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Mapping of the Rsd Contact Site on the Sigma 70 Subunit of *Escherichia coli* RNA Polymerase

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Rsd (regulator of sigma D) is an anti-sigma factor for the *Escherichia coli* RNA polymerase σ^{70} subunit. The contact site of Rsd on σ^{70} was analyzed after mapping of the contact-dependent cleavage sites by Rsd-tethered iron-*p*-bromoacetamidobenzyl EDTA and by analysis of the complex formation between Ala-substituted σ^{70} and Rsd. Results indicate that the Rsd contact site is located downstream of the promoter −35 recognition helix-turn-helix motif within region 4, overlapping with the regions involved in interaction with both core enzyme and σ^{70} contact transcription factors.

The survival of bacterial cells in various environments depends on their abilities to sense the external conditions and adapt their internal metabolic systems by turning on and off the expression of specific genes (for reviews, see references 12 and 21). For quick change of the global gene expression pattern in response to sudden environmental changes, bacteria carry modulation systems for the specificity and activity of transcription apparatus. The transcription apparatus of *Escherichia coli* is composed of the RNA polymerase core enzyme (subunit composition, $\alpha_2\beta\beta'$) with the catalytic activity of RNA polymerization and one of seven species of the σ subunit with the promoter recognition activity (reviewed in references 14, 15, 19, and 22). The major σ subunit, σ^{70}, is responsible for transcription of most genes expressed during steady-state cell growth under laboratory culture conditions. The other six species of the σ subunit are required only during certain growth stages or under specific stress conditions. In agreement with their functional roles, the levels of these alternative σ subunits vary depending on the cell growth conditions (25, 26, 42), and all the σ subunits compete with each other for binding to a fixed amount of the core enzyme (41). In addition to the level control, the activities of at least some *E. coli* σ subunits are under a control system in which the unused σ subunits are stored in inactive forms by forming complexes with another set of proteins, often designated as anti-σ factors, with the regulatory activity of σ functions (for reviews see references 18 and 21).

Subunit σ^F is involved in transcription of the genes needed for flagellum formation and chemotaxis. The *flgM* gene product is an anti-σ^F factor that acts by directly binding to σ^F and thereby preventing its interaction with the core RNA polymerase (33). Subunit σ^E is a member of the ECF family of σ subunits for transcription of the genes related to extracytoplasmic functions (39) as well as those required for high temperature survival or thermotolerance (9). The σ^E activity is regulated by the *rseA* (regulator of sigma E) gene product or anti-σ^E factor, which is associated with the inner membrane and inhibits the activity of σ^E by directly interacting with σ^E (7, 43). FecI also belongs to the ECF σ family and is involved in transcription activation of the ferric-citrate transport genes (*fec*) (1). Genetic studies revealed that FecR, an inner membrane protein, negatively regulates the activity of the FecI σ subunit (49). FlgM, RseA, and FecR are classified as members of anti-sigma factors for σ^F, σ^E, and σ^{FecI}, respectively. A heat shock protein, DnaK, can be an anti-σ factor for the heat shock σ^H subunit (18), which is induced following heat shock and is involved in transcription of the genes encoding heat shock proteins, including DnaK, DnaJ, and GrpE (13). After returning from the transient adaptation period to heat shock to steady-state growth at high temperatures, unused σ^H becomes stored as DnaJ-DnaK-σ^H complexes (38), which are dissociated by the action of GrpE to release σ^H for reuse or for degradation by HflB (FtsH) protease (10).

Recently we discovered a novel *E. coli* protein, referred to as Rsd (regulator of sigma D), which forms a complex with σ^{70}, the major σ^{70} subunit for growth-related gene transcription, and prevents its function (24). Purified Rsd protein formed complexes in vitro with σ^{70} but not with other σ subunits and inhibited transcription in vitro by the holoenzyme containing σ^{70} to various extents, depending on the promoters used (24). Since Rsd is induced in the stationary phase of cell growth, where σ^{70} is not used, we proposed that Rsd is an anti-σ factor for the major σ^{70} subunit for storage in the stationary phase. In *E. coli* mutants lacking the *rsd* gene, the expression of σ^{70}-dependent genes increases while transient overproduction of Rsd leads to a reduction in σ^{70}-dependent gene expression (23). Based on these results, taken together, we proposed that Rsd is an anti-sigma factor for the σ^{70} subunit.

