THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 30, Issue of July 26, pp. 27282–27287, 2002

Printed in U.S.A.

Interaction of the Conserved Region 4.2 of σ^E with the RseA Anti-sigma Factor*

Received for publication, March 25, 2002, and in revised form, May 8, 2002
Published, JBC Papers in Press, May 16, 2002, DOI 10.1074/jbc.M202881200

Christina Tam, Bruno Collinet, Gary Lau, Satish Raina‡, and Dominique Missiakas§

From the Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637 and the ‡Departement de Biochimie Médicale, Centre Médical Universitaire, 1 Rue Michel Servet, 1211 Geneva 4, Switzerland

Eσ^E RNA polymerase transcribes a regulon of folding factors for the bacterial envelope and is induced by physical and chemical stresses. The RseA anti-sigma factor inhibits the activity of Eσ^E RNA polymerase. It is shown here that the N-terminal portion of σ^E, residues 1–153, binds core RNA polymerase. RseA interacts with residues 154–191 of σ^E, a site that is homologous to region 4, the sigma factor binding site for promoter DNA. Mutations that reduce transcription of Eσ^E RNA polymerase map to σ^E residues 178, 181, and 183. Variant σ^E proteins with amino acid substitutions at residues 178, 181, or 183 do not associate with RseA. A regulatory mechanism is proposed whereby RseA binds to a C-terminal peptide of σ^E and inhibits the transcription of Eσ^E RNA polymerase by blocking promoter recognition.

σ^E (RpoE)[1] is a member of the extracytoplasmic function (ECF) subfamily of sigma factors, which transcribes genes that encode protein folding factors in response to extracytoplasmic stress stimuli (1, 2). In *Escherichia coli*, the unfolding of proteins in the cell envelope appears to be a primary stimulus that activates the σ^E-dependent response (1, 3). Previous work identified genes that are transcribed by Eσ^E RNA polymerase. The Eσ^E regulon controls at least two cellular processes, folding of polypeptides in the bacterial envelope and biosynthesis/transport of lipopolysaccharides (4). σ^E is encoded by the *rpoE* gene and in part regulates its own expression, as Eσ^E RNA polymerase transcribes *rpoE* as well as the downstream genes *rseA/B/C*.

Conditions that cause unfolding of polypeptides in the envelope signal the Eσ^E response by a mechanism that requires RseA and RseB (5). The genes for these two regulators of Eσ^E are located immediately downstream of *rpoE*. RseA is a short polypeptide that integrates into the cytoplasmic membrane. The N-terminal cytoplasmic domain of RseA is known to bind σ^E, whereas the C-terminal domain of RseA protrudes into the periplasm. The C-terminal domain of RseA interacts with RseB in the periplasm, a compartment that is located between the inner and outer membranes of *E. coli* (6, 7). RseB binding to RseA increases the affinity of the RseA/RseB complex for σ^E. Stresses that cause unfolding of proteins in the bacterial envelope lead to the dissociation of RseA/RseB, thereby reducing the affinity of RseA for σ^E.

The domain structure of sigma factors has been probed with trypsin cleavage of the peptide backbone under conditions of limited proteolysis (8). σ^{70}, the major sigma factor of *E. coli* RNA polymerase, is a 613-amino acid residue polypeptide with two preferred trypsin cleavage sites, suggesting that it is assembled from folded subdomains (see diagram in Fig. 1). The N-terminal cleavage fragment of σ^{70}, residues 1–114, has not yet been analyzed in depth. The central domain, $\sigma^{70}{}_2$ (residues 104–448), is capable of associating with core RNA polymerase in a manner that allows binding of the $\sigma^{70}{}_2$ holoenzyme to single stranded DNA oligonucleotides that encompass the promoter binding site for σ^{70} RNA polymerase. Despite this DNA binding activity, $\sigma^{70}{}_2$ holoenzyme does not promote transcription *in vitro*. The C-terminal trypsin fragment of σ^{70}, $\sigma^{70}{}_{3,4}$ (residues 449–613), binds to the coliphage T4 anti-sigma factor AsiA (9–11). The formation of a complex between AsiA with the C-terminal domain 4.2 of σ^{70}-(551–608) prevents the association of RNA polymerase holoenzyme with −35 promoter sequences and blocks σ^{70} RNA polymerase transcription during T4 coliphage infection. Rsd, the anti-sigma factor of *E. coli*, also binds to region 4.2, suggesting that inactivation of σ^{70} may occur by a similar mechanism (12–14). Promoters that are composed of an extended −10 binding site for σ^{70} RNA polymerase but lack the canonical −35 sequence can be transcribed by a truncated RNA polymerase, σ^{70}-(1–529) (15). Although this has not yet been tested experimentally, σ^{70}-(1–529) is presumably refractory to AsiA- or Rsd-mediated inhibition.

By using limited trypsin digestion and peptide mapping, it is reported here that an N-terminal portion of σ^E, residues 1–153, binds core RNA polymerase but not RseA. The remaining portion of σ^E, residues 154–191, is homologous to region 4.2, the sigma factor binding site for promoter DNA. In order to establish the biochemical activity of the putative region 4.2, mutations that reduce transcription of Eσ^E RNA polymerase were isolated and mapped to σ^E residues 178, 181, and 183. The variant σ^E proteins failed to associate with RseA. These findings suggest that binding of RseA to region 4.2 of σ^E prevents transcription of Eσ^E RNA polymerase by blocking promoter recognition.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Strains used in this study are listed in Table I. Sequences of primers used in this study can be obtained from the authors upon request. When necessary, Luria Bertani (LB) medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml). Induction of $_{His}$RseA^N

* This work was supported by United States Public Health Service Grant GM58266. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, The University of Chicago, 920 East 58th St., Chicago IL 6063. Tel.: 773-834-8161; E-mail: dmissiak@bsd.uchicago.edu.

