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

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Received 3 May 2001/Accepted 6 August 2001

A number of transcriptional regulators mediate their effects through direct contact with the $\sigma^{70}$ subunit of *Escherichia coli* RNA polymerase (RNAP). In particular, several regulators have been shown to contact a C-terminal portion of $\sigma^{70}$ that harbors conserved region 4. This region of $\sigma$ contains a putative helix-turn-helix DNA-binding motif that contacts the $-35$ element of $\sigma^{70}$-dependent promoters directly. Here we report the use of a recently developed bacterial two-hybrid system to study the interaction between the putative anti-$\sigma$ factor Rsd and the $\sigma^{70}$ subunit of *E. coli* RNAP. Using this system, we found that Rsd can interact with an 86-amino-acid C-terminal fragment of $\sigma^{70}$ and also that amino acid substitution R596H, within region 4 of $\sigma^{70}$, weakens this interaction. We demonstrated the specificity of this effect by showing that substitution R596H does not weaken the interaction between $\sigma$ and two other regulators shown previously to contact region 4 of $\sigma^{70}$. 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 $\sigma^{70}$ subunit of RNAP from this organism. We found that amino acid substitution R600H in $\sigma^{70}$ from *P. aeruginosa*, corresponding to the R596H substitution in *E. coli* $\sigma^{70}$, specifically weakens the interaction between AlgQ and $\sigma^{70}$. Taken together, our findings suggest that Rsd and AlgQ contact similar surfaces of RNAP present in region 4 of $\sigma^{70}$ 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 $\sigma$ factors, and $\sigma^{70}$ is the principal $\sigma$ factor (27). The presence of different types of $\sigma$ 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 $\sigma$ 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-$\sigma$ factors, which are regulatory proteins that bind $\sigma$ factors and often prevent their association with the RNAP core enzyme (19, 26). Anti-$\sigma$ factors ultimately inhibit transcription from the class of promoters recognized by the $\sigma$ factors that they sequester.

The $\sigma^{70}$ 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 $\sigma^{70}$ often contact a region of $\sigma$ that contains a putative helix-turn-helix DNA-binding motif responsible for contacting the $-35$ elements of $\sigma^{70}$-dependent promoters (18, 23, 37). This DNA-binding region of $\sigma$ is conserved in members of the $\sigma^{70}$ 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 $\sigma^{70}$ 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 $\sigma^{70}$ (but not with several alternative $\sigma$ factors) and was shown to be capable of inhibiting $\sigma^{70}$-dependent transcription from certain promoters in vitro (28). The binding site for Rsd on $\sigma^{70}$ 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 $\sigma^{70}$-dependent transcription that occurs as cells enter the stationary phase of growth, Jishage and Ishihama suggested that Rsd might be an anti-$\sigma$ factor (28). Subsequent work has shown that consistent with this idea, Rsd may facilitate the replacement of $\sigma^{70}$ by the stationary-phase-specific $\sigma$ factor $\sigma^{38}$ in functional RNAP holoenzyme complexes as cells go from the exponential phase to the stationary phase of growth (29).

The sequence of putative anti-$\sigma$ 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
responses and antibiotics (10). AlgQ was originally identified as a positive transcriptional regulator of the key alginate bio-
synthetic 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^70 (AlgU/AlgT) and the other of which is rec-
ognized by RNAP containing σ^34 (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 mecha-
nism 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 specifi-
cally weakens the interaction of Rsd and AlgQ with the σ moe-
ity.