Cleavage sites of σ^{70} by Rsd-tethered FeBABE. Previously we estimated the contact site of Rsd on σ^{70} to be downstream from residue 500, including regions 3.2, 4.1, and 4.2, after analysis of complex formation between Rsd and σ^{70} fragments (24). For detailed mapping of the contact site of Rsd on the σ^{70} subunit, we employed the contact-dependent cleavage of target proteins by FeBABE (iron-*p*-bromoacetamidobenzyl EDTA)-conjugated pairing proteins (6, 20, 22). In this study, FeBABE

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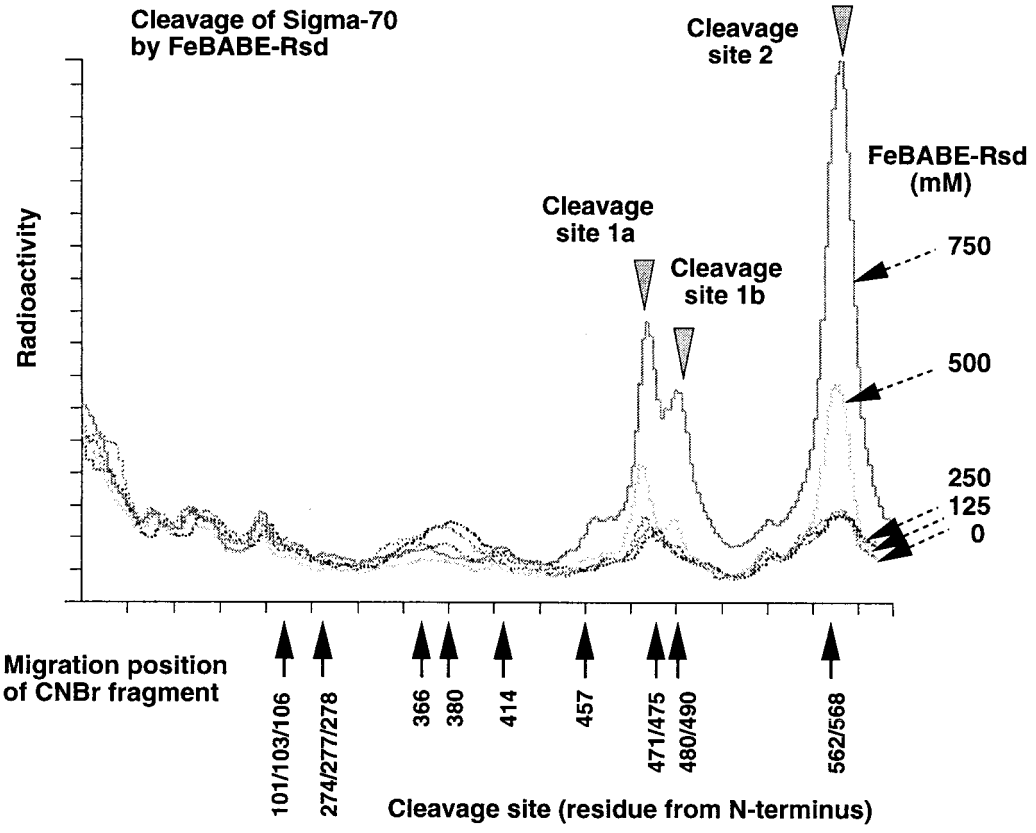

FIG. 1. Cleavage of σ^{70} by Rsd-tethered FeBABE. Purified Rsd was conjugated with FeBABE (Dojin, Kumamoto, Japan) in the presence of 2-iminothiolane according to Traviglia et al. (48), while σ^{70} with a PK tag at the C terminus was labeled in vitro with ^{32}P with protein kinase A. Mixtures of a fixed amount of ^{32}P-labeled σ^{70} (final concentration, 250 nM) and the indicated amounts of FeBABE-tethered Rsd were incubated for 30 min at 37°C and then were subjected to contact-dependent protein cleavage reaction by adding ascorbate and H_2O_2 followed by SDS-PAGE. CNBr-treated ^{32}P-σ^{70} was run on the same gel as size markers. The gel was exposed to an imaging plate, and the plate was analyzed with the BAS 2000 Image Analyzer (Fuji, Tokyo, Japan). Migration is from left to right. The migration positions of CNBr fragments are indicated at the bottom.

