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Binding of the Unorthodox Transcription Activator, Crl, to the Components of the Transcription Machinery*

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The small regulatory protein Crl binds to σ^S, the RNA polymerase stationary phase σ factor. Crl facilitates the formation of the σ^S-associated holoenzyme ($E\sigma^S$) and thereby activates σ^S-dependent genes. Using a real time surface plasmon resonance biosensor, we characterized in greater detail the specificity and mode of action of Crl. Crl specifically forms a 1:1 complex with σ^S, which results in an increase of the association rate of σ^S to core RNA polymerase without any effect on the dissociation rate of $E\sigma^S$. Crl is also able to associate with preformed $E\sigma^S$ with a higher affinity than with σ^S alone. Furthermore, even at saturating σ^S concentrations, Crl significantly increases $E\sigma^S$ association with the *katN* promoter and the productive isomerization of the $E\sigma^S$-*katN* complex, supporting a direct role of Crl in transcription initiation. Finally, we show that Crl does not bind to σ^{70} itself but is able at high concentrations to form a weak and transient 1:1 complex with both core RNA polymerase and the σ^{70}-associated holoenzyme, leaving open the possibility that Crl might also exert a side regulatory role in the transcriptional activity of additional non-σ^S holoenzymes.

In Enterobacteria, σ^S, encoded by *rpoS*, is the master regulator of the general stress response and is also responsible for the transcription of stationary phase-specific genes. σ^S accumulates at the onset of the stationary phase and in response to harsh environmental conditions, including carbon and phosphate starvation and acidic and osmotic stress (1, 2). When associated with the RNA polymerase core enzyme (*E*),[3] the σ^S-associated holoenzyme ($E\sigma^S$) transcribes *rpoS*-dependent genes and endows the cells with the ability to endure stationary phase and tolerate a multitude of stress conditions (3–5). The acquisition of this multiple stress resistance status, which is dependent on σ^S, has an energetic cost and decreases bacterial fitness in environments containing nutrients at low concentrations (6). Therefore, σ^S abundance and activity are tightly controlled by the interplay of a complex set of regulators that affect transcription, translation, and the stability of the protein. Indeed, the levels of σ^S are controlled primarily by the ClpXP protease, which, together with the catalytic adaptor protein RssB, targets free σ^S for degradation during exponential growth in the absence of stress (7, 8). σ^S concentration is not the sole parameter controlling the expression of *rpoS*-dependent genes. Another important checkpoint is the formation of $E\sigma^S$, which in *Escherichia coli* is restricted by the competition of σ^S with six other σ factors for binding to a limited amount of *E* (9–11). Even in stationary phase, when σ^S is most abundant, the concentration of the primary σ factor, σ^{70}, remains 3-fold higher (12). Furthermore, of all of the *E. coli* σ factors, σ^S exhibits the lowest affinity for *E* (13). *E. coli* has developed different strategies that enable σ^S to capture enough *E* to transcribe its target genes, and several factors favor the formation of $E\sigma^S$ during stationary phase or in response to toxic insult. First, by preventing ribosomal gene transcription, the alarmone ppGpp, together with DksA, dissociates the σ^{70}-associated holoenzyme ($E\sigma^{70}$) from rRNA loci releasing *E* for binding to other σ factors (14–16). Second, by directly binding to σ^{70}, Rsd, the level of which increases 2-fold in stationary phase, captures and inhibits free σ^{70} (17–20). Finally, in the late stationary phase, the small 6S RNA sequesters a large fraction of $E\sigma^{70}$, thereby restricting σ factor competition to the remaining six σ factors (21, 22). These mechanisms offer a partial solution, and clearly other elements are needed to assist σ^S in its competition with other σ factors and to directly facilitate the binding of σ^S to *E*. One such factor is Crl, a protein that was initially described as an activator of curli formation (23). Crl binds to σ^S (24) and positively regulates σ^S-dependent genes, especially when σ^S levels are low (25–29). Biochemical analyses confirm the direct effect of Crl on σ^S-dependent transcription (26, 27, 29, 30) and furthermore demonstrate that Crl favors $E\sigma^S$-dependent transcription over $E\sigma^{70}$-dependent transcription (29). Gel filtration experiments with crude *E. coli* cell extracts indicate that Crl enhances the binding of σ^S to *E* (29). Intriguingly, a recent biochemical study demonstrated that Crl could also enhance the transcriptional activity of $E\sigma^{70}$ and $E\sigma^{32}$, albeit significantly less than that of $E\sigma^S$ (30). Two alternative, but not exclusive, models have been proposed to describe the action of Crl in activating transcription. First, Crl may promote the unfurling of free σ, allowing it to adopt the appropriate conformation for efficient

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[3] The abbreviations used are: *E*, RNA polymerase core enzyme; $E\sigma^S$, σ^S-associated holoenzyme; SPR, surface plasmon resonance; BACTH, bacterial adenylate cyclase two-hybrid; RU, response units.

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binding to *E*, and second, Crl may function by stabilizing the $E\sigma$ once formed.

$E\sigma$ formation is a multistep process that involves major rearrangements in both E and σ (31). Both σ^{70} and σ^{S} are members of the σ^{70} family of σ factors, which are composed of four highly conserved domains that can adopt different conformations relative to one another (32–34). The minimal structural scaffold of all σ^{70} family members is composed of two large globular domains: σ domain 2 (σ_2) and σ domain 4 (σ_4). Domains σ_2 and σ_4, which are likely to interact with each other in the context of free σ (34), possess the main determinants for binding both *E* and promoter DNA. σ_2 binds the β' subunit coiled-coil and promoter −10 element, and σ_4 binds the β subunit flap and the promoter −35 element (35–38). These interactions are crucial for orientating the σ_2 and σ_4 DNA-binding determinants with the correct spacing for binding to the promoter −10 and −35 elements, respectively (31). High resolution structural studies of thermophilic bacterial $E\sigma$ reveal that σ is spread out along one face of *E*, forming an extensive protein-protein interface with the β and β' subunits (36–38). Both free σ^{70} and σ^{S} are unable to recognize promoter DNA (39, 40),[4] most likely a result of the intramolecular interactions between σ_2 and σ_4 (34). However, variants of σ^{70} and several alternative σ factors with the N-terminal domain 1.1 ($\sigma_{1.1}$) deleted exhibit poor but detectable promoter-specific binding (41). These results suggest that $\sigma_{1.1}$, which is not resolved in any structure, inhibits the DNA-binding properties of σ^{70} and several alternative σ factors, including σ^{S}, by locking them in an inactive closed conformation. Upon binding to *E*, dramatic conformational changes in free σ ensue, resulting in a 15-Å increase in the distance between σ_2 and σ_4 and the unmasking of the DNA-binding determinants, as well as the displacement of $\sigma_{1.1}$ by 20 Å (42).