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 lacO_62-61 driving expression of a linked lacZ reporter
gene and has been described previously (14). E. coli SFI harbors an F′ episome
containing the lac promoter derivative p lacO_5-55/Cons – 35 driving expression of
a linked lacZ reporter gene and has also been described previously (13).
Plasmids pACcIc, pACcIcR, pBRcR, pBRcR-σ^34, pBRcR-σ^70, and pBRcR-σ^34-70 (R596h) have all been described previously (13, 14). Plasmid pACcIc-Rsd encodes
lacI (residues 1 to 236) fused to Rsd (residues 1 to 236) via three alanine
residues. pACcI-Rsd was cloned by using the appropriate Not I-Bam HI-di-
gested PCR product into Not I-Bam HI-digested pACcIβ32 (24); expression of the
cI-rad fusion gene was therefore under the control of the lacUV5 promoter. Plasmid pACcIc-AlgQ encodes lacI (residues 1 to 286) fused to AlgQ (residues 1 to 160) via three alanine residues, and it was made in a manner similar to the manner in which pACcIc-Rsd was made. Expression of the cI-rad fusion gene on
pACcIc-AlgQ is also under the control of the lacUV5 promoter. Plasmid pACcIc-Asia encodes lacI (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 pACcIc-Rsd was made. Plasmid pACcIc-35cI-Asia contains the cI-asia fusion gene from plasmid pACcIc-Asia under the control of a lacUV5 promoter variant in which the 35-fragment of the promoter has been deleted. Plasmid pACcIc-35cIc-Asia, therefore, expresses less of the
lacI-Asia fusion protein than plasmid pACcIc-Asia expresses under identical
conditions. pACcIc-35cIc-Asia was made by cloning the appropriate HindIII-Not I fragment from plasmid pACcIc-Asia into plasmid pAB32 (57) cut with
both HindIII and Not I. Plasmid pBRcR-σ^70 harbors 1 to 248 of the σ 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 HindIII-Bam HI-digested pBRcR. Expression of the chimeric gene
on pBRcR-σ^70 is, therefore, under the control of tandem bsd and lacUV5
promoters. pBRcR-σ^70 (R600H) is a derivative of pBRcR-σ^70 in which the
R600H substitution in the σ moiety of the chimera was introduced by PCR.
The lac promoter derivative p lacCP3-93+ O_2-62 was made by the PCR and
contains two λ operators; a near-consensus λ operator (TACACCCGGCGGT-
GATA) and Ω2 (CAACACCCGCAGAGATA) are centered 93 and 62 bp,
respectively, upstream of the transcriptional start site of the lac core promoter. Plasmid pFW11-COP-93+ O_2-62 was constructed by cloning an EcoRI-HindIII-
cut PCR product containing p lacCP3-93+ O_2-62 into pFW11 (56) cut with
EcoRI and HindIII. Plasmid pFW11-COP-93+ O_2-62 was then transformed
into strain CS1010, and the promoter-lacZ fusion was recombined onto an F′ episome and mated into strain FW102 (56) to create reporter strain F^R632.
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 kana-
mycin (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-CHCl₃, and assayed for β-galactosi-
dase 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

A. coli-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 λcl-Rsd
dimer (Fig. 1A). Having fused the entire Rsd protein (residues 1 to 158) to the C-terminal of λcI, we placed the gene encoding this chimeric protein on a plasmid vector down-
stream of the IPTG-inducible lacUV5 promoter, thus creating
plasmid pACcIc-Rsd. We used plasmid pBRcR-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 σ subunit of RNAP (13). We introduced plasmids
pACcIc-Rsd and pBRcR-σ^70 into E. coli KS1 (14), which
harbors on its chromosome the lac promoter derivative p lacO_2-
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 transcrip-
tion from the p lacO_2-62 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 con-
taining only wild-type α. An additional control revealed that
λcI (lacking the fused Rsd moiety) did not activate transcrip-
tion 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^70 chimera (comprising residues 1 to 248 of α fused to
residues 243 to 330 of σ^38 ) encoded by plasmid pBRcR-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 ge-
etic tool for dissecting this interaction. We were particularly
interested in identifying mutant forms of σ^70 that were specif-
activity.

of different concentrations of IPTG and assayed for
/H9252
plasmids expressing the indicated proteins were grown in the presence
/H11011
dependent activation (to a factor of
/H9261
substitutions on the ability of the
/H9261
moiety of the
/H9261
lac
/H9261
cI-Rsd on transcription in vivo from p
/H9251
gene. The N-terminal domain of
/H9261
lacZ
/H409
located on the chromosome and drives expression of a linked
/H9247
lac
/H409
core promoter. In reporter strain KS1 the p
/H9251
OR2-62 test promoter is
/H9261
lac
/H418
moiety of the
/H9261
OR2-62 chimera. (A) Replacement of the RNAP α-CTD by a C-terminal
/H9251
fragment of
/E. coli
α70
(residues 528 to 613) permits interaction with the Rsd moiety of a
/H9268
lcl-Rsd chimera bound to DNA. The diagram depicts the test promoter
/H9268
placO2-62, which bears the λ operator O2 centered 62 bp upstream from the transcriptional start site of the lac
/H9247
core promoter. In reporter strain KS1 the placO2-62 test promoter is
/H9261
located on the chromosome and drives expression of a linked
/H9247
lacZ
/H436
gene. The N-terminal domain of α is designated αNTD. (B) Effect of
/H9268
lcl-Rsd on transcription in vivo from placO2-62 in the presence of the
/H9261
α-σ70
or α-σ70
(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.

phyzically defective in the ability to interact with Rsd. We therefore
introduced a variety of amino acid substitutions into the σ
moiety of the α-σ70 chimera and tested the effects of these
substitutions on the ability of the lcl-Rsd fusion protein to
mediate transcriptional activation from the test promoter. Figure
1B shows that substitution R596H in the σ moiety of the
/H9261
α-σ70
chimera strongly reduced the magnitude of lcl-Rsd-dependent activation (to a factor of ~5). Substitution R596A in the σ moiety of the α-σ70 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 lcl-Rsd-dependent activation (data not shown).

