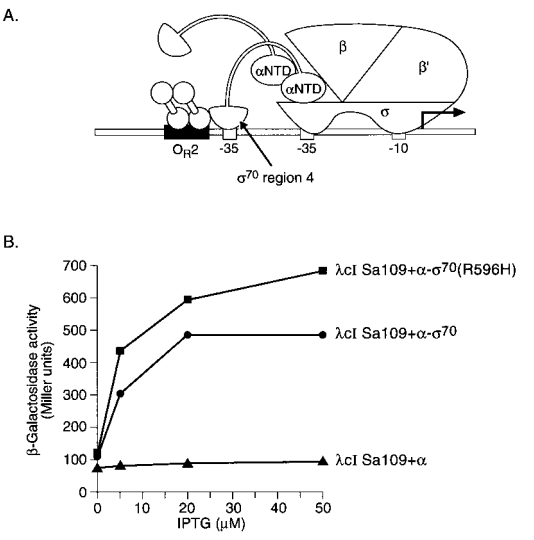

FIG. 2. Transcriptional activation by λcI superactivator in the presence of the α-σ^{70} chimera. (A) λcISa109 stabilizes the binding of region 4 of σ^{70} to an ectopic −35 element. The diagram depicts the test promoter p*lac*O_R2-55/Cons-35, which bears an ectopic −35 element and the λ operator O_R2 centered 45.5 and 55 bp, respectively, upstream from the transcriptional start site of the *lac* core promoter. In reporter strain SF1 the p*lac*O_R2-55/Cons-35 test promoter is located on an F′ episome and drives expression of a linked *lacZ* gene. (B) Effect of substitution R596H in the σ moiety of the α-σ^{70} chimera on the ability of λcISa109 to activate transcription in vivo from p*lac*O_R2-55/Cons-35. SF1 cells harboring compatible plasmids expressing the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity.

ically defective in the ability to interact with Rsd. We therefore introduced a variety of amino acid substitutions into the σ moiety of the α-σ^{70} chimera and tested the effects of these substitutions on the ability of the λcI-Rsd fusion protein to mediate transcriptional activation from the test promoter. Figure 1B shows that substitution R596H in the σ moiety of the α-σ^{70} chimera strongly reduced the magnitude of λcI-Rsd-dependent activation (to a factor of ~5). Substitution R596A in the σ moiety of the α-σ^{70} chimera had a nearly identical effect on the magnitude of the activation (data not shown). In contrast, substitutions L573A, E591A, E591Q, H600A, and H600R did not decrease the magnitude of λcI-Rsd-dependent activation (data not shown).

Substitution R596H in the σ moiety of the α-σ^{70} chimera does not compromise the ability of a superactivating variant of λcI to stimulate transcription from an appropriate test promoter. To determine whether the R596H substitution affects the interaction of σ^{70} with Rsd specifically, we tested its effect on the ability of another protein to interact with the σ moiety of the chimera. We showed recently that the λcI protein (a transcriptional activator as well as a repressor) can interact specifically with the σ moiety of the α-σ^{70} chimera and stabilize its binding to a promoter −35 element (13). The in vivo assay which we designed to detect the interaction between λcI and region 4 of σ^{70} is shown in Fig. 2A. In this experimental setup a DNA-bound λcI dimer activates transcription from test promoter p*lac*O_R2-55/Cons-35 by stabilizing the binding of the σ moiety of the α-σ^{70} chimera to the ectopic −35 element present upstream of the core promoter elements (13) (Fig. 2A). Transcriptional activation from this test promoter is dependent not only on the protein-protein interaction between λcI and the tethered σ moiety but also on the protein-DNA interaction between the tethered σ moiety and the ectopic −35 element (13).