To date, the binding properties of Crl to the components of the transcription machinery have not been characterized in a quantitative manner; nor has the mechanism by which Crl increases transcriptional activity been fully understood. In this study, we have used a real time surface plasmon resonance (SPR) microfluidic biosensor to decipher the specificity and mechanism of Crl-dependent activation. First, we investigated the binding of Crl to the components of the transcription machinery (σ^{S}, σ^{70}, *E*, $E\sigma^{S}$, and $E\sigma^{70}$), and second, we examined the promoter binding properties of $E\sigma^{S}$ and $E\sigma^{70}$, formed in the presence or absence of Crl. Our studies indicate that Crl binds to σ^{S} and not to σ^{70} and that Crl acts most likely by promoting a conformational change in σ^{S} that enhances its association rate to *E*. Furthermore, Crl is also able to bind to preformed $E\sigma^{S}$, resulting in a stable ternary complex. Finally, we find that Crl is able to bind directly to *E*, forming a transient complex that might be related to the reported Crl-dependent activation of transcription by other $E\sigma$ species (30).

EXPERIMENTAL PROCEDURES

Cloning Procedures—The DNA encoding a His_{12} tag was appended to the 5′ end of both *rpoD*, encoding σ^{70}, and *rpoS*, encoding σ^{S}, in a multistep procedure. First, the XbaI-HindIII fragment of pGEMHis*rpoD* (43) was cloned between the XbaI and HindIII sites of pET21a, generating pET21His_6*rpoD* and the internal *rpoD* NdeI site was removed by site-directed mutagenesis. Second, oligonucleotides, 5′-T ATG CAT CAC CAT CAC CAC CA-3′ (K81) and 5′-T ATG GTG GTG ATG GTG ATG CA-3′ (K82), encoding an NsiI site and a His_6 tag, were annealed and cloned into the unique NdeI site of pET21His_6*rpoD*, creating pET21His_{12}*rpoD*. Finally, the BamHI-HindIII fragment from pET21His_{12}*rpoD* was replaced with the wild type *rpoD* BamHI-HindIII fragment. Similarly, the K81/K82 fragment was inserted into the unique NdeI site of pFC0, that encodes σ^{S} (44), resulting in pFC0His_6*rpoS*, after which the vector-encoded NsiI-HindIII fragment was replaced by the NsiI-HindIII vector-encoded fragment of pET21His_{12}*rpoD*, generating pET21His_{12}*rpoS*.

The DNA encoding *Salmonella enterica* Crl was amplified by PCR using primers L73 5′-GTT GCT TCA TTA AAG GAG ATC CAT ATG ACG TTA CCG AGT GGA CAC C-3′ and L74 5′-GGC ATG GCA GAA TTC TTA TGC CGA CAG TTT TAC CGG C-3′ using *S. enterica* genomic DNA as a template. The resulting fragment was cleaved with NdeI and EcoRI and cloned between the NdeI and EcoRI sites of a pET28a-based plasmid (20), creating pSKB2ppX*crl*.

Derivatives of pUT18 encoding N-terminal Crl-T18 fusion proteins used in the bacterial adenylate cyclase two-hybrid (BACTH) assays were constructed by cloning PCR-amplified DNA fragments encoding Crl between the XbaI and KpnI sites of pUT18. The DNA encoding *E. coli* Crl was amplified using primers M75 (5′-CG ACT CTA GAG ATG ACG TTA CCG AGT GGA CAC CCG-3′) and M76 (5′-GCT CGG TAC CCG TTA TTA TGC CGA CAG TTT TAC CGG CTC GTC G-3′) and plasmid pQE*crl* as the template (26). The DNA encoding *S. enterica* Crl was amplified by PCR using primers M82 (5′-C CCT CTA GAA ATG ACG TTA CCG AGT GGA CAC CCG-3′) and M83 (5′-C TCG GTA CCC GCG CCG TTA ACT TCA CCG G-3′) and plasmid pSKB2ppX*crl* as the template. The resultant PCR products were cloned between the XbaI and KpnI sites of pUT18. All plasmids were confirmed to be correct by DNA sequencing.

Purification of Proteins—Expression plasmids were transformed into BL21 (DE3) *E. coli* cells, and the transformants were selected in the presence of the appropriate antibiotic. Cultures were grown at 37 °C to an A_{600} ~0.6 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h at 30 °C. Cells containing overexpressed proteins were harvested by centrifugation and stored at −80 °C.

His_{12}-σ^{S} and His_{12}-σ^{70} were prepared using an adapted protocol (45). The untagged σ^{70} and σ^{S} factors were purified from the M5219/pMRG8 and BL21(DE3)/pLysS/pFC0 strains, respectively, according to previously described protocols (46, 47). *E* was prepared according to Ref. 48.

The His_6-ppXCrl protein, containing a vector-encoded N-terminal His_6 tag and a PreScission protease cleavage site, was purified by HiTrap Ni^{2+}-charged affinity chromatography (GE Healthcare). The N-terminal His_6 tag was removed using the PreScission protease (GE Healthcare). Crl produced in this manner contains three additional N-terminal amino acids, followed by the methionine residue of the wild type 133-amino

[4] A. Kolb, unpublished data.

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acid protein. The sample was further purified by a second subtractive HiTrap Ni^{2+}-charged affinity chromatography step to remove uncleaved His_6-ppXCrl protein and the His_6 tag, followed by an ion exchange chromatography step (HiTrap Q Sepharose; GE Healthcare) and a gel filtration chromatography step (Superdex 75; GE Healthcare). The peak fractions were dialyzed against storage buffer (10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 200 mM KCl and 50% (v/v) glycerol) and stored at −20 °C. His_6-Crl was purified as previously described (26) and used in place of untagged Crl with identical results. Aliquots were passed over a Microcon YM-3 to exchange the storage buffer for buffer A (40 mM Hepes (pH 8.0), 10 mM $MgCl_2$ and 100 mM potassium glutamate) prior to SPR analysis.

