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Short Conceptual Overview

Paola Cavaliere[a] and Françoise Norel*

Recent advances in the characterization of Crl, the unconventional activator of the stress sigma factor σ^S/RpoS

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Abstract: The bacterial RNA polymerase (RNAP) holoenzyme is a multisubunit core enzyme associated with a σ factor that is required for promoter-specific transcription initiation. Besides a primary σ responsible for most of the gene expression during active growth, bacteria contain alternative σ factors that control adaptive responses. A recurring strategy in the control of σ factor activity is their sequestration by anti-sigma factors that occlude the RNAP binding determinants, reducing their activity. In contrast, the unconventional transcription factor Crl binds specifically to the alternative σ factor σ^S/RpoS, and favors its association with the core RNAP, thereby increasing its activity. σ^S is the master regulator of the general stress response that protects many Gram-negative bacteria from several harmful environmental conditions. It is also required for biofilm formation and virulence of *Salmonella enterica* serovar Typhimurium. In this report, we discuss current knowledge on the regulation and function of Crl in *Salmonella* and *Escherichia coli*, two bacterial species in which Crl has been studied. We review recent advances in the structural characterization of the Crl-σ^S interaction that have led to a better understanding of this unusual mechanism of σ regulation.

Keywords: Crl; RNA polymerase; RpoS; *Salmonella*; sigma S.

[a]Present address: Weill Cornell Medicine, 1300 York Avenue, New York, NY 10065, USA
***Corresponding author: Françoise Norel,** Institut Pasteur, Laboratoire Systèmes Macromoléculaires et Signalisation, Département de Microbiologie, 25 rue du Docteur Roux, F-75015 Paris, France; and CNRS ERL3526, rue du Docteur Roux, F-75015 Paris, France, e-mail: francoise.norel@pasteur.fr
Paola Cavaliere: Institut Pasteur, Laboratoire Systèmes Macromoléculaires et Signalisation, Département de Microbiologie, 25 rue du Docteur Roux, F-75015 Paris, France; and CNRS ERL3526, rue du Docteur Roux, F-75015 Paris, France

Introduction

Bacterial cells encountering multiple environments are constantly exposed to suboptimal conditions, such as nutrient starvation and variations in physical and chemical parameters, to which they adapt by regulating gene expression. One major strategy employed by bacteria to modify expression of their genome is the use of alternative sigma (σ) subunits of the RNA polymerase (RNAP), directing transcription initiation at different classes of promoters (1–4). Sigma factors direct the expression of specific sets of genes by interacting with the catalytically active RNAP core enzyme (E, $\alpha_2\beta\beta'\omega$) and enabling the holoenzyme Eσ to bind to specific promoters and initiate transcription. Replacement of one σ factor in the RNAP holoenzyme by another one changes the transcription pattern. All bacteria have a housekeeping σ factor essential for transcription of the majority of cellular genes during growth, and one or more alternative σs, which allow transcription of specific sets of genes in response to environmental conditions. Bacterial σ factors have been divided into two structurally and functionally distinct families, the σ^{70} and σ^{54} families, named after the *Escherichia coli* housekeeping and nitrogen-stress σ factors respectively (3–5). The alternative sigma factor σ^S, closely related to σ^{70} and encoded by the *rpoS* gene, is the master regulator of the general stress response in *E. coli* and many other Gram-negative bacteria. σ^S remodels the transcriptional program and the cell physiology to promote multiple stress resistance and long-term survival. σ^S also plays important roles in biofilm formation and virulence of the food-borne pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (6–10).