[1] The abbreviations used are: RpoE or σ^E, sigma E transcription factor; E or $\alpha_2\beta\beta'$, core RNA polymerase; Eσ^E or $\alpha_2\beta\beta'$ σ^E, holoenzyme complexed to sigma E; Rse, regulator of σ^E; *rpoE*R178G, *rpoE*I181A, and *rpoE*V185A, alleles of *rpoE* encoding mutants of σ^E with severely impaired transcriptional activity; $_{His}$RseA^N and RseA$^N{}_{His}$, N-terminal domain of RseA fused to a 6-histidine tag at the N terminus or C terminus, respectively; NTA, nitrilotriacetic acid; DTNB, dithionitrobenzoate.

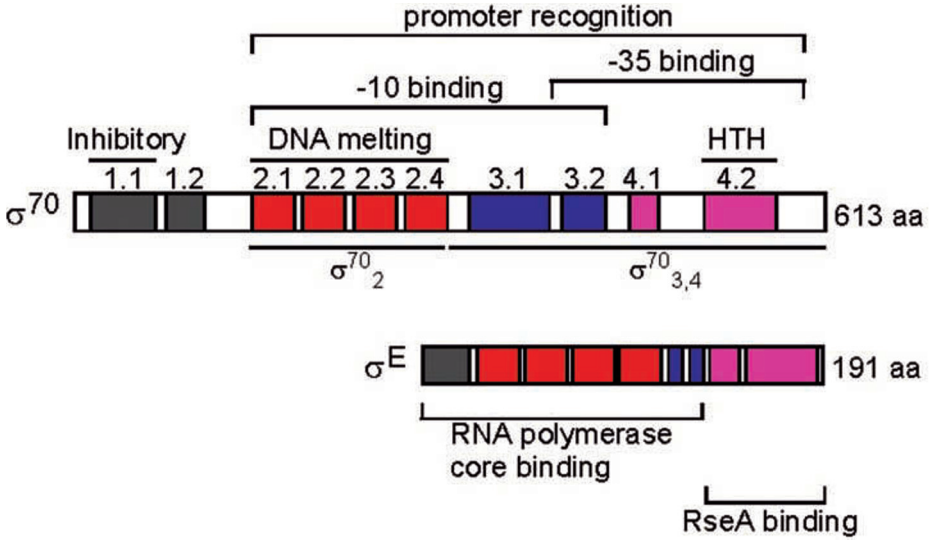

FIG. 1. **Domain structure of σ^{70} and σ^E.** Regions 1, 2, 3, and 4 of σ^E were defined by Lonetto *et al.* (2) using BLAST searches with σ^{70}. *HTH* indicates the position of a helix-turn-helix motif. $\sigma^{70}{}_2$ (residues 104–448) and $\sigma^{70}{}_{3,4}$ (residues 449–613) are two fragments obtained by limited trypsin digestion of σ^{70}. $\sigma^{70}{}_2$ is capable of associating with core RNA polymerase but does not promote transcription *in vitro*. $\sigma^{70}{}_{3,4}$ binds to Rsd and to the coliphage T4 anti-sigma factor, AsiA. This study examines the σ^E domains involved in binding to RNA polymerase core or the anti-sigma factor RseA; the results are summarized in the drawing.

TABLE I
Bacterial strains used in this study

Strains	Relevant characteristics	Source
MC4100	F$^-$ *araD*139 Δ(*argF-lac*) U169	(28)
LMG194	F$^-$ *lacX*74 *galE thi rpsL phoA ara*714 *leu*::Tn10	Invitrogen
DM1917	MC4100 *rpoH*P3-*lacZ*	(18)
DM1856	MC4100 *htrA-lacZ*	(29)
SR4965	MC4100 *rpoE*P2-*lacZ*	(7)
SR1502	MC4100 *rpoE*P2-*lacZ nadB*::Tet *rpoE*R178G	This study
SR1503	MC4100 *rpoE*P2-*lacZ nadB*::Tet *rpoE*I181A	This study
SR1504	MC4100 *rpoE*P2-*lacZ nadB*::Tet *rpoE*V185A	This study

and $RseA^N{}_{His}$ and the various $_{His}\sigma^E$ truncations or mutants was accomplished by addition of arabinose (0.2%) or isopropyl-1-thio-β-D-galactopyranoside (1 mM) to cultures of *E. coli* LMG194 or BL21, respectively, harboring the appropriate plasmids. *rpoE* alleles were transduced along with the linked *nadB*::Tet marker to the desired background using P1 bacteriophage as described (16).

Cloning Procedures—The DNA regions corresponding to fragments or mutants of σ^E polypeptide were amplified by PCR using appropriate primers and cloned into pBAD-B vector (Invitrogen) using the appropriate restriction sites. Plasmids were verified by DNA sequence analysis. In this manner, the cloned DNA fragments were fused to generate a translational fusion between a 6-histidine tag followed by a cleavable enterokinase site. To improve production yields, the DNA fragments were subcloned into pET-24d vector, using *Nco*I and *Eco*RI restriction sites. Such plasmids were transformed in strain BL21, and gene expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside.

Protein Purification—The histidine-tagged N-terminal RseA proteins were purified from cell extracts of *E. coli* strain LMG194 (pBAD-$B\text{-}_{His}RseA^N$; the 6-histidine tag can be removed by enterokinase cleavage) or *E. coli* strain BL21 (pET-24d-$RseA^N{}_{His}$). Cells of 2L culture were harvested by centrifugation at 3,000 × *g* for 10 min, suspended in buffer A (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, pH 8.0), and lysed in a French pressure cell at 14,000 p.s.i. Unbroken cells were removed by centrifugation at 3,000 × *g* for 10 min, and the supernatant was centrifuged at 100,000 × *g* for 45 min at 4 °C. $RseA^N$ pelleted with the insoluble material. The protein was recovered from the pellet using buffer A containing 8 M urea. This treatment solubilized the protein that could be recovered after centrifugation at 100,000 × *g* for 45 min at 4 °C to 90% homogeneity. The supernatant was subjected to affinity chromatography using 1 ml of nickel-NTA resin (Qiagen). The resin was washed with buffer A supplemented with 10 mM imidazole, and $RseA^N$ with a C-terminal histidine tag was eluted using an imidazole gradient. The N-terminal tagged $RseA^N$ protein was recovered by treatment of the beads with enterokinase as indicated by the manufacturer (Invitrogen). Purification of $_{His}\sigma^E$ full-length and the various protein mutants and fragments was performed as described for $_{His}\sigma^E$ (5).