BACTH Assays—The *E. coli cya* strain BTH101 was transformed with derivatives of plasmids pKT25 and pUT18 encoding the T25 and T18 fragments of *Bordetella pertussis* adenyl cyclase. Plasmids pKT25-σ^{70}, pKT25-σ^S, and pUT18-Rsd have been described previously (19). Co-transformants were plated onto MacConkey maltose plates supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. After incubating the plates at 30 °C for 3 days, colonies were collected and lysed with chloroform and 0.05% (w/v) SDS, and their β-galactosidase activities were measured as described by Miller (49). Each transformation was performed twice, and the S.E. values on the β-galactosidase activities were below 15%.

SPR Experiments—All SPR binding assays were conducted on a Biacore 2000 instrument (GE Healthcare), equilibrated at 25 °C in buffer A supplemented with 0.034% (v/v) Tween 20.

Binding of Crl to σ—5000 RU of penta-His monoclonal antibody (Qiagen) were covalently immobilized through their solvent-accessible primary amine groups to the carboxymethylated dextran matrix of a CM5 sensor chip, using the Amine Coupling Kit (GE Healthcare), according to the manufacturer's instructions. Briefly, each flow cell, equilibrated at a flow rate of 5 μl/min in phosphate-buffered saline (pH 7.4), supplemented with 0.005% (v/v) Tween 20, was activated for 12 min with a solution of 50 mM *N*-hydroxysuccinimide and 200 mM *N*-ethyl-*N*′-(3 dimethylaminopropyl)carbodiimide, followed by an injection of the antibody (5 μg/ml) in 10 mM sodium acetate (pH 4.5). The surface was finally deactivated for 12 min with 1 M ethanolamine (pH 8.5).

330–350 RU of either His_{12}-σ^S or His_{12}-σ^{70} were noncovalently captured on the penta-His antibody surface. Another surface was left unliganded and used as a reference flow cell. Crl (50 nM to 15 μM) was then injected for 1 min at a flow rate of 100 μl/min, and the dissociation was followed for 3 min. Regeneration of the surfaces was performed by successive injections of 10 mM glycine-HCl (pH 2) and 0.05% (w/v) SDS.

Binding of σ to Core RNA Polymerase—10,000 RU of 4RA2 monoclonal antibody (Neoclone), purified on a Protein A-Sepharose column (GE Healthcare), were covalently immobilized on the surface of a CM5 sensor chip, as described above. 2000–2500 RU of *E* were captured on a 4RA2 antibody surface. Another surface was left unliganded and used as a reference flow cell.

σ^S (0.4–250 nM) or σ^{70} (0.25–15 nM), in the presence or absence of Crl, was then injected for 7 min at 20 μl/min, and the dissociation was followed for 5 min. Control experiments were performed by injecting Crl alone. Regeneration of the surfaces was performed by successive injections of 10 mM glycine-HCl (pH 1.5) and 0.1% (w/v) SDS.

Binding of Crl to Core RNA Polymerase or Holoenzymes—1500–2000 RU of *E* were captured on a 4RA2 antibody surface, whereas a reference flow cell was left unliganded, followed by 130–150 RU of σ^S or σ^{70} (or running buffer for the study of direct *E*-Crl interactions). Crl (0.1–25 μM) was then injected for 5 min at 20 μl/min, and the dissociation was followed for 10 min. Regeneration of the surfaces was performed by successive injections of 10 mM glycine-HCl (pH 1.5) and 0.1% (w/v) SDS.

SPR Data Analysis—All of the association and dissociation profiles were double-referenced using the Scrubber 2.0 software (BioLogic Software) (*i.e.* both the signals from the reference surfaces and from blank experiments using running buffer instead of Crl or σ factors were subtracted). The binding curves were globally analyzed with a nonlinear least squares algorithm implemented in the BIAevaluation 4.1 software (Biacore), using single exponential (Langmuir model) or coupled double-exponential functions of time ("conformation change" model). Kinetic parameters (k_{on} and k_{off}), half-lives ($t_{1/2}$), equilibrium dissociation constants (K_d), and maximal binding capacities (R_{max}) were determined based on at least two experiments. Steady-state signals (R_{eq}; measured or extrapolated) were plotted against the Crl or σ^S concentration (C). Fitting was performed with the following equations,

$$R_{eq} = (R_{max} \times C)/(K_d + C) \quad \text{(Eq. 1)}$$

(single class of binding sites) or

$$R_{eq} = (R_{max1} \times C)/(K_{d1} + C) + (R_{max2} \times C)/(K_{d2} + C) \quad \text{(Eq. 2)}$$

(two independent classes of binding sites). The stoichiometry of binding of Crl or σ^S was determined with the equation,

$$n = (R_{max}/R_{immo})/(M/M_{immo}) \quad \text{(Eq. 3)}$$

where M and M_{immo} represent the molecular weight of the injected molecule (Crl or σ^S) and of the tethered molecule (*E* or σ^S), respectively, and R_{immo} is the density of tethered molecules on the sensor chip surface.

Gel Retardation Assays—The 123-bp labeled wild-type *katN* fragment was generated by PCR using primers 5′-[^{32}P]CGA GCT CGT GTT CCT CGT TGC TTG C-3′ and 5′-biotin-TTA CGC GGT AAA TCA CAA CTA TTT CCG-3′, and pJCD*katN* (27) as a template. A variant of the *katN* promoter with substitutions in the −35 element (TTGA to CCAG) and −10 region (CTAATTTTA to GAGCTCGGC) was synthesized using the QuikChange multisite-directed mutagenesis kit (Stratagene). The fragments were purified on a 7.5% polyacrylamide gel run in Tris-borate-EDTA buffer. *E* (16 nM) was preincubated in buffer A supplemented with 0.034% Tween 20 with σ^S (0, 16, 80, or 2000 nM). 5 μl of the protein complexes were then added to 5 μl of ^{32}P-labeled *katN* fragment in buffer A supplemented with 0.034% Tween 20 and 0.5 μg/ml of heparin and incubated at 25 °C for 20 min. After the addition of 2 μl of loading buffer (buffer A containing 50% sucrose, 0.025% xylene cyanol blue), the mixture was loaded onto a 6% native polyacrylamide gel run