σ^S is tightly regulated at the transcriptional, translational and posttranslational levels to restrict its expression and activity under inappropriate conditions (6, 7). This is because σ^S has a negative effect on the expression of several housekeeping genes, making σ^S expression a disadvantage for bacterial growth (7, 9, 11, 12). σ^S expression is

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blocked during active growth, both by inefficient translation and by rapid degradation of the σ^S protein through its interaction with the adaptor protein RssB, which targets σ^S to the ClpXP protease (6, 7) (Figure 1). Under stress conditions, translation of the *rpoS* mRNA is facilitated by small regulatory RNAs, and the stability of the σ^S protein is increased due to sequestration of RssB by anti-adaptors (6, 7) (Figure 1). Furthermore, σ^S activity is regulated by an unusual mechanism. In order to become active, a sigma factor has to associate with the core RNAP. The efficiency of formation of the housekeeping and alternative Eσ is another target step for regulation, which can be modulated by regulatory factors that bind E and/or σ (1–4, 6). A recurring strategy to inhibit σ factors activity is their sequestration by anti-σ proteins that prevent σ binding to the core RNAP (1, 3, 4). In contrast, the small regulatory protein Crl binds to σ^S and favors its association with the core RNAP, thereby increasing its activity (Figure 1). This review focuses on this unique transcription factor that has been characterized so far in two closely related bacterial species, *E. coli* and *S.* Typhimurium. We will present recent knowledge on its structural characterization that has led to a better, but still far from complete, understanding of its mechanism of action.

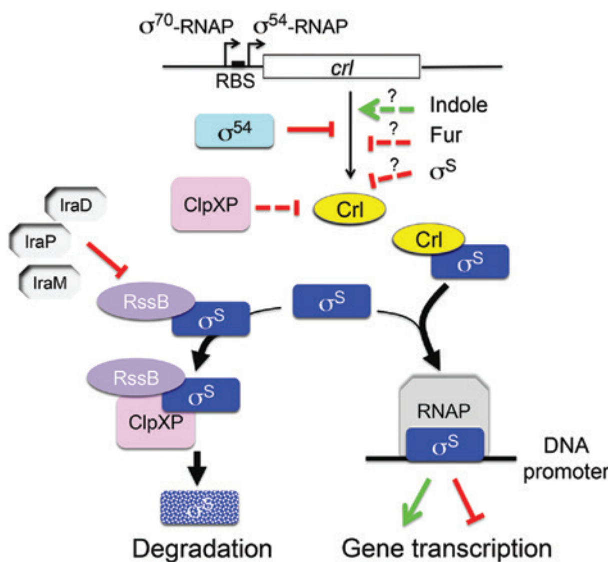

Figure 1: Main features for Crl expression and function.
The two transcriptional start sites and the ribosome binding site (RBS) of *crl* are shown. Crl binds to σ^S to favor its association with the core RNAP, thereby increasing its activity. The RssB protein binds σ^S to favor its degradation by the ClpXP protease, unless one of the anti-adaptors (IraP, M or D) is produced and interferes with RssB. Known and potential regulators of Crl expression are indicated. Green arrows and blunt red arrows indicate positive and negative regulation, respectively. Dashed lines indicate unclear regulatory effect. See text for details and references.

Crl: an unconventional transcription activator

The *crl* gene was named as such because, initially, it was thought to encode a protein that forms fibers called curli at the cell surface of *E. coli* K12 (13), and was found later to be a regulator of the *csg* genes encoding the curli protein subunits and secretion apparatus (14–16). Transcription of the *csg* genes was shown to be dependent on σ^S in the stationary phase of growth (17). Later on, the finding that a *crl* mutation decreases expression of many other σ^S-regulated genes and does not lower σ^S expression, led to the suggestion that Crl stimulates the activity of σ^S (18). *In vitro* experiments demonstrated a direct role of Crl in activating σ^S-dependent transcription initiation (19). Crl activation was unusual because Crl stimulated σ^S-dependent transcription at different promoters without binding to the promoter DNA (18–21). Instead, to promote σ^S transcriptional activity, Crl physically interacts with σ^S (22) and enhances the formation of Eσ^S (20, 21, 23) (Figure 1). Crl increases the affinity of σ^S for E by increasing the association constant rate of the binding reaction and has no significant effect on the stability of the Eσ^S complex (23). Even though it was initially suggested that Crl affects transcription initiation *in vitro* by other sigmas, such as σ^{70} and σ^{32} (20), it is now established that Crl is exclusively dedicated to σ^S (21, 24–27). In particular, Crl does not bind σ^{70} and does not modify the affinity of σ^{70} for the core RNAP enzyme (23).