Binding Assays—For affinity measurements, 50% slurry of nickel-NTA was prepared that contained bound 6-histidine-tagged proteins ($RseA^N$, wild-type σ^E, or mutant σ^E or σ^E fragments each at 1–2 pmol protein/μl slurry). Increasing concentrations of substrate proteins (only the 5- or 10-fold molar excess are shown in the figures) were added to a 1-ml suspension of 50 μl of nickel-NTA-Sepharose charged with bait protein in buffer A containing 0.2% octylglucoside. Samples were incubated for 2 h at 20 °C and centrifuged at 3,000 × *g* for 5 min. The supernatant (850 μl) was removed and protein precipitated with 10% trichloroacetic acid. Sediments were washed with acetone, solubilized in 50 μl of 0.1 M Tris-HCl, 4% SDS, pH 7.0, and heated at 95 °C for 5 min. The beads were washed three times with 1 ml of buffer A containing 0.2% octylglucoside. Washes were completed within 10 min, and no significant elution was observed during washes. Samples were separated on 12% SDS-PAGE, electrotransferred to polyvinylidene difluoride membrane and immunoblotted with anti-σ^E, anti-RseA, or anti-RpoA (the α subunit of core polymerase) antibodies. Immune complexes were detected using a secondary antibody linked to horseradish peroxidase.

Biochemical Assays—β-Galactosidase activity was determined as described previously (16). Modification of sulfhydryl groups using Ellman's reagent (dithionitrobenzoate (DTNB)) was performed at 20 °C using 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 8 μM σ^E protein in the presence or absence of $RseA^N$ (a 2–160 μM range of concentration was used; only the 10-fold molar excess is shown in Fig. 4). Upon addition of DTNB (250 μM) to a 1-ml reaction volume, formation of 5-thio-2-nitrobenzoate was monitored at 412 nm and quantified using the extinction coefficient value of 14150 $M^{-1}cm^{-1}$ (17).

Trypsin digestion reactions contained 20 mM Tris-HCl (pH 7.9), 50 mM NaCl, 5% glycerol, 0.1 mM EDTA, 200 pmol of σ^E, and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at either a 1:200, 1:400, or 1:800 ratio of trypsin over σ^E. The reactions were incubated at 25 °C and stopped by addition of 1 mM phenylmethylsulfonyl fluoride and 10% trichloroacetic acid. Proteins were separated on 15% SDS-PAGE and analyzed by immunoblotting using anti-σ^E antibodies. Edman degradation and electrospray-ionization mass spectrometry measurements of trypsin digests were performed by the Rockefeller University Protein Sequencing Facility and the Mass Spectrometry Facility at the University of California, Los Angeles.

RESULTS

Trypsin Digestion of σ^E—We sought to probe the domain structure of the *E. coli* extracellular sigma factor, σ^E, by trypsin cleavage. σ^E protein was purified from an engineered *E. coli* strain that overexpressed a His-tagged variant of the sigma factor using affinity chromatography. The N-terminal histidine tag was removed by enterokinase cleavage, causing σ^E elution from nickel-NTA-Sepharose. The eluate was subjected to ion exchange chromatography yielding a more than 95% pure preparation of σ^E. Purified σ^E is soluble and stable at 4 °C. It is capable of binding core RNA polymerase or the RseA anti-sigma factor (see below). These results suggested that our purified σ^E was correctly folded and functional. Incubation of purified σ^E with dilute amounts of trypsin caused peptide cuts at many different sites, generating a large spectrum of cleavage fragments. A 10-kDa fragment of σ^E appeared to be resistant to

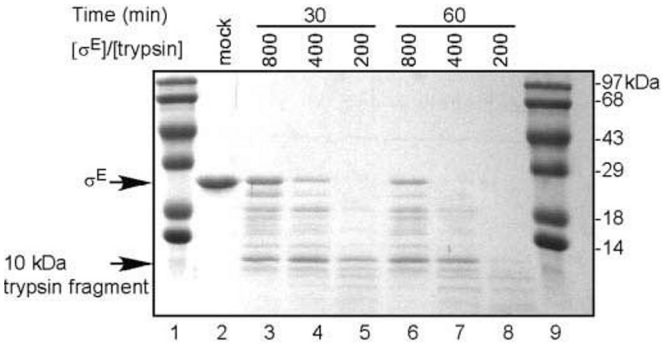

FIG. 2. **Trypsin digestion of σ^E.** Purified σ^E was incubated with trypsin for 30 and 60 min at 200, 400, or 800 molar excess σ^E over trypsin (*lanes 3–8*). Proteolysis was quenched by the addition of phenylmethylsulfonyl fluoride and precipitation with trichloroacetic acid. Protein was separated on 15% SDS-PAGE and analyzed by Coomassie Blue staining. Electrospray-ionization mass spectrometry revealed an average compound mass of 10506.0 for the10-kDa cleavage fragment. Edman degradation revealed the presence of the N-terminal methionine as well as the predicted peptide sequence, indicating that trypsin had cleaved the C-terminal of σ^E and that the 10-kDa peptide comprises σ^E residues 1–91. Prestained molecular weight markers were separated in *lanes 1* and *9*; the *numbers* indicate the molecular mass in kDa. Purified undigested σ^E was separated in *lane 2* (*mock*).