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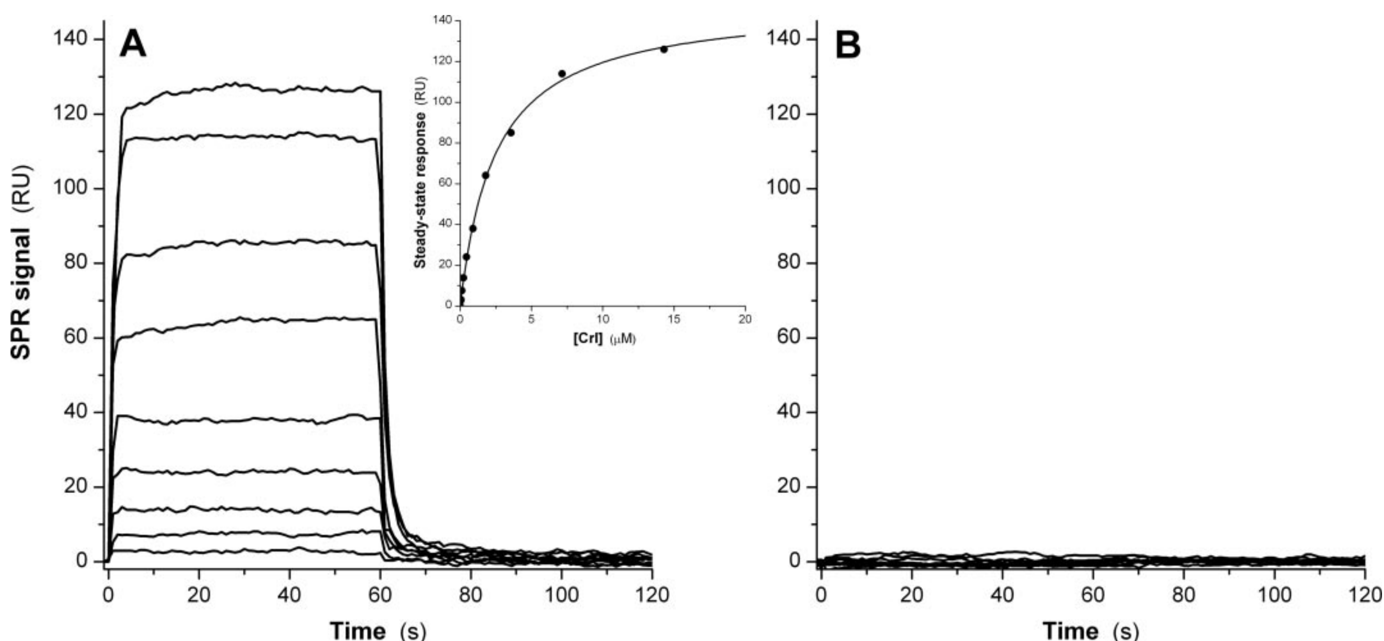

FIGURE 1. **Binding of Crl to immobilized His$_{12}$-tagged σ factors.** Association and dissociation real time profiles are shown for the following Crl concentrations: 50 nM, 110 nM, 225 nM, 450 nM, 890 nM, 1.79 μM, 3.58 μM, 7.15 μM, and 14.3 μM. *A*, immobilized σ^S surface. The *inset* shows the steady state response as a function of Crl concentration. *B*, immobilized σ^{70} surface. No binding is observed.

in TG buffer (25 mM Tris, 192 mM glycine, pH 8.5) at 10 V/cm. The gel was dried before being autoradiographed and quantified using a PhosphorImager (GE Healthcare).

Potassium Permanganate Reactivity—Complex formation was initiated by adding a mixture of $E\sigma^S$ with or without Crl to the labeled wild-type *katN* fragment at 37 °C or at 20 °C in buffer A containing 500 μg/ml bovine serum albumin. At various times, 10-μl aliquots were withdrawn and allowed to react with 3 μl of 18 mM potassium permanganate solution. The reaction was stopped after 15 s by adding 6 μl of 200 mM dithiothreitol, and the samples were phenol-extracted, precipitated with ethanol, and rinsed with 70% (v/v) ethanol. The ethanol precipitates were resuspended in 100 μl of piperidine (1 M), heated at 90 °C for 30 min, and evaporated until dry. Then 20 μl of water was added and evaporated (twice). The samples were resuspended in 5 μl of 20 mM EDTA in formamide containing xylene cyanol and bromphenol blue and loaded onto a 9% (w/v) denaturing polyacrylamide gel.

RESULTS

SPR instruments sense small refractive index changes, which are proportional to local concentration variations in the vicinity of a solid surface. SPR can be used to monitor protein-protein and protein-DNA interactions in real time and measure association and dissociation rates (50–54). SPR is especially appropriate for analyzing the properties of Crl, which in contrast to many transcription activators, exhibits no DNA binding activity but has been shown to bind directly to σ^S and affect the rate of open complex formation.

Crl Binds to σ^S but Not to σ^{70}—Real time SPR assays were first conducted to investigate the direct binding of Crl to either σ^S or σ^{70}. Variants of σ^S with the N-terminal portion of the protein deleted are responsive to Crl (24); hence, to immobilize σ^S and σ^{70} to the solid surface (sensor chip), we used a noncovalent orientated strategy, relying on the stable interaction between the His$_{12}$ moiety appended to the N termini of both σ^S and σ^{70}, and an anti-His$_5$ monoclonal antibody attached to the dextran surface of the sensor chip. We observed that Crl readily bound to σ^S, at concentrations as low as 60 nM, and reached a steady state within a few seconds (Fig. 1*A*). The analysis of the concentration dependence of the steady-state responses allowed us to determine a dissociation equilibrium constant (K_d) of 2.46 ± 0.13 μM and a 1:1 stoichiometry for the Crl-σ^S complex (Fig. 1*A*, *inset*). The dissociation rate (k_{off}) of the complex was ~0.3 s^{-1} (corresponding to a $t_{1/2}$ of ~3 s), and the association rate (k_{on}) was 1.5 × 10^5 M^{-1} s^{-1} (calculated from the k_{off}/K_d ratio). Under the same conditions, no binding of Crl to σ^{70} could be detected, even at the highest Crl concentration used (15 μM; Fig. 1*B*), indicating a minimal K_d threshold of 750 μM for a hypothetical Crl-σ^{70} interaction. The inability of Crl to bind σ^{70} was not due to the quality of the σ^{70} preparation used, since it was at least 50% active in abortive and run-off transcription assays using the *lac*UV5 promoter, and furthermore, when immobilized as above, it was able to interact tightly with its specific ligand, Rsd (regulator of sigma *D*; data not shown).