The *in vitro* affinity of purified σ^S for RNAP core is the lowest of all six *E. coli* sigma factors (28, 29), and σ^S levels are lower than that of σ^{70}, even in the stationary phase of growth when σ^S reaches its highest concentration (30, 31). Therefore, by increasing σ^S affinity for the core RNAP, Crl increases the competitiveness of σ^S and thereby stimulates expression of σ^S-dependent genes (18, 19, 21, 24, 25). However, the magnitude of Crl activation is promoter-specific both *in vivo* and in *in vitro* transcription assays (24). These differences in the levels of responsiveness of different promoters to Crl activation may reflect differences in the intrinsic binding constants of these promoters for Eσ^S RNAP. Promoters that recruit Eσ^S inefficiently would be more affected by an increase in the Eσ^S concentration caused by Crl. Also, feedforward regulatory loops are likely very sensitive to Eσ^S levels and thus to Crl activation (24, 32, 33). However, at particular promoters, Crl might affect downstream steps in the transcription initiation pathway, including Eσ^S-DNA open complex formation (22, 23). It is possible that Crl modifies the positioning of Eσ^S on the promoter region, resulting in an

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altered architecture of the complex, and/or increases the ability of Eσ^S to melt DNA. One point that is unclear yet is whether Crl remains associated with Eσ^S. On one hand, Crl co-purifies with RNAP under certain conditions (34) and binds preformed Eσ^S *in vitro* (23). On the other hand, the half-life of the Crl-σ^S complex is very short *in vitro* (23), and likely *in vivo* since fractionation by gel filtration of free and bound Crl from cellular extracts of *E. coli* (21) and *Salmonella* (Monteil and Norel unpublished) showed that Crl is found mainly in a free state. Furthermore, there is no data so far demonstrating the participation of Crl in a quaternary complex with Eσ^S bound to DNA.

The effects of Crl on expression of σ^S-dependent genes are greatest at low levels of σ^S, *in vitro* and *in vivo,* and increased levels of σ^S can complement a *crl* knockout mutation (19–21, 24). Consistently, the physiological impact of Crl on σ^S-dependent gene transcription is the highest at the entry into stationary phase when σ^S begins to accumulate (19, 24). In addition, Crl effects have been also revealed in the exponential phase of growth when σ^S levels are very low (35). Unexpectedly, while a *crl* knockout mutation lowers σ^S activity, levels of σ^S are slightly higher in *crl* mutants, compared to wild-type strains (18, 19, 21). This finding results from two antagonistic effects of Crl (21). By activating Eσ^S-dependent expression of *rssB*, Crl increases σ^S degradation (Figure 1). However, by stimulating σ^S association with E, Crl indirectly has a stabilizing effect on σ^S by limiting its interaction with RssB and its subsequent degradation by ClpXP (Figure 1). This dual effect of Crl likely contributes to the tight control of kinetics and levels of Eσ^S formation in the cell.

Whereas σ^S is essential for cell viability under non optimal growth conditions, it also has negative effects on expression of several housekeeping genes and bacterial growth (7, 10–12), explaining why *rpoS* mutants, which show growth advantages, are selected in populations of *E. coli* and *Salmonella* in the absence of environmental stress (7, 12, 36). For bacterial populations living in changing environments, diversification into individuals with variable levels of σ^S activity is likely a bet-hedging strategy, in which Crl plays a role. By favoring Eσ^S formation, Crl contributes to the negative effects of σ^S on gene expression and bacterial growth (24, 37, 38). Thus, it is not unexpected that Δ*crl* mutants of *Salmonella* and *E. coli* have a competitive advantage over wild type strains during stationary phase (24, 38). *crl* mutants have also been detected among *E. coli* and *Salmonella* isolates and Crl was shown to rescue *rpoS* mutants with reduced σ^S activity (39–41). These findings suggest that Crl contributes to the fitness advantages of mutants with reduced σ^S activity in particular environments.