We tested whether the R596H substitution in the α-σ^{70} chimera has an effect on the ability of a superactiviating variant of λcI to activate transcription from test promoter p*lac*O_R2-55/Cons-35 (Fig. 2A). Reporter strain SF1 carries promoter p*lac*O_R2-55/Cons-35 fused to the *lacZ* gene in single copy on an F′ episome (13). We assayed the ability of λcI superactivator 109 (λcISa109) (5, 13) to activate transcription from this reporter in the presence of the α-σ^{70} chimera with or without the R596H substitution in the σ moiety. λcISa109 activated transcription a maximum of ~5.5-fold from the test promoter in the presence of the α-σ^{70} chimera and a maximum of ~7.5-fold in the presence of the chimera harboring the R596H substitution (Fig. 2B). (Although the R596H substitution has previously been shown to inhibit the ability of wild-type λcI to activate transcription from P_{RM} [35], we have observed that this substitution does not inhibit the ability of λcISa109 to activate transcription from P_{RM} [see below].) We concluded that substitution R596H in the σ moiety of the α-σ^{70} chimera

does not result in a general defect in the ability of the σ moiety to interact with other proteins.

We also tested the effect of the R596A substitution in the σ moiety of the α-σ^{70} chimera on the ability of λcISa109 to activate transcription from the same test promoter. Unlike the R596H substitution, the R596A substitution significantly reduced the magnitude of the activation by λcISa109 (data not shown). Using another assay, however, we were able to determine that this reduction in activation was not due to a general defect caused by the R596A substitution (see below).

λcI-AlgQ activates transcription from a test promoter in the presence of a chimeric α-subunit harboring region 4 of *P. aeruginosa* σ^{70}. Jishage and Ishihama (28) noted that Rsd exhibits 31% identity with the alginate regulatory protein AlgQ (also known as AlgR2) from *P. aeruginosa*. AlgQ was originally identified as a positive regulator of alginate production in *P. aeruginosa* (8, 32) but has subsequently been shown to regulate several other gene products in this organism (see below).

Very little is known about how AlgQ mediates its effects on gene expression. In order to test the hypothesis that AlgQ, like Rsd, can interact with region 4 of σ^{70}, we made a *cI-algQ* fusion gene analogous to the *cI-rsd* fusion gene. We also made a fusion gene encoding a protein analogous to the α-σ^{70} chimera that contained region 4 of σ^{70} from *P. aeruginosa*. We fused the entire AlgQ protein (residues 1 to 160) to the C terminus of λcI. We placed the gene encoding this chimeric protein on a plasmid vector downstream of the IPTG-inducible *lac*UV5 promoter, creating plasmid pACλcI-AlgQ. We then fused residues 532 to 617 of *P. aeruginosa* σ^{70} (equivalent to residues 528 to 613 of *E. coli* σ^{70}) to residues 1 to 248 of α. The resulting chimera was called α-σ^{70}PA. The hybrid α-σ^{70}*PA* gene encoding this chimera was placed on a plasmid vector downstream of tandemly arranged *lpp* and *lac*UV5 promoters, creating plasmid pBRα-σ^{70}PA. We introduced plasmids pACλcI-AlgQ and pBRα-σ^{70}PA into *E. coli* KS1. We then tested the ability of the λcI-AlgQ chimera to activate transcription from p*lac*O_R2-62 in the presence of the α-σ^{70}PA chimera (Fig. 3A). Figure 3B shows that λcI-AlgQ activated transcription from the test promoter a maximum of ~17-fold in cells containing the α-σ^{70}PA chimera compared to control cells containing only wild-type α. An additional control revealed that λcI without the fused AlgQ moiety did not activate transcription from the test promoter in the presence of the α-σ^{70}PA chimera (Fig. 3B).