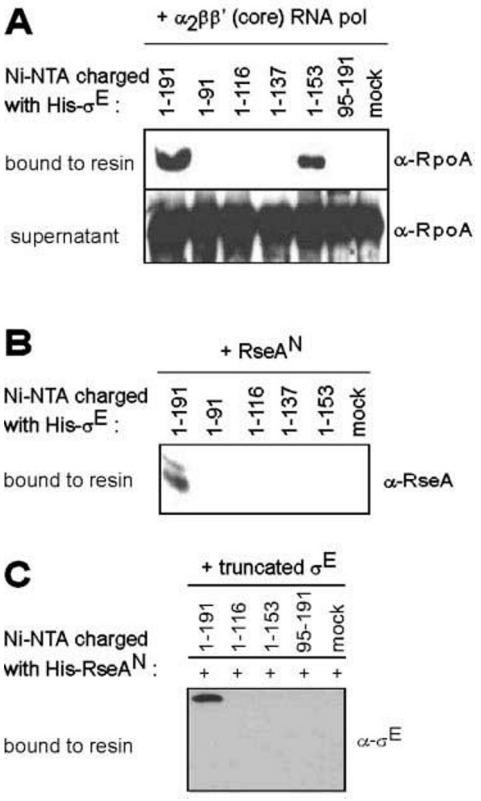

FIG. 3. **Interaction of σ^E fragments with core polymerase and RseA^N.** Binding of core RNA polymerase or RseA^N to the σ^E fragments was assessed in a co-sedimentation assay using nickel-NTA-Sepharose. Sepharose resin was charged with histidine-tagged polypeptides and used as bait to capture proteins in solution (50% slurry of beads). Proteins that are bound to the resin were collected by slow speed centrifugation, washed three times, and eluted with imidazole. Both bound and unbound proteins were precipitated with trichloroacetic acid, washed in acetone, and analyzed on 12% SDS-PAGE followed by immunoblotting with antibodies directed against RpoA (the α subunit of RNA polymerase) (*A*), RseA^N (*B*), or σ^E (*C*). Full-length σ^E spans residues 1–191. The fragments σ^E_{1-91}, σ^E_{1-116}, σ^E_{1-137}, σ^E_{1-153}, and σ^E_{95-191} are depicted as *1–91*, *1–116*, *1–137*, *1–153*, and *95–191*. σ^E proteins were used at a final concentration of 1 pmol/μl resin and incubated with purified core RNA polymerase (10-fold excess) for 2 h at room temperature (*A*) or 5-fold excess of purified RseA^N for 2 h at room temperature (*B*). As RseA^N did not co-sediment with histidine-tagged σ^E fragments (*B*), the reciprocal experiment was performed (*C*) by charging nickel-NTA resin with RseA$_{His}$ (2 pmol/μl resin) and incubating RseA^N with 10-fold excess of σ^E.

cleavage by dilute amounts of trypsin (Fig. 2). Nevertheless, an increase in the molar ratio of trypsin to σ^E to 1/200 caused cleavage of the entire polypeptide into fragments of small sizes that eluded further analysis on SDS-PAGE. Thus, in contrast to the trypsin-resistant σ^{70}, σ^E seems sensitive to protease cleavage, suggesting a folded structure that is less compact and more accessible to protease.

Electrospray-ionization mass spectrometry and Edman degradation were used to characterize the 10-kDa fragment of σ^E that resisted trypsin cleavage at dilute or intermediate enzyme concentrations. The 10-kDa fragment corresponded to amino acid residues 1–91 of σ^E. This portion of σ^E carries a peptide with sequence homology to region 1 and region 2 of σ^{70} (Fig. 1). Region 2 of σ^{70} contains four functional subdomains, designated 2.1, 2.2, 2.3, and 2.4. Homologous subdomains were also identified within σ^E using BLAST homology searches (Fig. 1). Subdomain 2.1 represents the binding region for RNA polymerase core enzyme, whereas subdomain 2.3 contributes to DNA melting. The subdomain 2.4 is involved in promoter recognition in the -10 nucleotides region upstream of the transcriptional start site. The N-terminal trypsin-resistant fragment of σ^E (residues 1–91) binds neither to core RNA polymerase nor to RseA (see below). Together these results indicate that, unlike σ^{70}, trypsin cleavage of σ^E does not separate folded domains with distinct function.

A Subdomain of σ^E Binds Core RNA Polymerase—We sought to map the binding sites of σ^E for both RNA polymerase core enzyme and the anti-sigma factor RseA. N- and C-terminal truncations of σ^E were generated by PCR amplification using specific primers and *rpoE* template DNA (*rpoE* encodes σ^E). $_{His}\sigma^E$ (amino acids 1–191), $_{His}\sigma^E_{1-91}$, $_{His}\sigma^E_{1-116}$, $_{His}\sigma^E_{1-137}$, $_{His}\sigma^E_{1-153}$, and $_{His}\sigma^E_{95-191}$ are *rpoE* variants of variable lengths bearing an N-terminal histidine tag. The subscript numbers indicate the RpoE amino acid positions at the beginning and the end of each fragment, respectively. His-tagged σ^E variants were purified by affinity chromatography, and their ability to interact with either RseA^N or core RNA polymerase was tested *in vitro*.

Aliquots of nickel-NTA-Sepharose precharged with σ^E protein were dispensed as a 50% slurry into buffer containing 10-fold excess of purified *E. coli* core RNA polymerase. $_{His}\sigma^E$-$\alpha_2\beta\beta'$ binding was measured as the amount of RNA polymerase α subunit that sedimented during 4,000 $\times$ *g* centrifugation with nickel-NTA-Sepharose. Full-length RpoE, $_{His}\sigma^E$, and the C-terminal truncated variant $_{His}\sigma^E_{1-153}$, caused co-sedimentation of core RNA polymerase with σ^E-charged resin (Fig. 3*A*). These data suggest that RpoE residues 154–191, a peptide sequence homologous to the σ^{70} 4.2 domain involved in binding the -35 region of promoter DNA, is not required for σ^E binding to RNA polymerase. As a control, mock-charged nickel-NTA-Sepharose did not associate with RNA polymerase. N-terminal truncation of the presumed binding region for core RNA polymerase, domains 1.2, 2.1, 2.2, and 2.3, indeed prevented the variant σ^E protein (σ^E_{95-191}) to capture the α subunit of RNA polymerase. A similar result was observed for all C-terminal truncations tested, as nickel-NTA-Sepharose charged with $_{His}\sigma^E_{1-91}$, $_{His}\sigma^E_{1-116}$, or $_{His}\sigma^E_{1-137}$ failed to promote co-sedimentation of RNA polymerase (Fig. 3*A*).