Regulation of Crl expression

In *S.* Typhimurium and *E. coli* grown in rich medium, maximal levels of Crl are found at the entry into stationary phase (19, 21, 24). Indole has been proposed to act as an extracellular signal for Crl expression in *E. coli* during the transition between the exponential and stationary phases (25) (Figure 1). However, this cannot be the case in *S.* Typhimurium, which, unlike *E. coli* K12, does not produce indole. In late stationary phase, when σ^S levels are high, σ^S exerts a negative effect on Crl production (18, 24) (Figure 1). The mechanism underlying this negative correlation is not yet understood but is consistent with the finding that Crl is required at low levels of σ^S. Crl levels are not limiting for σ^S activity in *Salmonella* grown in rich medium (33). Indeed, Crl is present in a 2- to 3-fold excess over σ^S in late stationary phase and the excess of Crl over σ^S is even greater at the entry into stationary phase (33).

The *crl* gene has two overlapping promoters, a σ^{70}-dependent promoter (15) and a downstream σ^{54}-promoter that is up-regulated under nitrogen limitation (42). However, Crl production is silenced under nitrogen limited conditions because the σ^{54}-promoter produces a *crl* transcript which lacks a ribosome binding site, and the Eσ^{54} holoenzyme occludes the σ^{70}-dependent *crl* promoter, thereby preventing the production of the translatable *crl* mRNA (42) (Figure 1). Under nitrogen-limiting conditions, σ^S production slows growth, and by reducing Crl synthesis this simple regulatory mechanism restrains the activity of σ^S and allows faster growth (42).

Little is known about other possible mechanisms of Crl regulation (Figure 1). Crl has been proposed to be a thermosensor favoring σ^S activity at 30°C because *crl* expression in *E. coli* K12 is increased at low temperature (22). However, in other studies *crl* expression in *E. coli* (15, 21, 26) and *S.* Typhimurium (33) was only mildly affected by temperature. The ferric uptake regulator Fur might both repress *crl* transcription and interact with Crl in *E. coli* K12 (43) but not in *S.* Typhimurium (39). Crl contains a potential ClpX recognition signal and has been captured in a trap for ClpXP substrates, suggesting a role for ClpXP in Crl proteolysis (44). However, a His-tagged Crl protein was not a substrate of the ClpXP proteolytic machinery in *in vitro* degradation assays (21) and Crl degradation by ClpXP remains to be monitored *in vivo*.

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Distribution and structural features of Crl

RpoS homologues are found in many Gram-negative bacteria of the γ, δ, and β subdivisions (7). In contrast, analysis of the protein sequence databases revealed the narrow distribution of Crl homologues in bacteria (39). Thus, *crl* is not as widely distributed as *rpoS*, and it is also less conserved at the sequence level. An alignment of 60 Crl sequences showed that only 17 residues are conserved among all Crl proteins (39). The low level of sequence conservation of Crl in bacterial species raised several questions: do all Crl family members have the same structure? Do they have the same σ^S-activator function? Do they bind to the same region of σ^S?