Substitution R600H in the σ moiety of the α-σ^{70}PA chimera weakens the interaction between σ and AlgQ. The σ^{70} subunits from *E. coli* and *P. aeruginosa* are very similar to one another, exhibiting ~83% identity over the length of the σ fragments (86 amino acids) that we used in our experiments (36). We wanted to test whether substitution R600H in σ^{70} from *P. aeruginosa*, which corresponds to the R596H substitution in *E. coli* σ^{70}, had any effect on the ability of λcI-AlgQ to activate transcription in the presence of the α-σ^{70}PA chimera. To do this, we made a version of the α-σ^{70}PA chimera harboring substitution R600H [α-σ^{70}PA(R600H)] and assayed the ability of λcI-AlgQ to activate transcription in KS1 cells expressing this chimera. Figure 3B shows that λcI-AlgQ activated transcription from the reporter gene a maximum of ~5-fold in the presence of the α-σ^{70}PA(R600H) chimera, compared to a maximum of ~17-fold with the chimera derived from the wild-type form of *P. aeruginosa* σ^{70}.

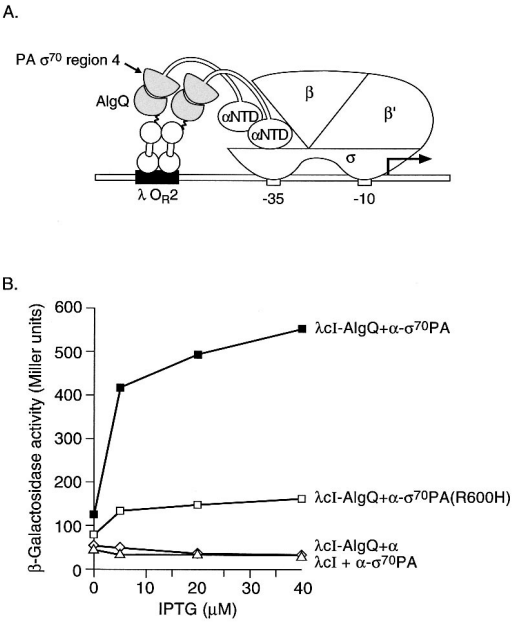

FIG. 3. Transcriptional activation by λcI-AlgQ in the presence of the α-σ^{70}PA chimera. (A) Replacement of the RNAP α-CTD by a C-terminal fragment of *P. aeruginosa* σ^{70} (residues 532 to 617) permits interaction with the AlgQ moiety of a λcI-AlgQ chimera bound to DNA. The diagram depicts the test promoter p*lac*O_R2-62, which bears the λ operator O_R2 centered 62 bp upstream from the transcriptional start site of the *lac* core promoter. In reporter strain KS1 the p*lac*O_R2-62 test promoter is located on the chromosome and drives expression of a linked *lacZ* gene. (B) Effect of λcI-AlgQ on transcription in vivo from p*lac*O_R2-62 in the presence of the α-σ^{70}PA or α-σ^{70}PA(R600H) chimera. KS1 cells harboring compatible plasmids expressing the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity.

Substitution R600H in the σ moiety of the α-σ^{70}PA chimera does not compromise the ability of a superactivating variant of λcI to stimulate transcription from an appropriate test promoter. In order to assess whether the effect of the R600H substitution was specific for the interaction between σ^{70}PA and AlgQ, we first tested whether λcISa109 could interact with the σ moiety of the α-σ^{70}PA chimera. We found that λcISa109 stimulated transcription from test promoter p*lac*O_R2-55/Cons-35 up to ~3.5-fold specifically in the presence of the α-σ^{70}PA chimera (Fig. 4). Furthermore, introduction of the R600H substitution into the σ moiety of the α-σ^{70}PA chimera did not abrogate the stimulatory effect of λcISa109 (instead it resulted in a modest increase in the observed activation), suggesting that the effect of the R600H substitution is specific for the λcI-AlgQ chimera (Fig. 4B).