RseA is a type II membrane protein with an N-terminal σ^E-binding domain that resides in the cytoplasm and a C-terminal RseB-binding domain within the periplasm. RseA^N is a truncated variant containing an N-terminal His tag and a C-

terminal truncation to the first residue of the membrane-spanning domain of wild-type RseA. $RseA^N$ was purified from the cytoplasm of *E. coli* by affinity chromatography and its N-terminal His tag removed by cleavage using enterokinase. Purified $RseA^N$ was added to Ni-NTA-Sepharose pre-equilibrated with $_{His}\sigma^E$ fragments and sigma factor binding was measured by immunoblotting as the amount of $RseA^N$ that sedimented with the Sepharose beads during 4,000 × *g* centrifugation. Only full-length RpoE, $_{His}\sigma^E$, but not $_{His}\sigma^E_{1–91}$, $_{His}\sigma^E_{1–116}$, $_{His}\sigma^E_{1–137}$, or $_{His}\sigma^E_{1–153}$, caused co-sedimentation of $RseA^N$ with the pre-charged resin (Fig. 3*B*). To confirm these findings, a reciprocal experiment was conducted (Fig. 3*C*). Ni-NTA-Sepharose charged with $_{His}RseA^N$ was incubated with the σ^E and its variants $\sigma^E_{1–91}$, $\sigma^E_{1–116}$, $\sigma^E_{1–137}$, $\sigma^E_{1–153}$, or $\sigma^E_{95–191}$. The His tag of each variants was removed using enterokinase, and proteins were purified by ion exchange chromatography. Control experiments showed that the purified σ^E variants ($\sigma^E_{1–91}$, $\sigma^E_{1–116}$, $\sigma^E_{1–137}$, $\sigma^E_{1–153}$, or $\sigma^E_{95–191}$) did not co-sediment with mock-charged nickel-NTA-Sepharose beads (Fig. 3*C*). Our assay could detect the binding of $RseA^N$ to full-length σ^E. All C-terminal truncations of σ^E abolished the association with $RseA^N$, suggesting that C-terminal σ^E sequences are required for RseA binding. Failure to observe binding between $\sigma^E_{95–191}$ and $RseA^N$ can be attributed to a lack of structure or stability of $\sigma^E_{95–191}$. Indeed when $RseA^N$ was overproduced along with $\sigma^E_{95–191}$ in the same cell, a soluble $\sigma^E_{95–191}$-$RseA^N$ complex could be extracted from total cell extracts (data not shown).

Accessibility of Cysteine 165 of σ^E to Ellman's Reagent—Amino acids 1–153 of σ^E polypeptide (fragment $\sigma^E_{1–153}$) are sufficient to promote interaction between the sigma factor and core RNA polymerase, but fail to associate with $RseA^N$. We entertained the possibility that interaction with RseA occurs at a C-terminal portion of σ^E. *rpoE* codon 165 encodes cysteine, a sulfhydryl-containing amino acid, within the C-terminal peptide sequence (amino acids 154–191). Cysteine 165, Cys^{165}, is the only cysteine residue within σ^E. Reaction of Ellman's reagent (DTNB) with sulfhydryl residues results in the formation of 5-thio-2-nitrobenzoate, a product that absorbs light at 412 nm. Cys^{165} is accessible to Ellman's reagent because incubation of σ^E with this compound led to the rapid generation (less than 30 s) of the product with absorbance at 412 nm (Fig. 4). The extent of release of thionitrobenzoate did not yield a molar ratio of DTNB over σ^E, as the amount of reacting thiol was 12 μM and the calculated σ^E concentration was 8 μM. Either the preparation of σ^E was contaminated with thiol-containing proteins or the concentration of σ^E, deduced from the calculated molar extinction coefficient $\epsilon_{278\ nm}$ 15,400 M^{-1} cm^{-1}, was underestimated. Nonetheless, further incubation of this reaction did not increase the absorbance at 412 nm, a finding that is consistent with the notion that all available sulfhydryl moieties had been modified within 30 s. Incubation of Ellman's reagent with complexes formed from equimolar amounts of σ^E and $RseA^N$ generated only a modest amount of absorbance at 412 nm. Further, the absorbance at 412 nm during this experiment increased slowly with a steady rate while incubating for 800 s (Fig. 4). These data are consistent with a model in which the binding of RseA to the C-terminal portion of σ^E blocks the access of Ellman's reagent to Cys^{165}. Further, the slow increase in absorbance is likely caused by the dissociation of σ^E-$RseA^N$, thereby liberating the sulfhydryl at position 165 for modification with Ellman's reagent. $RseA^N$ does not contain cysteine residues, and incubation of $RseA^N$ alone with Ellman's reagent did not yield a significant absorbance at 412 nm (data not shown). Covalent modification of σ^E with Ellman's reagent does not prevent the association of the sigma factor with either $RseA^N$ or core RNA polymerase, suggesting that Cys^{165} is not an essential residue for the formation of the σ^E-$RseA^N$ complex (data not shown).

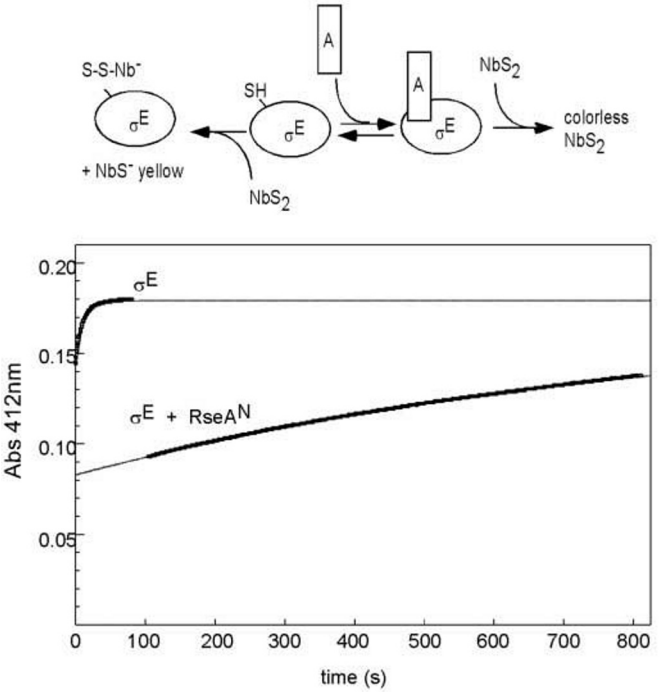

FIG. 4. **Accessibility of C_{165} of σ^E to Ellman's reagent in the presence or absence of $RseA^N$.** Ellman's reagent (DTNB) was added at a final concentration of 250 μM to σ^E (8 μM) in the absence or presence of $RseA^N$ (8 μM). The total volume of the reaction was 1 ml. Change in absorbance was recorded for 800 s at 412 nm using a Varian spectrophotometer thermostatted at 20 °C.