In all SPR experiments, we used σ^S and σ^{70} from *E. coli* and the Crl protein from *S. enterica*. Crl proteins from both species share 95% similarity and 85% identity, and the *S. enterica* protein supports transcription activation of *E. coli* $E\sigma^S$ at each promoter tested (26, 27). In order to probe the interplay of these proteins with one another *in vivo*, we utilized the BACTH system. The BACTH system is dependent upon the functional reconstitution of the *B. pertussis* adenyl cyclase T18 and T25 subdomains by two interacting partners (55, 56). The resultant cAMP binds to and activates the transcription activator CRP, a positive regulator of β-galactosidase expression. C-terminal fusions of *E. coli* σ^{70} and *E. coli* σ^S to T25 and N-terminal

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fusions of *E. coli* Crl and *S. enterica* Crl to T18 were constructed. Table 1 shows the different fusion proteins, which were overproduced in the *E. coli cya lac*$^+$ strain BTH101, and the resultant β-galactosidase activities. As shown previously (19) and confirmed by our experiments, the T25-σ^{70} fusion interacted with the Rsd-T18 fusion protein (Table 1, row 4). However, T25-σ^{70} did not interact with either *E. coli* or *S. enterica* Crl fused to T18 (Table 1, rows 2 and 3). In contrast, both the *E. coli* and *S. enterica* Crl-T18 fusion proteins interacted with T25-σ^S (rows 6 and 7), showing that Crl from both species was functionally selective for σ^S but unable to recognize σ^{70}. Taken together, our SPR and BACTH data clearly show that Crl has exquisite binding specificity for free σ^S but does not bind to σ^{70}.

Crl Specifically Facilitates the Formation of the σ^S-associated Holoenzyme—We reasoned that the N-terminally oriented immobilization of σ might not be optimal for the study of the interactions of σ with *E*, since it might restrict the mobility of $\sigma_{1.1}$. Therefore, to monitor the effect of Crl on σ-E interactions, we resorted to an alternative strategy that utilized a monoclonal antibody specific for the C terminus of the RNA polymerase α subunit (α-CTD; which plays no role in the association of σ with E) to noncovalently tether *E* on the surface of the chip. In the absence of Crl, σ^S bound to E with a stoichiometry of 1:1 and a K_d value of 68.2 ± 8.4 nM (Fig. 2*A*). The association process followed a complex mechanism best described by an initial encounter step between σ^S and *E*, followed by subsequent isomerizations of the $E\sigma^S$ complex, as previously reported for the formation of the $E\sigma^{70}$ holoenzyme (57). The dissociation curves were biphasic, corresponding to two different populations of σ^S-*E* complexes dissociating with markedly different k_{off} rates of $2.5 \pm 0.2 \times 10^{-2}\ s^{-1}$ and $2.3 \pm 0.3 \times 10^{-3}\ s^{-1}$. The experiment was repeated in the presence of a range of Crl (40 nM to 5 μM), showing that the K_d of the σ^S-*E* interaction steadily decreased as a function of Crl concentration, reaching an optimum for 5 μM Crl, with a K_d of 9.41 ± 1.86 nM, more than 7-fold lower than in the absence of Crl. Interestingly, the presence of Crl did not affect the biphasic nature of the σ^S-E dissociation curves or the corresponding k_{off} rates, indicating that the difference in K_d was primarily due to a 7-fold increase in the association rate (k_{on}) of σ^S with *E* (Fig. 2*B*). A concomitant increase in the maximum steady-state response (R_{max}) could be observed, reaching 285 ± 13 RU at [Crl] = 5 μM, against 205 ± 9 RU in the absence of Crl (Fig. 2*C*). This 1.4-fold increase can be directly correlated to the fact that, at 5 μM Crl, σ^S should essentially be present in the form of a σ^S-Crl complex (1.4 times heavier than σ^S).

In contrast, no effect of Crl on the binding of σ^{70} to *E* could be detected, regardless of the σ^{70} concentrations tested (0.25–15 nM; we determined a K_d of 0.3 nM for the σ^{70}-E interaction). The total absence of effect of Crl on σ^{70} incorporation (Fig. 3*B*, [σ^{70}] = 0.25 nM) can be compared with its large effect on the formation of $E\sigma^S$ (Fig. 3*A*, [σ^S] = 5 nM).

Crl Is Able to Bind Directly to the Core Enzyme and to the σ^S-associated Holoenzyme—Interestingly, we demonstrated that Crl could bind directly to E with a 1:1 stoichiometry and a low affinity (K_d = 13.8 ± 1.8 μM; Fig. 4*A*). The dissociation of the Crl-*E* complex was too fast (half-life < 1 s) to determine reliably its dissociation rate. Crl also bound transiently to preformed $E\sigma^{70}$ (Fig. 4*B*) with a 1:1 stoichiometry and an affinity

TABLE 1

BACTH analysis of Crl-σ interactions

The table lists measured β-galactosidase activities in BTH101 *cya* cells carrying pKT25 derivatives encoding a T25-σ^{70} or a T25-σ^S fusion protein and pUT18 derivatives encoding Crl-T18 and Rsd-T18 fusions. Cells were grown on MacConkey maltose plates containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 30 °C before measuring β-galactosidase activities. No difference in activity was detected between *E. coli* Crl-T18 and *S. enterica* Crl-T18.

	Protein fusion 1	Protein fusion 2	β-Galactosidase activity
			Miller units
1	T25-σ^{70}	T18	63
2	T25-σ^{70}	*E. coli* Crl-T18	60
3	T25-σ^{70}	*S. enterica* Crl-T18	62
4	T25-σ^{70}	*E. coli* Rsd-T18	1022
5	T25-σ^S	T18	60
6	T25-σ^S	*E. coli* Crl-T18	696
7	T25-σ^S	*S. enterica* Crl-T18	698
8	T25-σ^S	*E. coli* Rsd-T18	72