The *E. coli* protein Rsd can interact with region 4 of σ^{70} from *P. aeruginosa*, and the *P. aeruginosa* protein AlgQ can interact with region 4 of σ^{70} from *E. coli*. Given the high degree of similarity between the regions of σ^{70} from *E. coli* and *P. aeruginosa* used in our experiments, we thought that each regulator might be able to contact region 4 of σ^{70} from either *E. coli* or *P. aeruginosa*. We explicitly tested whether Rsd could interact with region 4 of σ^{70} from *P. aeruginosa* and also whether AlgQ could interact with region 4 of σ^{70} from *E. coli*. Figure 5A shows that the λcI-Rsd chimera activated transcrip-

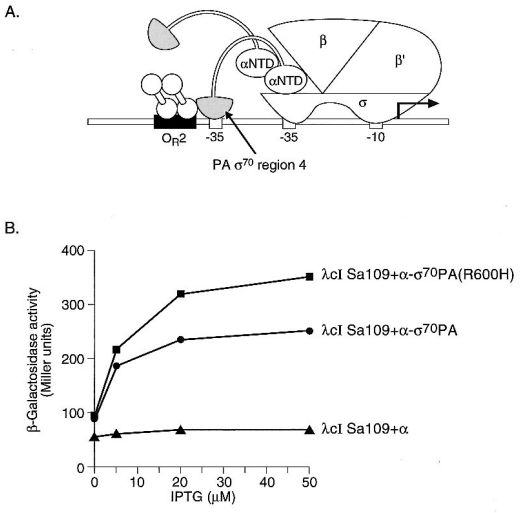

FIG. 4. Transcriptional activation by λcI superactivator in the presence of the α-σ^{70}PA chimera. (A) λcISa109 stabilizes the binding of region 4 of σ^{70} from *P. aeruginosa* to an ectopic −35 element. The diagram depicts the test promoter p*lac*O_R2-55/Cons-35, which bears an ectopic −35 element and the λ operator O_R2 centered 45.5 and 55 bp, respectively, upstream from the transcriptional start site of the *lac* core promoter. In reporter strain SF1 the p*lac*O_R2-55/Cons-35 test promoter is located on an F′ episome and drives expression of a linked *lacZ* gene. (B) Effect of substitution R600H in the σ moiety of the α-σ^{70}PA chimera on the ability of λcISa109 to activate transcription in vivo from p*lac*O_R2-55/Cons-35. SF1 cells harboring compatible plasmids expressing the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity.

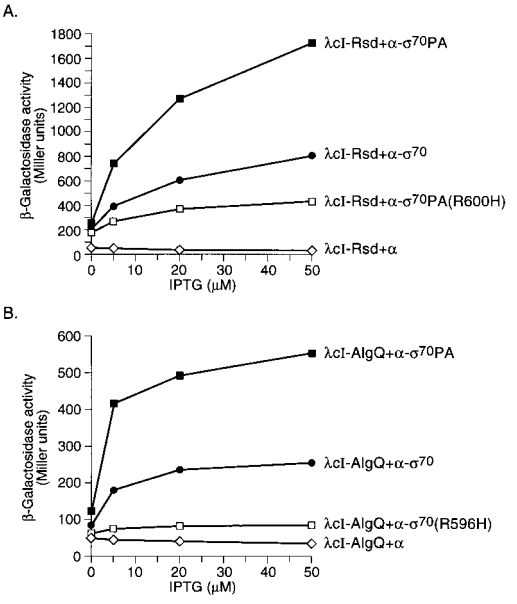

FIG. 5. Rsd can interact with region 4 of σ^{70} from *P. aeruginosa*, and AlgQ can interact with region 4 of σ^{70} from *E. coli*. (A) Effect of λcI-Rsd on transcription in vivo from p*lac*O_R2-62 in the presence of the α-σ^{70}PA, α-σ^{70}PA(R600H), or α-σ^{70} chimera. KS1 cells harboring compatible plasmids expressing the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity. (B) Effect of λcI-AlgQ on transcription in vivo from p*lac*O_R2-62 in the presence of the α-σ^{70}, α-σ^{70}(R596H), or α-σ^{70}PA chimera. KS1 cells harboring compatible plasmids expressing the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity.

tion from the test promoter in KS1 cells a maximum of ~52-fold in the presence of the α-σ^{70}PA chimera, compared to ~24-fold in the presence of the *E. coli* α-σ^{70} chimera. We also found that introduction of substitution R600H into the α-σ^{70}PA chimera reduced the ability of λcI-Rsd to stimulate transcription from the test promoter to a factor of ~13 (Fig. 5A).