RseA Interacts with Region 4.2 of σ^E Polypeptide—Cys^{165} is located in a region of σ^E that is homologous to the 4.2 domain of σ^{70} (Fig. 1). Region 4.2 of σ^{70} contains a putative helix-turn-helix DNA-binding motif that interacts with the −35 element of σ^{70}-dependent promoters. We assumed that mutations within the 4.2 region of σ^E may likely display defects in the transcription at Eσ^E-dependent promoters. To test this prediction, we sought to isolate mutations that decrease the transcription of Eσ^E-dependent promoters. A strain carrying the *nadB*::tet allele was mutagenized with hydroxylamine (18). The *nadB* gene is linked to the *rpoE* operon. Bacteriophage P1 lysates of mutated cultures were transduced into *E. coli* MC4100 carrying the *rpoE*P2-*lacZ* transcriptional fusion. Mutations linked to the Tet^R marker were selected for by plating on agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 20 μg/ml) and tetracycline. Light blue Tet^R transductants were retained. Only mutations that co-transduced with *tet*^{R} at a frequency greater than 90% linked to the Tet^R marker and that conferred temperature-sensitive growth above 37 °C were retained. The location of *rpoE* mutations was determined by DNA sequencing.

Three of the isolated mutations changed the specificity of single codons within the 4.2 domain of σ^E: R178G, I181A, and V185A. Each of the three mutations decreased transcriptional activity of σ^E RNA polymerase at multiple promoters (*htrA-lacZ*, *rpoE*P2-*lacZ* and *rpoH*P3-*lacZ*), suggesting that the regulatory defects were neither allele- nor promoter-specific (Fig. 5).

We wondered whether *rpoE* mutations that affect promoter selection and/or transcriptional activity by σ^E RNA polymerase caused a simultaneous effect on the binding of σ^E to RseA. This possibility was tested in a biochemical experiment. Three mutant *rpoE* alleles were amplified by polymerase chain reaction,

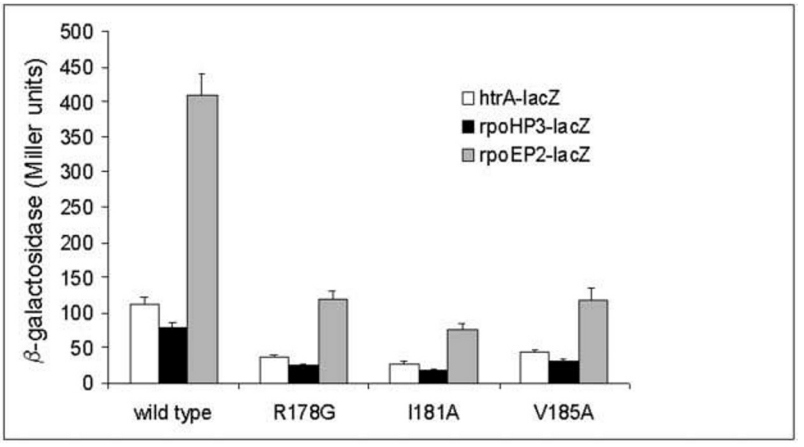

FIG. 5. **Transcriptional activity of various σ^E mutants *in vivo*.** To test the activity of the σ^E mutants *in vivo*, the *rpoE*R178G, *rpoE*I181A, and *rpoE*V185A alleles were transduced into *E. coli* strain MC4100 carrying chromosomal insertions of the reporter genes *rpoH*P3-*lacZ*, *rpoE*P2-*lacZ*, or *htrA-lacZ*, respectively. Bacteria were grown overnight at 30 °C, diluted 1:100, and allowed to reach $A_{595\ nm}$ between 0.5 and 0.7. β-Galactosidase activity measurements were performed in duplicate and calculated in Miller units (16). Data represent the average of at least three independent experiments.

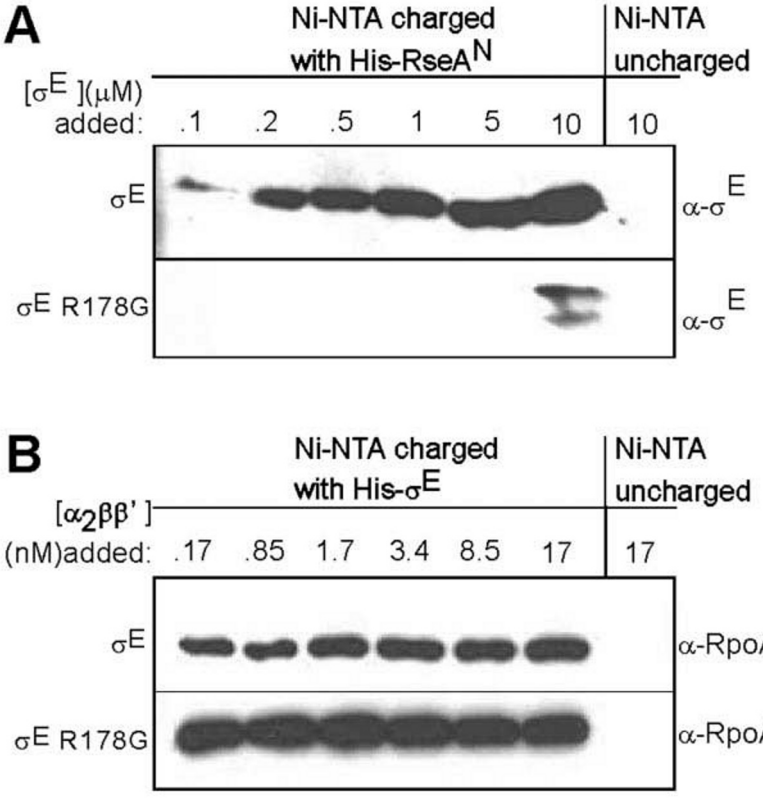

FIG. 6. **Interaction of σ^E and σ^E_{R178G} with RseA^N and core RNA polymerase.** *A*, nickel-NTA resin was charged with 1 μM RseA^N and incubated with increasing concentrations of σ^E or σ^E_{R178G}. *B*, nickel-NTA resin was charged with 1 μM $_{His}\sigma^E$ or $_{His}\sigma^E_{R178G}$ and incubated with increasing concentrations of core RNA polymerase. Bound proteins were collected by low speed centrifugation, washed three times with the resin, and eluted with imidazole. Protein was precipitated with trichloroacetic acid, washed in acetone, and subjected to 12% SDS-PAGE and immunoblotting with antibodies raised against σ^E (α-σ^E) or the α subunit of RNA polymerase (α-RpoA).