Substitution R596H in the σ moiety of the α-σ70 chimera
do not compromise the ability of a superactivating variant of
lcl to stimulate transcription from an appropriate test promoter.
To determine whether the R596H substitution affects the
interaction of σ70 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 lcl protein (a
transcriptional activator as well as a repressor) can interact
specifically with the σ moiety of the α-σ70 chimera and stabilize
its binding to a promoter −35 element (13). The in vivo assay
which we designed to detect the interaction between lcl and
region 4 of σ70 is shown in Fig. 2A. In this experimental setup
a DNA-bound lcl dimer activates transcription from test promoter
placO2-55/Cons-35 by stabilizing the binding of the σ
moiety of the α-σ70 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
lcl 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-σ70
chimera has an effect on the ability of a superactivating variant of
lcl to activate transcription from test promoter placO2-55/Cons-35
(Fig. 2A). Reporter strain SF1 carries promoter
placO2-55/Cons-35 fused to the lacZ
/H420
gene in single copy on an F'
/H11002
episode (13). We assayed the ability of lcl superactivator
109
(lclSa109) (5, 13) to activate transcription from this
reporter in the presence of the α-σ70 chimera with or without
the R596H substitution in the σ moiety. lclSa109 activated
transcription a maximum of ~5.5-fold from the test promoter
in the presence of the α-σ70 chimera with 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 lcl to
activate transcription from P
/H35
[35], we have observed that
this substitution does not inhibit the ability of lcl Sa109 to
activate transcription from P
/H35
[see below].) We concluded that
substitution R596H in the σ moiety of the α-σ70 chimera

FIG. 1. Transcriptional activation by lcl-Rsd in the presence of the
α-σ70 chimera. (A) Replacement of the RNAP α-CTD by a C-terminal
fragment of E. coli α70 (residues 528 to 613) permits interaction with the Rsd moiety of a lcl-Rsd chimera bound to DNA. The diagram depicts the test promoter placO2-62, which bears the λ operator O2 centered 62 bp upstream from the transcriptional start site of the lac core promoter. In reporter strain KS1 the placO2-62 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 lcl-Rsd on transcription in vivo from placO2-62 in the presence of the α-σ70 or α-σ70 (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 lcl superactivator in the presence of the α-σ70 chimera. (A) lclSa109 stabilizes the binding of region 4 of σ70 to an ectopic −35 element. The diagram depicts the test promoter placO2-55/Cons-35, which bears an ectopic −35 element and the λ operator O2 centered 45.5 and 55 bp, respectively; upstream from the transcriptional start site of the lac core promoter. In reporter strain SF1 the placO2-55/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-σ70 chimera on the ability of lclSa109 to activate transcription in vivo from placO2-55/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.
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 α°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 cI. We placed the gene encoding this chimeric protein on a plasmid vector downstream of the IPTG-inducible lacUV5 promoter, creating plasmid pACcI-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°70PA. The hybrid α-σ°70PA gene encoding this chimera was placed on a plasmid vector downstream of tandemly arranged lpp and lacUV5 promoters, creating plasmid pBRα-σ°70PA. We introduced plasmids pACcI-AlgQ and pBRCα-σ°70PA into E. coli KS1. We then tested the ability of the λcI-AlgQ chimera to activate transcription from placO2-62 in the presence of the α-σ°-70PA chimera (Fig. 3A). Figure 3B shows that cI-AlgQ activated transcription from the test promoter a maximum of ~17-fold in cells containing the α-σ°-70PA 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°-70PA chimera (Fig. 3B).

Substitution R600H in the σ moiety of the α-σ°-70PA 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°-70PA chimera. To do this, we made a version of the α-σ°-70PA chimera harboring substitution R600H [α-σ°-70PA(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°-70PA(R600H) chimera, compared to a maximum of ~17-fold with the chimera derived from the wild-type form of P. aeruginosa σ°70.