was tethered to Rsd at all possible Lys residues by using 2-iminothiolane, which links between Lys and FeBABE (48). For detection of the cleavage sites on σ^{70}, a protein kinase tag sequence was added at either its N or C terminus and the tag was phosphorylated using [γ-^{32}P]ATP and protein kinase A. Mixtures of a fixed amount of ^{32}P-labeled σ^{70} and increasing amounts of FeBABE-tethered Rsd were incubated for 10 min at 37°C to form binary complexes and then were subjected to cleavage reaction by adding ascorbate and H_2O_2. The reaction mixtures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. Fig. 1 shows a tracing of the SDS-PAGE pattern of the cleavage products. As size markers, the same ^{32}P-labeled σ^{70} was treated with CNBr, which induces cleavage at Met residues. Although several nonspecific cleavage products were generated by the addition of H_2O_2 and ascorbate even in the absence of FeBABE-tethered Rsd, the specific cleavage products increased concomitantly with the increase in Rsd-FeBABE addition. At least three such bands, cleaved at sites 1a, 1b, and 2, were identified, each migrating close to the C-terminal CNBr fragment (471/475–613, 488/490–613, or 562/568–613, respectively). Thus we concluded that the FeBABE tethered on the surface of σ^{70}-bound Rsd approached near the σ^{70} segment between residues 471 and 568. The cleavage sites 1a and 1b are located within σ^{70} region 3 while cleavage site 2 is within region 4 (see Fig. 3). The result, however, does not immediately indicate the location of the Rsd contact site on these regions because the spacer length between the BABE-tethered Lys and the BABE-associated Fe is about 18 Å (20, 48).

The cleavage reaction by Rsd-tethered FeBABE was also performed for all six alternative subunits, σ^N, σ^S, σ^H, σ^F, σ^E, and σ^{FecI}, but none of them were cleaved even after the addition of excess amounts of Rsd-FeBABE (data not shown). These observations confirm our previous finding that Rsd specifically interacts only with the σ^{70} subunit and not with the other σ subunits (24).

Rsd-binding activity of Ala-substituted σ^{70} mutants. For detailed mapping of the contact site on σ^{70} with Rsd, we next tested the complex formation in vitro between Rsd and Ala-substituted σ^{70} subunits. The library of Ala-substituted σ^{70} was constructed and used for mapping the σ^{70} contact sites with the core enzyme (46) or with σ^{70} contact transcription factors CRP and FNR (40). The mutant σ^{70} subunits with a glutathione *S*-transferase (GST) tag fused at the N termini were overproduced and were purified to near homogeneity. The GST-