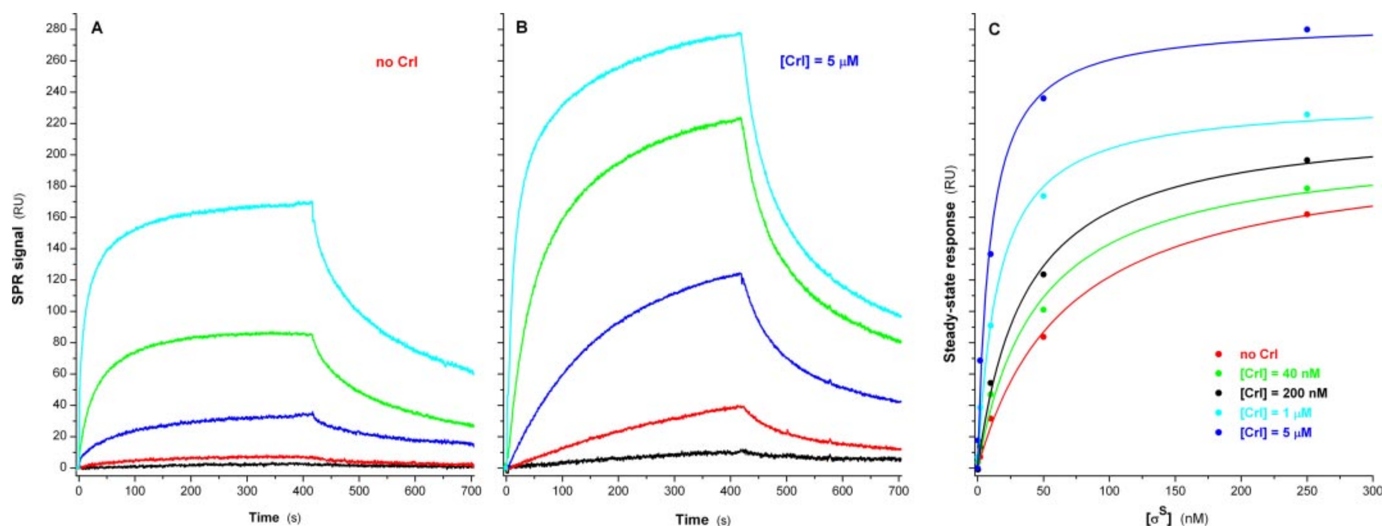

FIGURE 2. **Binding of σ^S to immobilized E.** Association and dissociation real time profiles are shown for the following σ^S concentrations: 0.4 nM (*black*), 2 nM (*red*), 10 nM (*blue*), 50 nM (*green*), and 250 nM (cyan). *A*, in the absence of Crl. *B*, in the presence of 5 μM Crl. *C*, plot representing the steady-state responses as a function of σ^S concentration in the presence of five different Crl concentrations.

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2-fold lower than that for E (K_d = 27.5 ± 1.8 μM). In contrast, the interaction between Crl and preformed $E\sigma^S$ exhibited several interesting characteristics (Fig. 4*C*). First, Crl bound to $E\sigma^S$ with a stoichiometry of 2:1, with two clearly distinct affinities (K_d1 = 227 ± 27 nM, and K_d2 = 40 ± 9 μM), which can be interpreted as two independent binding events per $E\sigma^S$ holoenzyme. K_{d1} is 10-fold lower than the K_d of the interaction between Crl and free σ^S, and could correspond to the binding of Crl to σ^S incorporated into $E\sigma^S$. The close match of K_{d2} with the K_d values determined for the Crl-*E* and Crl-$E\sigma^{70}$ interactions suggests that it could correspond to the direct binding of Crl to *E* within $E\sigma^S$. The sensorgrams (Fig. 4*C*) were best fitted assuming that an isomerization of the ternary Crl-$E\sigma^S$ complex occurred after the initial encounter between Crl and $E\sigma^S$. Accordingly, the dissociation curves were biphasic, with two distinct k_{off} rates, $4.1 \pm 1.0 \times 10^{-2}$ s^{-1} and $4.2 \pm 1.3 \times 10^{-3}$ s^{-1}. The half-life of the ternary Crl-$E\sigma^S$ was ~50 s, more than 15-fold higher than the half-life of the Crl-σ^S binary complex. Altogether, these data suggest that Crl not only favors the incorporation of σ^S within $E\sigma^S$ but that it could bind to and persist within the $E\sigma^S$ holoenzyme long enough to play an active role during the transcription initiation process.

Crl Increases the DNA Binding Ability of $E\sigma^S$—We therefore investigated by electrophoretic mobility

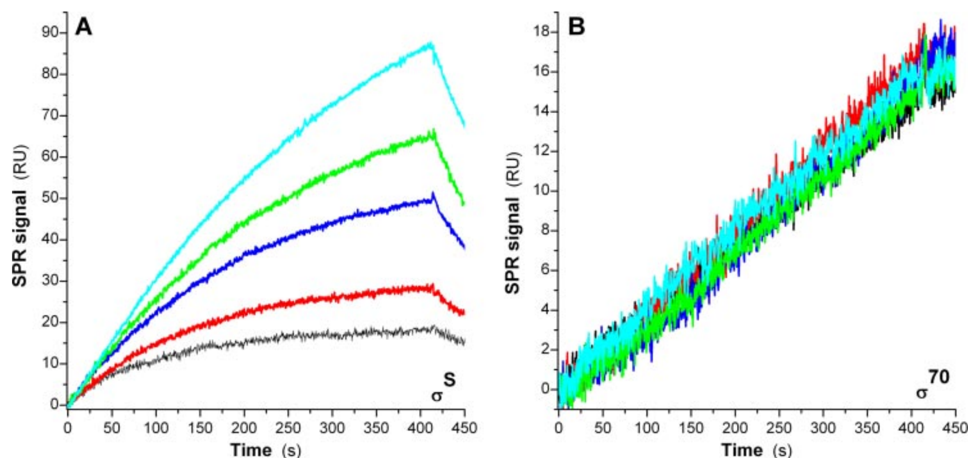

FIGURE 3. **Effect of Crl on the binding of either σ^S or σ^{70} to *E*.** The sensorgrams shown in *black*, *red*, *blue*, *green*, and *cyan* correspond to Crl concentrations of 0, 0.2, 1, 5, and 25 μM. *A*, σ^S, 5 nM; *B*, σ^{70}, 0.25 nM.

FIGURE 4. **Binding of Crl to *E*, $E\sigma^S$, and $E\sigma^{70}$.** Injection of Crl (1.5–25 μM) over an *E* (*A*) or an $E\sigma^{70}$ (*B*) surface. *C*, interaction of Crl (0.1–25 μM) with immobilized $E\sigma^S$. *D*, steady state responses measured on the *E*, $E\sigma^{70}$, and $E\sigma^S$ surfaces as a function of Crl concentration.