The first X-ray crystal structure of Crl, from *Proteus mirabilis,* was released in the protein data bank (PDB) by the Midwest Center for Structural Genomics consortium [PDB code 3RPJ, later reported in (45)]. Cavaliere et al. also solved the crystal structure of Crl from *Proteus mirabilis* [PDB code 4Q11, (46)] and the solution nuclear magnetic resonance (NMR) structure of Crl from *S*. Typhimurium (47, 48) (Figure 2). These structural studies, and complementary biophysical and functional analyses, demonstrated that Crl proteins from different bacterial species display similar structural features and σ^S-enhancer activity (45, 46). Moreover, they bind to the same region of σ^S, suggesting a common functionality in Crl family members (45, 46). Although Crl forms dimers in the X-ray crystal structures (45, 46), biophysical analyses and the NMR studies have demonstrated the monomeric state of Crl in solution (46–48). Crl has a globular fold with a single α/β-domain in which an exposed cavity, formed by antiparallel β-sheets, is enclosed by flexible loops (Figure 2). Both the cavity and the flexible loops have a fundamental role in the recognition and binding to σ^S (45–48). Conserved residues important for Crl activity have been identified in the cavity and loop 2 (39, 45, 46, 48).

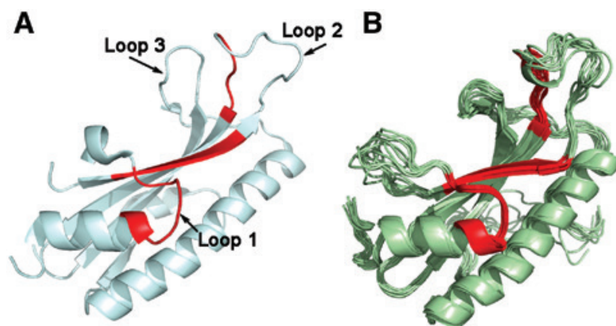

Figure 2: Tridimensional structures of Crl.
The structure of Crl is characterized by a single α/β-domain in which an exposed cavity, formed by antiparallel β-sheets, is enclosed by three flexible loops (loops 1, 2 and 3). In panel (A) the X-ray crystal structure of Crl from *Proteus mirabilis* [4Q11 (46)] is shown and in panel (B) are shown 10 conformers of *Salmonella* Crl obtained by NMR [2MZ8 (47, 48)]. The σ^S binding regions are highlighted in red.

σ^S-Crl binding interface

There is no tridimensional structure available for σ^S and for other isolated full-length σ factors. However, crystal structures were solved for housekeeping σ factors in the RNAP holoenzyme, and for other σ factors in complex with anti-sigmas (2, 4). For the couple σ^S-Crl, crystallization trials have failed [Ref. (48) and unpublished works], probably in part because of the instability of the Crl-σ^S complex. Indeed, the interaction between σ^S and Crl is not strong. The K_d value of 0.8 μM, measured by isothermal titration calorimetry (ITC) for the *Salmonella* proteins (46), is very high compared to K_d values obtained for interaction between sigma factors and the core RNAP that are in the nanomolar range (28). Moreover, surface plasmon resonance (SPR) experiments have shown that the half-life of this complex is of about 3 s (23). The σ^S-Crl binding reaction is characterized by negative values of enthalpy changes ($\Delta_b H$), suggesting that mainly electrostatic interactions drive the formation of this complex (46).

σ^S belongs to the σ^{70}-family of σ factors whose members contain at least two structural domains connected by flexible linkers: domain 2 and domain 4 (4, 5). The Crl binding region on σ^S was initially spotted within domain 2 by using the bacterial two-hybrid system (41). Domain 2 is the most highly conserved domain of σ factors, and is composed of five regions (1.2, 2.1, 2.2, 2.3 and 2.4) with specific roles in RNAP and promoter DNA binding (2, 4, 5). Biochemical, biophysical and mutational analyses have identified two noncontiguous regions in σ^S domain 2 required for Crl binding, one in region 1.2 and one in region 2.3 (27, 41, 48). The Crl binding motif in σ^S region 2.3 is not conserved in σ^{70}, and the other Crl binding site is at a position in σ^S region 1.2 where a large non-conserved region (NCR) interrupts the sequence of σ^{70}, explaining why Crl does not recognize σ^{70} (23, 27). Indeed, a σ^{70} chimeric protein lacking NCR but containing the Crl-binding motifs of σ^S interacts with Crl (27).