Figure 5B shows that the λcI-AlgQ chimera activated transcription from the test promoter in KS1 cells a maximum of ~7-fold in the presence of the α-σ^{70} chimera, compared to ~15-fold in the presence of the *P. aeruginosa* α-σ^{70}PA chimera. Introduction of substitution R596H into the σ moiety of the *E. coli* α-σ^{70} chimera reduced the ability of λcI-AlgQ to activate transcription from the test promoter to a factor of ~2 (Fig. 5B).

λcI-AsiA activates transcription from a test promoter in the presence of either α-σ chimera. We took advantage of another protein known to interact with region 4 of *E. coli* σ^{70} to test further the specificity of the effect of the R596H substitution on the interaction of σ^{70} with either Rsd or AlgQ. The AsiA protein encoded by bacteriophage T4 is an anti-σ factor that has been shown to interact specifically and very strongly with region 4 of *E. coli* σ^{70} (1, 7, 22, 40, 50, 51). AsiA is thought to work by interacting with σ^{70} that is free in solution rather than with σ^{70} that is already complexed with the RNAP core enzyme (22). The interaction between AsiA and *E. coli* σ^{70} inhibits the activity of RNAP holoenzyme (containing the AsiA-σ^{70} complex) by preventing region 4 of σ from contacting the −35 element of σ^{70}-dependent promoters (7, 51).

We therefore sought to detect the interaction between AsiA and region 4 of σ^{70} by fusing AsiA to λcI and measuring the ability of the resulting λcI-AsiA chimera to activate transcription from p*lac*O_R2-62 in the presence of either the α-σ^{70} chimera or the α-σ^{70}PA chimera. We fused the entire AsiA protein (residues 1 to 90) to the C terminus of λcI. We placed the gene encoding this chimeric protein on a plasmid vector downstream of the IPTG-inducible *lac*UV5 promoter, creating plasmid pACλcI-AsiA. Initial experiments demonstrated that the λcI-AsiA chimera was extremely toxic to cells at the levels provided by the expression vector pACλcI-AsiA (data not shown). For subsequent experiments we constructed a different expression vector, pACΔ-35λcI-AsiA, in which the chimeric *cI-asiA* gene was under control of an IPTG-inducible variant of the *lac*UV5 promoter that lacked the normal −35 element and therefore directed the synthesis of smaller amounts of the fusion protein. Since pACΔ-35λcI-AsiA made insufficient levels of λcI-AsiA to saturate the λ operator present on the p*lac*O_R2-62 promoter construct, we also constructed a new test promoter bearing two relatively strong λ operators in the upstream region (Fig. 6A; also see Materials and Methods). Figure 6B shows that the λcI-AsiA chimera activated transcription from the modified test promoter in the presence of the α-σ^{70} chimera derived from σ^{70} of either *E. coli* or *P. aeruginosa* (similar findings with the α-σ^{70} chimera