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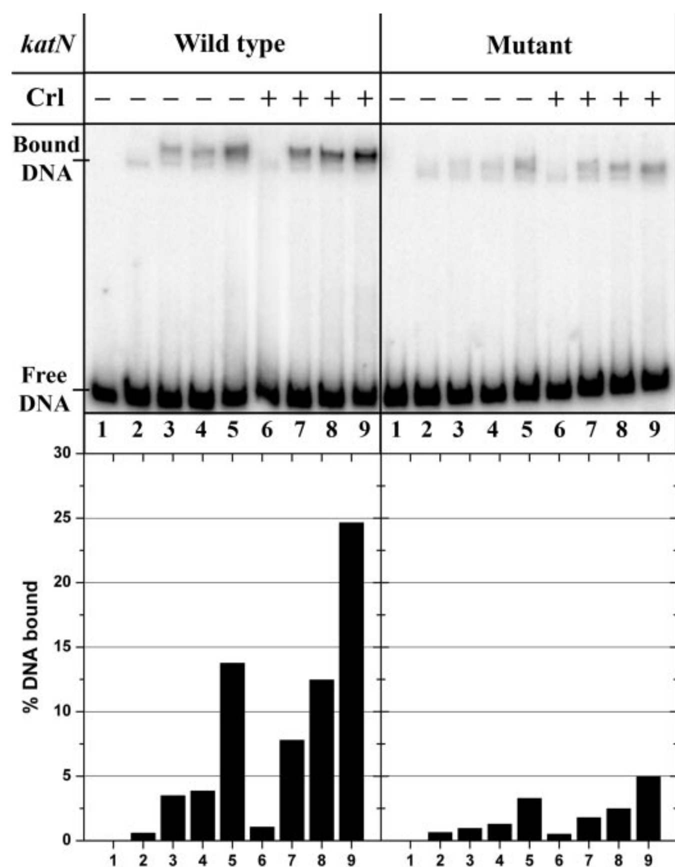

FIGURE 5. **Band shift analysis of *E* and *E*σ^S binding to wild type (*left*) or mutant (*right*) *katN*-labeled fragments in the presence (*lanes 6–9*) or absence of Crl (*lanes 2–5*) at 8 nM *E* and [σ^S] = 0 (*lanes 2* and *6*), 8 nM (*lanes 3* and *7*), 40 nM (*lanes 4* and *8*), and 1 μM (*lanes 5* and *9*); *lane 1*, no protein.** A typical autoradiogram is shown. The bands corresponding to free and bound DNAs are indicated, and the percentage of bound DNA is quantified in the histogram *below* each *lane*.

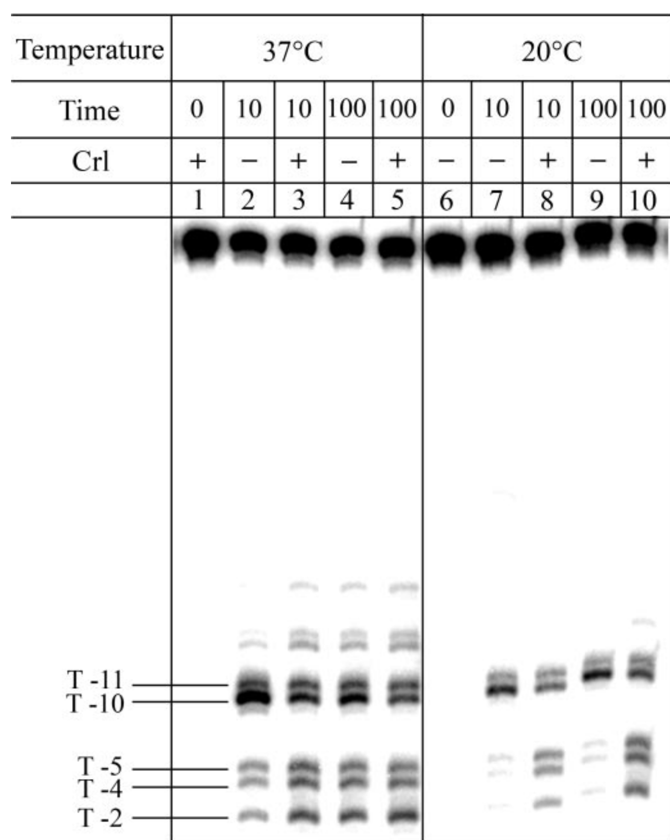

FIGURE 6. **Potassium permanganate reactivity of the *E*σ^S-*katN* promoter complexes formed at 37 °C (*lanes 2–5*) and 20 °C (*lanes 7–10*) with or without Crl (1 μM).** *Lanes 1* and *6*, controls without Eσ^S. *Lanes 2*, *4*, *6*, *7*, and *9*, without Crl. *Lanes 1*, *3*, *5*, *8*, and *10*, with Crl. *T*, thymine.

shift assays whether Crl had an effect on E and *E*σ^S binding to a *katN* promoter fragment, which is transcribed by *E*σ^S exclusively *in vivo* and preferentially *in vitro* (27, 58). To limit the nonspecific binding of *E* to the DNA fragment, heparin was added to the *E*σ and *E* solutions, at a very low concentration (0.25 μg/ml), which does not affect the σ-E interactions. In all cases, we used a Crl concentration of 1 μM, at which direct binding of Crl to *E* is virtually negligible. Interestingly, although we could not detect any effect of Crl on the binding of *E*, a 2–3-fold increase was observed for *E*σ^S binding, even at concentrations of σ^S (1 μM), at which *E* should be fully saturated (Fig. 5). This strongly suggests that Crl may selectively promote DNA binding by *E*σ^S. To probe the specificity of this effect, we constructed a variant of the *katN* fragment where both the −10 and the −35 regions were severely mutated; the −35 element was changed from TTGACT to CCAGCT, and the −10 region was changed from CTAATTTTA to GAGCTCGGC. The mutant fragment bound *E*σ^S 4–5-fold less than the wild-type fragment, and the positive effect of Crl on this residual binding was lower than that on the specific binding to wild-type *katN* (Fig. 5).