and the corresponding genes were cloned in the pET expression vector. The mutant proteins, $_{His}\sigma^E_{R178G}$, $_{His}\sigma^E_{I181A}$, and $_{His}\sigma^E_{V185A}$, were purified by affinity chromatography on nickel-NTA-Sepharose. When measured with a co-sedimentation assay, $_{His}\sigma^E_{R178G}$, $_{His}\sigma^E_{I181A}$, and $_{His}\sigma^E_{V185A}$ bound to purified RNA core polymerase in a manner that was indistinguishable from the binding of wild-type σ^E. Fig. 6 shows the results of these experiments for wild-type σ^E and σ^E_{R178G}. To measure binding of σ^E to RseA, nickel-NTA-Sepharose beads were charged with RseA^N and incubated with enterokinase-cleaved σ^E protein. Although co-sedimentation of wild-type σ^E and RseA^N was observed, neither σ^E_{R178G} nor σ^E_{I181A} or σ^E_{V185A} interacted with RseA^N. Fig. 6 shows the results of the experiments for wild-type σ^E and σ^E_{R178G}. Alanine substitution of Cys^{165}, the sulfhydryl residue, did not affect the *in vitro* binding of σ^E to core polymerase or to RseA^N (data not shown).

DISCUSSION

σ^E is a member of the σ^{70} family of proteins. These sigma factors are modular proteins, consisting of four conserved regions and their subregions (Fig. 1; reviewed in Ref. 19). Regions 2.4 and 4.2 encode two DNA binding determinants that allow for recognition of the conserved −10 and −35 regions of promoters, respectively. Region 1.1 is an autoinhibitory domain that masks the DNA binding determinants of σ^{70} when the transcription factor is not associated with RNA polymerase (20). Interaction with core polymerase relieves region 1.1-mediated autoinhibition (20) and reorients regions 3.1–4.2 of σ^{70} relative to the central 1.2–2.4 domains. These movements permit the selective recognition of −10 region promoter sequences by regions 2.3–2.4 (21). Although region 1.1 is present in many primary σ factors (σ^{70}), this domain is absent in σ^E. Mutational changes in regions 2, 3, and 4 of σ^{70} have been shown to decrease binding to RNA polymerase, and it has been proposed that putative contact sites exist between regions 2, 3, 4, and the $\alpha_2\beta\beta'$ core (22).

We have used limited trypsin digestion to probe the domain organization of σ^E. One fragment resisted digestion by dilute amounts of trypsin and contained region 1.2 as well as region

2:σ^E amino acids 1–91. These data correlate well with preliminary NMR experiments in which σ^E residues 92–191, unlike amino acids 1–91, generated little resonance (data not shown). Thus, the C-terminal part of σ^E (residues 92–191) does not assume a compact fold. The N-terminal peptide, σ^E_{1-91}, was cloned, expressed, and purified by affinity chromatography. Purified σ^E_{1-91} did not bind $RseA^N$ or core polymerase. The latter observation was a surprise to us, as fragment σ^{70}_2, comprising region 1.2 and region 2 of σ^{70} (39 kDa), is known to bind to core RNA polymerase in a manner that competes with the binding of wild-type σ^{70} (8). Although our measurements for binding of σ^E fragments to core polymerase employed an assay similar to that of the σ^{70} studies, pronounced differences were observed (8). Although all three fragments, σ^E_{1-116}, σ^E_{1-137}, and σ^E_{1-153}, comprise region 1.2, only the largest fragment, σ^E_{1-153}, was capable of binding to core polymerase.

The fact that RseA did not bind to σ^E_{1-153}, together with the observation that region 4.2 is located in the peptide sequence 153–191, suggested that RseA may bind region 4.2 of σ^E. A direct demonstration of this hypothesis could not be achieved as σ^E_{95-191} did not co-sediment with $RseA^N$. One explanation for the negative result is a lack of structure of the C-terminal domain of σ^E. Is it possible that RseA does bind to region 4.2 but only if this domain is tethered to the remainder of σ^E? This notion is corroborated by several observations. First, σ^E_{95-191} is unstable and rapidly forms aggregates that can be sedimented by centrifugation. Second, binding of RseA to σ^E masks Cys^{165} for modification with Ellman's reagent, as if RseA occupies residues 153–191 (region 4.2). Third, region 4.2 was also characterized by isolating σ^E variants that fail to promote $\alpha_2\beta\beta'$ σ^E-mediated transcription of σ^E-dependent promoters. Three amino acid substitutions mapped to region 4.2: R178G, I181A, and V185A. When tested in a biochemical experiment, all three σ^E variants failed to bind RseA although the association with core RNA polymerase was similar to that of wild-type σ^E. Together these data suggest that RseA indeed binds to region 4.2 of σ^E.