Substitution R600H in the σ moiety of the α-σ°-70PA 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°70PA and AlgQ, we first tested whether λcIsa109 could interact with the σ moiety of the α-σ°-70PA chimera. We found that λcIsa109 stimulated transcription from test promoter placO2-62/55/Cons-35 up to ~3.5-fold specifically in the presence of the α-σ°-70PA chimera (Fig. 4). Furthermore, introduction of the R600H substitution into the σ moiety of the α-σ°-70PA 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 α°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-σ⁷₀PA chimera. (A) λcI-AsiA stabilizes the binding of region 4 of σ⁷₀ from P. aeruginosa to an ectopic −35 element. The diagram depicts the test promoter placO₂₉-55/Cons-35, which bears an ectopic −35 element and the λ operator O₉ centered 45.5 and 55 bp, respectively, upstream from the transcriptional start site of the lac core promoter. In reporter strain SF1 the placO₂₉-55/Cons-35 test promoter is located on an F’ episome and drives expression of a linked lacZ gene. (B) Effect of substitution R₆₀₀H into the σ moiety of the α-σ⁷₀PA chimera on the ability of λcI-AsiA to activate transcription in vivo from placO₂₉-55/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 σ⁷₀ from P. aeruginosa, and AlgQ can interact with region 4 of σ⁷₀ from E. coli. (A) Effect of λcI-Rsd on transcription in vivo from placO₂₉-62 in the presence of the α-σ⁷₀PA, α-σ⁷₀PA(R₆₀₀H), or α-σ⁷₀PA 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 placO₂₉-62 in the presence of the α-α-σ⁷₀PA(R₅₉₆H), or α-σ⁷₀PA chimeras. 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.
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 λcl-AsiA chimeras to activate transcription from placCOP-93+O2-2-62. Similarly, the R596A substitution in the E. coli σ moiety did not reduce the stimulatory effect of the λcl-AsiA chimeras (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 σ70 is at or near the contact surface for Rsd and the equivalent residue of P. aeruginosa σ70, R600, is at or near the contact surface for AlgQ (see Discussion). Furthermore, the finding that substitution R596A in E. coli σ70 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 σ70 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 σ70 subunit of E. coli and P. aeruginosa RNAP, respectively. This fragment of σ70 encompasses conserved region 4, which contains a DNA-binding domain that mediates recognition of the −35 element of σ70-dependent promoters (18). Furthermore, each protein can also interact with the corresponding σ70 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 σ70, respectively) that inhibits the binding of both Rsd and AlgQ to either σ70 fragment. The interchangeability of the E. coli and P. aeruginosa σ70 fragments in our experiments suggests that regulators from P. aeruginosa or E. coli that interact with region 4 of σ70 might be expected to function in either organism. In fact, a previous in vitro study showed that the λcl protein (which contacts region 4) can activate transcription from the λ promoter PRM by the P. aeruginosa RNAP (16).

**Analysis of the interaction between Rsd and region 4 of σ70.** Our bacterial two-hybrid results for Rsd and region 4 of σ70 are consistent with the biochemical data of Jishage and Ishihama (28). These authors found that Rsd could interact with σ70 in vitro but not with σ28 (or several other alternative σ factors), and they showed that Rsd could interact with a tryptic fragment of σ70 composed of residues −550 to 613. We found that Rsd can interact in vivo with an 86-amino-acid C-terminal fragment of σ70 that contains region 4 but cannot interact with the equivalent 88-amino-acid C-terminal fragment of σ28. 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 σ70 in vitro. However, in contrast with our results, they reported that substitution R596A in σ70 did not prevent association of Rsd with σ70 (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-σ70 chimeras 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 σ70 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 λclIsa109 to activate transcription from our artificial test promoter in the presence of the α-σ70 chimeras (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 σ70 (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 λcl positive control mutation at the λ promoter PRM. Suppressor mutations in the rpoD gene were sought that would reverse the activation defect of a λcl mutant bearing substitution D38N in its activating region, and a single
shown to mediate strong activation of the algD gene (9), but mutations that activate the particular mechanism by which it regulates gene expression in DNA.

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 σ70. The two-hybrid system that we used to study the interactions of Rsd and AlgQ with region 4 of σ70 should facilitate studies of other regulators that interact with this region of σ70 from E. coli, P. aeruginosa, or other bacteria. Use of the α-σ70 chimeras, in particular, could facilitate genetic analysis of these interactions by providing a convenient vehicle for mutagenesis of region 4 of σ70. Whereas isolation and analysis of rpoD mutations are complicated by the fact that σ70 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.

ACKNOWLEDGMENTS

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This work was supported by National Institutes of Health grant GM44025, by an established investigatorship from the American Heart Association (to A.H.), and by a Charles A. King Trust postdoctoral fellowship (to S.L.D.).

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mutations specifying the R596H change was obtained (35). Although the R596H substitution in σ70 reduces the ability of wild-type λcI to activate transcription from P_RM, we have shown that this substitution actually enhances the ability of λcI and position 596 of σ70 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 σ70 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 σ70 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 σ70 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 σ70 are both weakened by the same substitution in σ70.

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, σE and σ54 (3, 9, 11, 21, 38, 44, 49, 59). In particular, mutations that activate σE (encoded by the algU gene) lead to increased expression of algD (reviewed in reference 9), but algD expression can also be activated by a σ54-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 σ70 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 σE or σ54), 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 σ54 (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 σ70 from E. coli as well as to σ70 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 σ70 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 secretible 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).

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