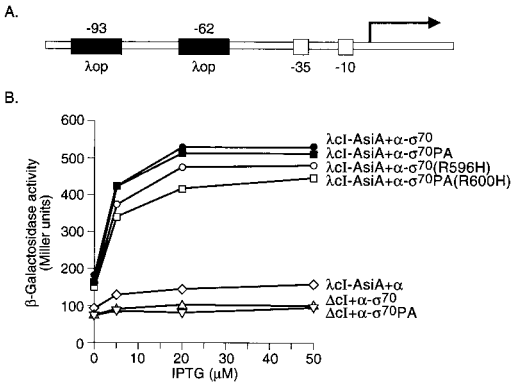

FIG. 6. Transcriptional activation by λcI-AsiA in the presence of the α-σ chimeras. (A) Schematic diagram of test promoter p*lac*COP-93+O_L2-62 used to determine the effects of substitutions in the α-σ chimeras on transcriptional activation by λcI-AsiA. The test promoter p*lac*COP-93+O_L2-62 bears both a consensus λ operator and O_L2 centered 93 and 62 bp, respectively, upstream from the transcriptional start site of the *lac* core promoter. In reporter strain F′93+62 the p*lac*COP-93+O_L2-62 test promoter is located on an F′ episome and drives expression of a linked *lacZ* gene. (B) Effect of λcI-AsiA on transcription in vivo from p*lac*COP-93+O_L2-62 in the presence of the α-σ chimeras. F′93+62 cells harboring compatible plasmids expressing the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity. ΔcI refers to the fact that no λcI was made by control plasmid pACΔcI.

from *E. coli* have been obtained by S. Pande and D. Hinton [submitted for publication]). The data also show that substitution R596H in the *E. coli* σ moiety or the corresponding R600H substitution in the *P. aeruginosa* σ moiety had only a modest effect on the ability of the λcI-AsiA chimera to activate transcription from p*lac*COP-93+O_L2-62. Similarly, the R596A substitution in the *E. coli* σ moiety did not reduce the stimulatory effect of the λcI-AsiA chimera (data not shown). Since these substitutions do not appear to cause nonspecific defects in the abilities of the σ moieties to interact with other proteins, we suggest that residue R596 of *E. coli* σ^{70} is at or near the contact surface for Rsd and the equivalent residue of *P. aeruginosa* σ^{70}, R600, is at or near the contact surface for AlgQ (see Discussion). Furthermore, the finding that substitution R596A in *E. coli* σ^{70} results in a strong defect in the ability of σ to interact with Rsd suggests that the arginine side chain may make an energetically significant contact with Rsd.

DISCUSSION

Rsd and AlgQ can interact with region 4 of σ^{70} in vivo. We found that the *E. coli* Rsd protein and the *P. aeruginosa* AlgQ protein can interact in vivo with a C-terminal fragment of the σ^{70} subunit of *E. coli* and *P. aeruginosa* RNAP, respectively. This fragment of σ^{70} encompasses conserved region 4, which contains a DNA-binding domain that mediates recognition of the −35 element of σ^{70}-dependent promoters (18). Furthermore, each protein can also interact with the corresponding σ^{70} fragment from the heterologous organism. We also identified a single amino acid substitution (R596H and the corresponding substitution R600H in *E. coli* and *P. aeruginosa* σ^{70}, respectively) that inhibits the binding of both Rsd and AlgQ to either σ^{70} fragment. The interchangeability of the *E. coli* and *P. aeruginosa* σ^{70} fragments in our experiments suggests that regulators from *P. aeruginosa* or *E. coli* that interact with region 4 of σ^{70} might in general be expected to function in either organism. In fact, a previous in vitro study showed that the λcI protein (which contacts region 4) can activate transcription from the λ promoter P_{RM} by the *P. aeruginosa* RNAP (16).

Analysis of the interaction between Rsd and region 4 of σ^{70}. Our bacterial two-hybrid results for Rsd and region 4 of σ^{70} are consistent with the biochemical data of Jishage and Ishihama (28). These authors found that Rsd could interact with σ^{70} in vitro but not with σ^{38} (or several other alternative σ factors), and they showed that Rsd could interact with a tryptic fragment of σ^{70} composed of residues ~500 to 613. We found that Rsd can interact in vivo with an 86-amino-acid C-terminal fragment of σ^{70} that contains region 4 but cannot interact with the equivalent 88-amino-acid C-terminal fragment of σ^{38}. Jishage et al. (30) have recently reported that alanine substitutions at two positions flanking residue 596 (L595 and L598) disrupt the association of Rsd with σ^{70} in vitro. However, in contrast with our results, they reported that substitution R596A in σ^{70} did not prevent association of Rsd with σ^{70} (in a glutathione *S*-transferase pulldown assay). To better compare the results of Jishage et al. with our results, we introduced substitutions L595A and L598A into the σ moiety of the α-σ^{70} chimera and tested their effects on the interaction with Rsd in vivo. We found that both the L595A substitution and the L598A substitution strongly inhibited the interactions with Rsd and that their effects were more severe than that of the R596A substitution (data not shown). We suggest, therefore, that the in vivo assay may be more sensitive than the in vitro assay, allowing us to detect the less severe effect of the R596A substitution. Moreover, a structural model of region 4 of σ^{70} based on the crystal structure of the NarL protein (see below) suggests that the side chain of residue 595, at least, is likely to be partially buried. Substitutions at position 595 and possibly also at position 598 may affect the interaction with Rsd indirectly; consistent with this possibility, we found that substitutions L595A and L598A both completely eliminated the ability of λcISa109 to activate transcription from our artificial test promoter in the presence of the α-σ^{70} chimera (data not shown).