The activity of several sigma factors is regulated by a cognate anti-sigma factor. Many pairs of sigma/anti-sigma factors have been identified, and their biological roles have been characterized (9, 23). Recently, the structural features of some anti-sigma factors and the binding sites for the cognate sigma factors have been elucidated (10, 11, 14, 24, 25). The two anti-sigma factors of σ^{70}, AsiA and Rsd, are about 10 kDa of mass. AsiA is encoded by the bacteriophage T4, and its association with the σ^{70} subunit of the *E. coli* RNA polymerase is one of the principal events controlling transcription of host cells and of the T4 genome. The recognition site of AsiA on σ^{70} has been mapped using NMR and alanine-scanning mutagenesis (10, 11). These studies showed that the highly conserved region 4.2 (and maybe 4.1) constitutes the AsiA-binding domain. Mutations that affect AsiA binding to σ^{70} are clustered in a stretch of 5 residues within the C-terminal half of region 4.2 ($KAL^{595}RK$). These residues belong to a loop of charged residues ($KAL^{595}RKLRHPS$) where Lys^{593}, Arg^{596}, Lys^{597}, Arg^{599}, and His^{600} contribute to the interaction with protein activators of transcription rather than directly to σ^{70} binding of DNA (26, 27). Modeling of residues 551 to 613 of σ^{70} on the structure of NarL and 434 Cro suggests that all of the charged residues are surfaced exposed in the HTH motif (26). Strikingly, RseA binding is dramatically affected by mutations clustered in the C-terminal half of region 4.2 of σ^E (R178G, I181A, and V185A). Sequence alignment between σ^E and σ^{70} shows that this region of σ^E ($RAR^{178}EAI^{181}DNKV^{185}$) corresponds to the stretch of charged residues described above for σ^{70} ($KAL^{595}RKLRHPS$). Within σ^E these residues are also important for interaction with the DNA as alterations of Arg^{178}, Ile^{181}, and Val^{185} lead to decreased σ^E activity *in vivo*. The recognition site of Rsd, a second anti-σ^{70} factor produced by *E. coli* during stationary phase (13), has also been mapped to region 4 of σ^{70} (12, 14). Alanine substitutions at two positions flanking arginine 596 (Leu^{595} and Leu^{598}) disrupt the association of Rsd with σ^{70} *in vitro* (14). Hence, the same region of σ^{70} that is targeted for recognition by two unrelated anti-sigma factors appears to be important for the σ^E-RseA interaction.

Acknowledgments—We thank K. Faull (University of California, Los Angeles) for mass spectrometry measurements and O. Schneewind (University of Chicago) for critical review of this manuscript.

REFERENCES

1. Missiakas, D., and Raina, S. (1998) *Mol. Microbiol.* **28,** 1059–1066
2. Lonetto, M., Brown, K. L., Rudd, K. E., and Buttner, M. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7573–7577
3. Raivio, T. L., and Silhavy, T. J. (1999) *Curr. Opin. Microbiol.* **2,** 159–165
4. Dartigalongue, C., Missiakas, D., and Raina, S. (2001) *J. Biol. Chem.* **276,** 20866–20875
5. Collinet, B., Yuzawa, H., Chen, T., Herrera, C., and Missiakas, D. (2000) *J. Biol. Chem.* **275,** 33898–33904
6. De Las Peñas, A., Conolly, L., and Gross, C. A. (1997) *Mol. Microbiol.* **24,** 3373–3386
7. Missiakas, D., Mayer, M., Lemaire, M., Georgopoulos, C., and Raina, S. (1997) *Mol. Microbiol.* **24,** 355–371
8. Severinova, E., Severinov, K., Fenyo, D., Marr, M., Brody, E. N., Roberts, J. W., Chait, B. T., and Darst, S. A. (1996) *J. Mol. Biol.* **263,** 637–647
9. Brody, E. N., Kassavetis, G. A., Ouhammouch, M., Sanders, G. M., Tinker, R. L., and Geiduschek, G. P. (1995) *FEMS Microbiol. Lett.* **128,** 1–8
10. Urbauer, J. L., Adelman, K., Urbauer, R. J., Simeonov, M. F., Gilmore, J. M., Zolkiewski, M., and Brody, E. N. (2001) *J. Biol. Chem.* **276,** 41128–41132
11. Minakhin, L., Camarero, J. A., Holford, M., Parker, C., Muir, T. W., and Severinov, K. (2001) *J. Mol. Biol.* **306,** 631–642
12. Dove, S. L., and Hochschild, A. (2001) *J. Bacteriol.* **183,** 6413–6421
13. Jishage, M., and Ishihama, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4953–4958
14. Jishage, M., Dasgupta, D., and Ishihama, A. (2001) *J. Bacteriol.* **183,** 2952–2956
15. Kumar, A., Grimes, B., Fujita, N., Makino, K., Malloch, R. A., Hayward, R. S., and Ishihama, A. (1994) *J. Mol. Biol.* **235,** 405–413
16. Miller, J. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
17. Riddles, P., Blakeley, R. L., and Zerner, B. (1979) *Anal. Biochem.* **94,** 75–81
18. Raina, S., Missiakas, D., and Georgopoulos, C. (1995) *EMBO J.* **14,** 1043–1055
19. Gross, C. A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., and Young, B. (1998) *Cold Spring Harbor Symp. Quant. Biol.* **63,** 141–155
20. Dombroski, A. J., Walter, W. A., and Gross, C. A. (1993) *Genes Dev.* 7, 2446–2455
21. Marr, M. T., and Roberts, J. W. (1997) *Science* **276,** 1258–1260
22. Sharp, M. M., Chan, C. L., Lu, C. Z., Marr, M. T., Nechaev, S., Merritt, E. W., Severinov, K., Roberts, J. W., and Gross, C. A. (1999) *Genes Dev.* **13,** 3015–3026
23. Hughes, K. T., and Mathee, K. (1998) *Annu. Rev. Microbiol.* **52,** 231–286
24. Campbell, E. A., and Darst, S. A. (2000) *J. Mol. Biol.* **300,** 17–28
25. Daughdrill, G. W., Chadsey, M. S., Karlinsey, J. E., Hughes, K. T., and Dahlquist, F. W. (1997) *Nat. Struct. Biol.* **4,** 285–291
26. Lonetto, M. A., Rhodius, V., Lamberg, K., Kiley, P., Busby, S., and Gross, C. A. (1998) *J. Mol. Biol.* **284,** 1353–1365
27. Landini, P., and Busby, S. J. (1999) *J. Bacteriol.* **181,** 1524–1529
28. Casadaban, M. J. (1976) *J. Mol. Biol.* **104,** 541–555
29. Missiakas, D., Georgopoulos, C., and Raina, S. (1993) *J. Bacteriol.* **175,** 2613–2624