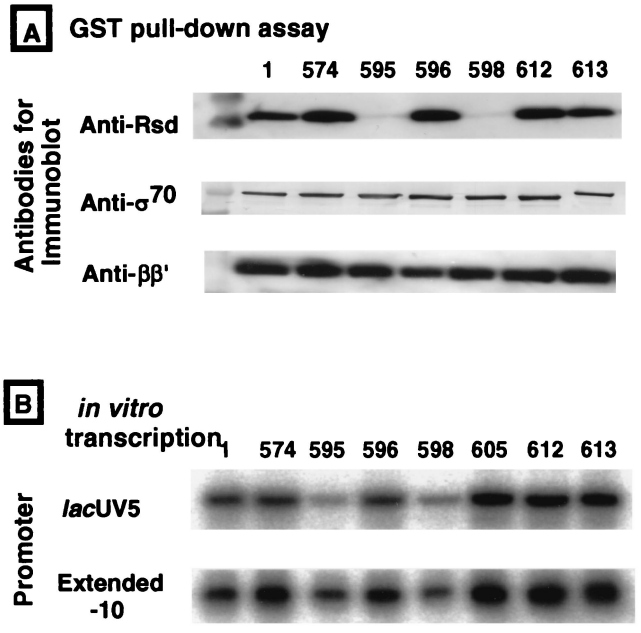

FIG. 2. Identification of the Rsd contact site on the σ^{70} subunit. (A) GST-σ^{70} Ala-substituted mutants were overexpressed and purified to near homogeneity. Each GST-σ^{70} mutant was mixed with an equal amount of Rsd, and after incubation for 5 min at 37°C, GST-σ^{70}-Rsd complexes were isolated by the GST pull-down assay using glutathione-Sepharose beads. The bead-bound proteins were eluted with 50 mM glutathione and were analyzed by SDS-PAGE. The gel was subjected to immunoblotting against anti-Rsd, anti-σ^{70}, or anti-ββ′ antibodies. (B) Activity of the Ala-substituted mutant σ^{70} subunits. In vitro transcription was carried out under the standard reaction conditions (28) using 1 pmol each of the GST-σ^{70} mutants, 1 pmol of the core enzyme, and 1 pmol of either *lac*UV5 or extended −10 promoter DNA fragment (31, 32).

tagged σ^{70} subunits were mixed with purified Rsd, and the complexes formed were recovered using glutathine-conjugated agarose beads. This GST pull-down assay indicated that two σ^{70} mutants with Ala substitutions at residues 595 and 598 were defective in binding to Rsd (Fig. 2A), indicating that the segment of σ^{70} including L595 and L598 is involved in molecular interaction with Rsd. The major determinant of core enzyme binding on σ^{70} is located in region 2.1 (37). L598 in region 4.2 also participates, at least in part, in binding of the core enzyme (46). The corresponding region of σ^{32} is also involved in core enzyme binding (27). In the case of core enzyme binding, multiple sites on the σ subunits are involved and thus a single mutation is often not so critical for overall functions of the σ subunits. In fact, under the assay conditions employed, the binding of L598A mutant σ^{70} with the core enzyme is stronger than that with the Rsd protein (Fig. 2A).

To analyze the role of these residues in the intrinsic σ^{70} function of promoting transcription initiation, holoenzymes were reconstituted from each of the Ala-substituted mutant σ^{70} subunits and the σ-free core enzyme and were used for in vitro transcription. The *lac*UV5-directed transcription was significantly reduced for only the same two mutants, L595A and L598A, which are required for Rsd interaction (Fig. 2B). Thus, the sites required for Rsd interaction are also critical for expression of the intrinsic σ^{70} activities, presumably at the step of core enzyme binding (46). The influence of Ala substitution at residues downstream of the −35 recognition helix-turn-helix (HTH) motif of σ^{70} was also observed in RNA I promoter-directed transcription in vitro (40). The reduction of σ^{70} activity for Ala-substituted mutant σ^{70} was, however, not observed when transcription was carried out using the extended −10 promoter (Fig. 2B), which is active in directing transcription even in the absence of −35 promoter -σ^{70} region 4 interactions (2, 4, 32).

Rsd contact site on the σ^{70} subunit. FeBABE cleavage experiments indicate the close location of Rsd near the σ^{70} segment between residues 471 and 568, including regions 3.1, 3.2, and 4.1 (see Fig. 1), while the mutant studies using an Ala-substituted σ^{70} library indicate that the Rsd contact site is downstream from region 4.2 (see Fig. 2). Since the reactive Fe^{3+} is located 18 Å apart from the Lys residue tethered with FeBABE with the use of a 2-iminothiolane linker (20, 48), it is unlikely that the region between 471 and 568 is the direct target of Rsd binding, but instead the Rsd-binding site includes the residues L595 and L598 identified by Ala scanning. The FeEDTA moiety of the FeBABE tethered at this region may be located close to regions 3 and 4.1, where the cleavage sites were identified (see Fig. 1). Thus we conclude that the direct contact site of Rsd is located on region 4 of the σ^{70} subunit downstream of the HTH motif of region 4.2 (Fig. 3), which is involved in recognition of the promoter −35 sequence (11, 47). The −35 contact is, however, not essential for promoter complex formation when the contact of σ^{70} region 2 with the promoter −10 sequence alone is strong enough, as in the case of an extended −10 signal (3, 4, 32).