Amino acid substitution R596H has been isolated previously. It was first identified based on its effect on expression of the arabinose operon (25, 53). Expression of the *ara* genes is positively controlled by two regulators, the global regulator cyclic AMP receptor protein (CRP) and the operon-specific regulator AraC (15). CRP is active only when it is complexed with the small molecule effector cyclic AMP, and consequently mutant strains that are not able to synthesize cyclic AMP (*cya* mutants) cannot induce expression of CRP-dependent operons, including the arabinose operon. A mutation in the σ^{70} (*rpoD*) gene specifying the R596H substitution was isolated as a suppressor that restored expression of the arabinose operon in a *cya* mutant background (25). It has been suggested that the R596H substitution enhances the ability of AraC to interact productively with RNAP (25, 37). This same amino acid substitution was subsequently isolated based on its ability to suppress the effect of a λcI positive control mutation at the λ promoter P_{RM}. Suppressor mutations in the *rpoD* gene were sought that would reverse the activation defect of a λcI mutant bearing substitution D38N in its activating region, and a single

mutation specifying the R596H change was obtained (35). Although the R596H substitution in σ^{70} reduces the ability of wild-type λcI to activate transcription from P_{RM}, we have shown that this substitution actually enhances the ability of λcISa109 (which bears a non-wild-type residue at position 38) to stimulate transcription from P_{RM} (unpublished data). Evidently, certain amino acid-amino acid combinations at position 38 of λcI and position 596 of σ^{70} permit efficient activation, while others do not.

Taken together, the data obtained in previous studies and data obtained in this study suggest that R596 of σ^{70} is exposed on the surface of the polypeptide such that it can be contacted by interacting proteins. This suggestion is supported by the results of structural modeling. Region 4 of σ^{70} contains a putative helix-turn-helix motif, and the structure of this DNA-binding domain has been modeled based on the three-dimensional crystal structures of two related helix-turn-helix proteins, the *E. coli* NarL protein and the bacteriophage 434 Cro protein (4, 37). In both structural models, residue 596 is solvent exposed and accessible when the protein domain is bound to DNA.

AlgQ and regulation of gene expression in *P. aeruginosa*. Our findings with AlgQ may be relevant to understanding the mechanism by which it regulates gene expression in *P. aeruginosa*. The finding that AlgQ, like Rsd, can interact with a C-terminal fragment of the σ^{70} subunit of RNAP provides strong support for the proposal that it is a functional homolog of Rsd. This conclusion is reinforced by our finding that the interactions of Rsd and AlgQ with region 4 of σ^{70} are both weakened by the same substitution in σ^{70}.