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Cavaliere et al. (48) recently added a new piece of information to the identification of the Crl-σ^S interface. They took advantage of the sequence evolution of conserved domain 2 of σ^S in bacterial species that do not contain a *crl* gene, such as *Pseudomonas aeruginosa*, to identify and assign a critical σ^S arginine residue to the σ^S-Crl interface. Whereas this arginine is conserved in σ^S proteins from *crl*-proficient species (R82, Figure 3), a leucine is present at the corresponding position in the *P. aeruginosa* σ^S protein. Remarkably, *P. aeruginosa* σ^S does not bind Crl unless the leucine is substituted by an arginine (48). The key arginine residue is located within the first Crl binding site, in region 1.2 of σ^S, which consists of an α-helix in the structural model of σ^S (27, 41, 48) (Figure 3). The loop just on top of this α-helix constitutes the second Crl binding site in region 2.3, which is formed by conserved residues D135, P136 and E137 (DPE motif) (27, 48).

Regarding Crl, mutational analyses and structural data have demonstrated that two conserved and surface exposed residues, D36 located in the cavity and R51 located in loop 2, are directly involved in the Crl-σ^S complex formation (39, 45, 46, 48) (Figure 3). Interestingly, NMR experiments using labeled $^{15}N/^{13}C$ Crl and unlabeled σ^S proteins from *Salmonella* revealed that chemical shift perturbations extend beyond the region directly involved in σ^S binding (48). These perturbations might be due to rearrangements in the flexible loops to allow breathing of the cavity and to accommodate σ^S. Using docking programs, Cavaliere et al. (48) proposed structural models for the σ^S-Crl complex, compatible with all the structural data and mutational analyses. In these models, salt bridges can be established between the two pairs of residues Crl-D36/σ^S-R82 and Crl-R51/σ^S-E137, leading to a binding interface with the α-helix of σ^S docking into the Crl cavity and the DPE motif in σ^S interacting with the Crl loop 2 (Figure 3). This interface, based on electrostatic interactions, endorses the finding of the electrostatic driven mode of σ^S-Crl complex formation (46).

Characterization of a Crl protein in which the key residue D36 is substituted by an alanine might shed light on the transient nature of the Crl-σ^S complex (48). In the native structure of Crl, residue D36 interacts intramolecularly with residue R24, which is not required for σ^S binding (46, 48) (Figure 3). Upon σ^S binding, this interaction is likely disrupted to allow interaction between D36 in Crl and R82 in σ^S (Figure 3). The NMR spectra of the variant Crl D36A showed how the disruption of the intramolecular contact D36-R24 is sensed by the whole Crl structure, in particular by loop 1, suggesting a scenario in which disruption of the D36-R24 interaction due to σ^S binding destabilizes the Crl structure, leading to a rapid dissociation of the Crl-σ^S complex.

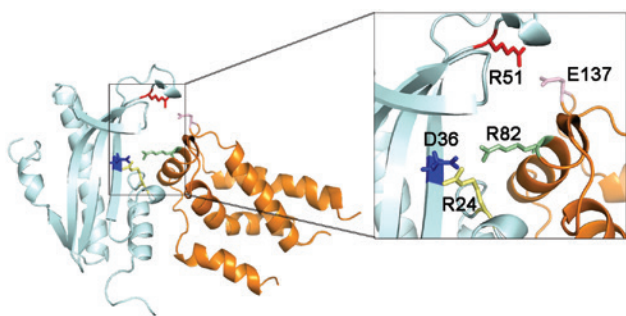

Figure 3: The σ^S-Crl binding interface model.
In the σ^S-Crl complex model obtained using docking programs, two possible electrostatic interactions involve conserved σ^S and Crl residues of paramount importance for complex formation: Crl R51-σ^S E137 and Crl D36-σ^S R82 (48). In the tridimensional structure of unbound Crl, residue R51 is free while D36 can establish an electrostatic interaction with the Crl residue R24 (46, 48). σ^S is depicted in orange and Crl in pale cyan.