The σ^{70} contact (or class II) transcription factors support the functional interaction of σ^{70} with promoters lacking the consensus −35 sequence (19). In these cases, the region upstream or downstream of the −35 contact HTH motif in σ^{70} region 4 is involved in interaction with class II factors (19, 45). Deletion mutant σ^{70} lacking region 4 is still functional with the extended −10 promoter, which alone has a high affinity to the σ^{70} region 2 but is defective in response to CRP (on class II promoters) and PhoB (31). Mutant studies indicate that the contact sites for several class II transcription factors, including λ cI (8, 30), PhoB (29), CRP (40), FNR (40), Ada (35, 36), AraC (17, 40), and RhaS (3) are all located upstream or downstream of the HTH promoter −35 recognition motif (Fig. 3). Transcription activation by λ cI becomes defective in a region 4 mutation of the σ^{70} subunit (30). At class II CRP-dependent promoters, CRP makes three different contacts, one of which, known as the activating region 3 (AR3), interacts with region 4 of the σ^{70} subunit (45). The positively charged residues K593, K597, and R599 on σ^{70} are required for this interaction. Mutations on these residues also affect the σ^{70} response to FNR (40). Most class I (or α contact) factors activate transcription by stabilizing the closed complex, while class II (or σ^{70} contact) factors such as λ cI activate the isomerization step of transcription initiation (8).

The contact site with Rsd is located on the same surface with those of AraC, CRP, FNR, RhaS, and λcI (Fig. 3). Thus, the region downstream from the promoter −35 binding HTH of σ^{70} seems to be involved in binding the core enzyme, the anti-sigma factor, and a group of class II transcription factors. The anti-σ^{70} factor Rsd should compete with both the core

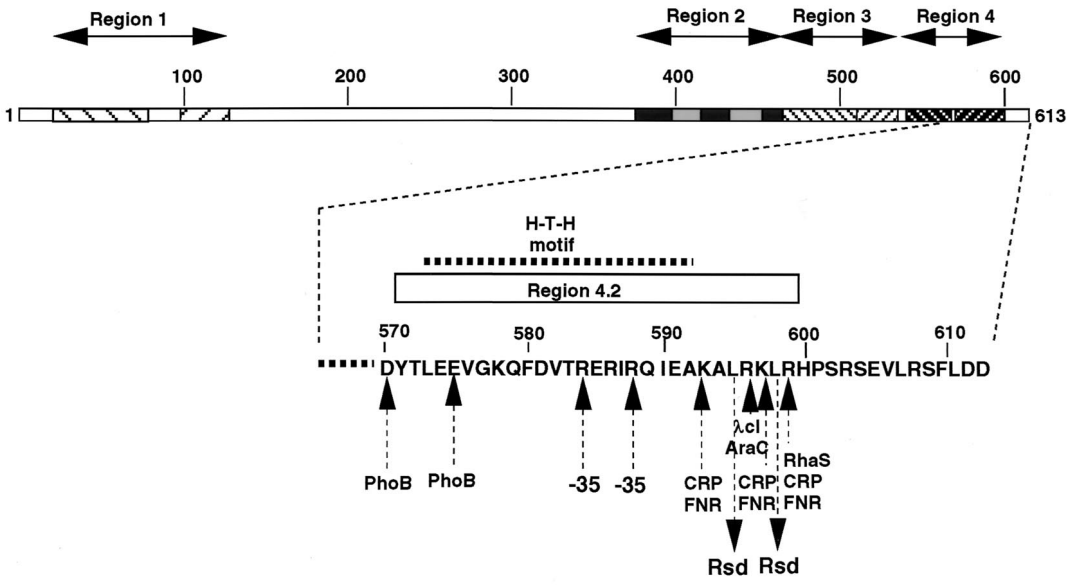

FIG. 3. The Rsd contact sites on the σ^{70} subunit. The predicted Rsd contact sites are compared with the known functional sites, including the sites involved in the molecular interaction with class II transcription factors. The HTH motif is involved in the promoter −35 recognition (11, 47). The contact sites for class II transcription factors are located upstream or downstream of this motif. For details, see the text.

enzyme and the class II transcription factors in binding with the σ^{70} subunit. Likewise, the contact site of FlgM, the anti-σ^F factor, has been mapped on region 4 of σ^F (34). The phage T4 AsiA protein is an anti-sigma factor against the host *E. coli* σ^{70} subunit to repress host cell gene transcription (5, 44). The contact site for the AsiA protein on σ^{70} is located within regions 3 and 4 (16). Thus, all the anti-σ factors so far analyzed seem to interact with the same σ region near the promoter −35 recognition surface.

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