The *algQ* (*algR2*) gene was originally identified as a regulatory gene implicated in activation of the *algD* promoter in experimentally derived mucoid strains *of P. aeruginosa* (8, 32). In particular, a putative *algQ* mutation was found to eliminate transcription from the *algD* promoter in a laboratory strain of *P. aeruginosa* (8). Furthermore, overexpression of *algQ* was shown to reverse the nonmucoid phenotype of spontaneous Alg^- mutants derived from mucoid cystic fibrosis isolates of *P. aeruginosa* (32). Interestingly, expression of *algQ* was also shown to mediate strong activation of the *algD* promoter in *E. coli* cells grown under high-osmolarity conditions (32). Activation of the *algD* promoter has been shown to occur by at least two different mechanisms involving one of two alternative sigma factors, σ^E and σ^{54} (3, 9, 11, 21, 38, 44, 49, 59). In particular, mutations that activate σ^E (encoded by the *algU* gene) lead to increased expression of *algD* (reviewed in reference 9), but *algD* expression can also be activated by a σ^{54}-dependent pathway (3). Our results support the idea that the effect of AlgQ on *algD* expression is likely to be indirect since there is no evidence that the σ^{70} form of RNAP can recognize the *algD* promoter. Possibly AlgQ functions as an anti-σ factor, increasing the amount of RNAP core that is available to bind the relevant alternative σ factor (either σ^E or σ^{54}), thereby increasing the occupancy of the *algD* promoter. It is interesting that *algD* gene expression is negatively controlled by an anti-σ factor (the product of the *mucA* gene), which is specific for σ^E (44, 48, 58). Thus, most mucoid cystic fibrosis isolates of *P. aeruginosa* have been found to bear mutations in the *mucA* gene, which result in constitutive alginate production (2).

Our finding that AlgQ can bind to σ^{70} from *E. coli* as well as to σ^{70} from *P. aeruginosa* could be relevant to its ability to activate the *algD* promoter in *E. coli* (32). Nevertheless, it is possible that the role of AlgQ in activation of the *algD* promoter is unrelated to its ability to bind to σ^{70} in either *P. aeruginosa* or *E. coli*. Although AlgQ was originally reported to have a kinase activity (45), the subsequent finding that it regulates production of a kinase (nucleoside diphosphate kinase) which has a similar molecular weight suggests that AlgQ is not itself a kinase (47).

The regulatory effects of AlgQ are not limited to alginate production. For example, AlgQ regulates production of a variety of secretable virulence factors, up-regulating a neuraminidase and a siderophore and down-regulating extracellular proteases and a rhamnolipid biosurfactant (6, 46, 54). AlgQ also regulates production of Ndk (see above) and succinyl coenzyme A synthetase, an enzyme of the tricarboxylic acid cycle that forms a complex with Ndk in *P. aeruginosa* (33, 46, 47). Finally, characterization of an *algQ* null mutant revealed a dramatic loss of viability in the stationary phase of growth, as well as reductions in the intracellular concentrations of GTP, ppGpp, and inorganic polyphosphate (34). Although the molecular basis for these regulatory effects has not been defined, the pleiotropic nature of AlgQ-dependent phenotypes and our results support the suggestion that AlgQ is a global regulator of transcription in *P. aeruginosa*. In addition to its similarity to Rsd, AlgQ exhibits 58% identity with PfrA, a positive regulator of siderophore biosynthetic genes in *Pseudomonas putida* (54). Interestingly, both PfrA and a putative member of the extracytoplasmic function family of alternative σ factors (PfrI) are required for transcriptional activation of siderophore biosynthetic genes under iron limitation conditions in *P. putida* (54, 55).

Two-hybrid assay for the interaction of transcriptional regulators with region 4 of σ^{70}. The two-hybrid system that we used to study the interactions of Rsd and AlgQ with region 4 of σ^{70} should facilitate studies of other regulators that interact with this region of σ^{70} from *E. coli*, *P. aeruginosa*, or other bacteria. Use of the α-σ^{70} chimeras, in particular, could facilitate genetic analysis of these interactions by providing a convenient vehicle for mutagenesis of region 4 of σ^{70}. Whereas isolation and analysis of *rpoD* mutations are complicated by the fact that σ^{70} is an essential protein that exerts global effects on cellular transcription, our α-σ chimeras exert their effects at specifically designed test promoters. Moreover, mutant chimeras can be assayed to determine their abilities to interact with a number of different regulators so that the specificities of their effects can be assessed.

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