Conclusion

Despite the greater knowledge accumulated recently on the structural determinants of the Crl-σ^S interaction, the mechanism by which Crl increases σ^S affinity for the core RNAP is still not fully understood. Crl binds to domain 2 (σ_2) of σ^S, the most highly conserved domain of σ factors. The σ-core RNAP interface involves several regions of σ, but the contact area of the σ_2-core interface is the largest among the σ domains (2, 4, 5). σ_2 interacts with the β′ subunit in core RNAP mainly through region 2.2 and to a lesser extent through region 2.1 (2, 4, 5). Thus, the Crl binding sites on σ^S are in close proximity with the RNAP binding regions. In some housekeeping σ, such as σ^{70}, the NCR inserted between regions 1.2 and 2.1 is also implicated in binding to the β′ subunit of RNAP (2, 4). In the structure of domain 2 of σ^{70} [PDB 1SIG, (49)], the C-terminus of region 1.2 is close to the N-terminus of conserved region 2.1. Crl binding to this position in σ^S_2 might facilitate interactions between the β′ subunit and σ^S_2, as suggested (27). The short half-life of the Crl-σ^S complex suggests a scenario where Crl acts as a ‘bind and deliver’ chaperone of σ^S, increasing the rate of σ^S association with the core RNAP and preventing σ^S to remain free in the cell. Crl might induce conformational changes in σ^S unmasking key β′ binding determinants and/or repositioning σ^S in the holoenzyme. Crl has no major effect on the stability

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of the Eσ^S holoenzyme, but a weak interaction has been detected between Crl and E (23). Moreover, Crl can bind Eσ^S and copurify with RNAP in some conditions (23, 34). In addition, Crl may aid Eσ^S assembly indirectly, by breaking σ^S intra-molecular or inter-molecular interactions, since Crl favors the solubility of σ^S and σ^S_2 proteins, which have a tendency to form dimers and aggregate at high concentrations (46). Altogether these findings suggest that our current knowledge on the Crl-Eσ^S interaction is the tip of the iceberg.

The NCR of housekeeping sigmas and the equivalent position in other σ factors (where Crl binds to σ^S) might constitute a target for transcription regulation (50). At least two transcriptional activators, GrgA and RbpA, interact with the NCR of σ factors (48–50). RbpA binding to this position in the housekeeping factor σ^A might facilitate interaction between RbpA and the -10 promoter element, and favor open complex formation (51, 52). Assuming that Crl stays associated with Eσ^S in the transcription initiation complex, and/or modifies the positioning of σ^S in the holoenzyme and in its complex with DNA, Crl could also affect the formation of open complexes at specific promoters, as suggested (22, 23). This activity of Crl might be facilitated by the close proximity on σ^S of the Crl binding sites and regions 1.2 and 2.3, which interact with the promoter discriminator and -10 elements and are involved in open complex formation in the context of housekeeping RNAP (2, 4, 5). For deeper understanding of the Crl mechanism, it would be important to determine whether and how Crl modifies the interaction between Eσ^S and the promoter DNA. *In vivo*, Crl appears to stimulate the expression of σ^S-dependent genes independently of any specific promoter motif (21). *In vitro* transcription profiling (53), using Eσ^S/Crl and the whole bacterial genome as DNA template, might reveal structural features of Crl-dependent promoters. Studies of transcriptional activators that show no sequence similarity to Crl, but bind to an equivalent region on σ factors and perform analogous tasks should provide important novel insights. It would be interesting to determine how widespread these unconventional transcription activators are, and to which extent their mechanisms of action share common features.

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List of abbreviations

RNAP	RNA polymerase
σ	sigma factor
E	core RNA polymerase
SPR	surface plasmon resonance
PDB	protein data bank
ITC	isothermal titration calorimetry
$\Delta_b H$	binding enthalpy change
NMR	nuclear magnetic resonance

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