Crl Stimulates Isomerization of the Eσ^S*-katN Promoter Complex en Route to Open Complex Formation*—To investigate *E*σ^S open complex formation at the *katN* promoter, potassium permanganate reactivity experiments (59) were performed at both 20 and 37 °C (Fig. 6, *lanes 2–5*). At both temperatures, thymines at positions −10 and −11 on the template strand were reactive, indicative of the nucleation of the transcription bubble. At 20 °C, in the presence of Crl (*lanes 8* and *10*), but not in its absence (*lanes 7* and *9*), the transcription bubble extended downstream to thymines at positions −5, −4, and −2. This experiment was repeated after incubating the *katN* promoter and *E*σ^S for long periods of time; even after 30 h of incubation at 20 °C, no reactivity of T −5, −4, or −2 could be detected in the absence of Crl. In contrast, after a prolonged incubation time at 37 °C, the patterns of permanganate reactivity were identical, irrespective of the presence or absence of Crl (Fig. 6, *lanes 4* and *5*). After only 10 min of incubation, a difference between the complexes formed with and without Crl could be noticed, especially in the intensity of the reactive bands (Fig. 6, *lanes 2* and *3*), suggesting that Crl at 37 °C enhanced the rate of open complex formation but did not affect the extent of the transcription bubble. Altogether, these data demonstrate that at 20 °C, the *katN-E*σ^S complex is trapped in a nonfunctional intermediate and that Crl is required to overcome the energetic barrier necessary to facilitate the transcription bubble formation around the transcription start site. Other steps subsequent to the opening of the transcription bubble to position −2 might be required to obtain the fully competent transcription complex. In support of this notion, we observed that the *katN*-

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$E\sigma^S$ complex formed at 20 °C in the presence of Crl led to only a few run-off transcripts (less than 5% of the amount synthesized at 37 °C).

DISCUSSION

This study aimed to elucidate the mechanism by which Crl promotes E-σ association and involved the two principal σ factors of Enterobacteria: σ^{70}, the σ factor required for vegetative growth, and σ^S, the σ factor required for stationary phase survival. Both σ factors exhibit a high degree of sequence similarity and bind to almost identical −35 and −10 promoter elements *in vitro* (60). Both σ^{70} and σ^S consist of four domains, which all have a role in mediating the binding of σ to both E and promoter DNA. However, σ^{70} is larger than σ^S and contains an extended $\sigma_{1.1}$ and a 265-residue insertion between $\sigma_{1.1}$ and region 1.2. These structural differences might explain why σ^{70} has a higher affinity for E than σ^S (12, 13, 61).

We used SPR to monitor the binding of Crl to either His_{12}-σ^{70} or His_{12}-σ^S tethered on a sensor chip through their His_{12} moiety. A 1:1 Crl-σ^S complex formed readily and the equilibrium was characterized by a K_d of ~2 μM. On the other hand, no Crl-σ^{70} complex could be observed, setting a minimal threshold of 750 μM for the K_d of a putative Crl-σ^{70} interaction, a value almost 50-fold higher than the intracellular concentration of σ^{70} (62). These data clearly show that Crl binds to σ^S but not to σ^{70}. The inability of Crl to bind to σ^{70} was unexpected, since previous biochemical studies demonstrated that Crl could activate $E\sigma^{70}$-dependent transcription (30). However, our observation was confirmed by two further experiments. First, σ^S, but not σ^{70}, bound to His_6-Crl tethered to a nickel-nitrilotriacetic acid sensor chip (data not shown); second, Crl interacted with σ^S, but not with σ^{70}, in a bacterial two-hybrid assay. Furthermore, our results are also fully consistent with the transcriptomic analysis of the *crl* regulon, where the only genes positively regulated by Crl are all dependent on σ^S for expression (29). Although we have not excluded that Crl might bind to σ factors other than σ^{70}, the specificity of Crl for σ^S is reminiscent of that exhibited by the RssB adaptor protein, which specifically binds and targets σ^S for proteolysis via the ClpXP proteolytic machine (63).

E-σ association involves major rearrangements in both E and σ (42), the most dramatic of which is the unmasking of the σ promoter DNA-binding determinants, which are occluded by extensive interdomain contacts within σ (64). Previous results on the formation of $E\sigma^{70}$ showed that the association mechanism involves a rapid bimolecular encounter step followed by isomerization(s) of the initial complex (57). Our data support a similar mechanism for $E\sigma^S$ formation in the presence and absence of Crl. In our study, we showed that Crl induced a 7-fold increase in the affinity of σ^S for E but had no effect on the affinity of σ^{70} for E. The effect of Crl originated solely from an increase in the rate of association (k_{on}) of σ^S, which, at saturating Crl concentrations (5 μM), appeared to bind to E almost exclusively as a σ^S-Crl complex. No significant effect of Crl could be detected on the rate of dissociation (k_{off}) of the $E\sigma^S$ complex. Crl therefore appears to function as a σ^S-specific chaperone, which most likely favors an "open" conformation of σ^S with a high E-binding propensity.

At high concentrations ([Crl] > 5 μM), we however observed that Crl could also bind directly and transiently to E or $E\sigma^{70}$ with a 1:1 stoichiometry. At similar concentrations, we consistently showed that the stoichiometry of binding of Crl to the preformed $E\sigma^S$ was 2:1 and that two distinct binding phases could be distinguished, which could correspond to the binding of Crl to two independent sites. The low affinity Crl-binding site (K_{d2} > 10 μM) is present on E, $E\sigma^{70}$, and $E\sigma^S$ and is therefore situated on E itself, whereas the high affinity one ($K_{d1} \approx 0.2$ μM) is specific to the σ^S molecule incorporated within $E\sigma^S$. Interestingly, K_{d1} is 10-fold higher than the affinity of Crl for free σ^S. Two explanations can be provided; either the conformation of σ^S in $E\sigma^S$ is more favorable for Crl binding than that of free σ^S, or the first Crl binding site on $E\sigma^S$ involves contacts with both σ^S and E. If Crl bound simultaneously to both σ^S and E, one would expect a stabilization of the E-σ^S-Crl ternary complex, contrary to our observations (Fig. 2). Therefore, our results rather indicate that the isomerization process that σ^S undergoes after its encounter with E leads to a conformation that is able to form a stable complex with Crl (half-life $t_{1/2} \approx$ 50 s), unlike that with free σ^S ($t_{1/2} \approx 3$ s). This long persistence within the $E\sigma^S$ holoenzyme opens up the possibility that Crl, beyond its chaperone-like role on the process of incorporation of σ^S, could also directly influence transcription initiation.

We indeed found that Crl increased 2–3-fold $E\sigma^S$ association with the *katN* promoter fragment at saturating (1 μM) concentrations of σ^S (Fig. 5). However, a lower Crl-induced increase could also be observed for a *katN* mutant fragment, suggesting that the stimulatory effect of Crl was not entirely promoter-specific at least under our electrophoretic mobility shift assay conditions. The $E\sigma^S$ residual binding to the mutant fragment might be attributed to nonspecific binding (65) or binding to fragment ends (66), as suggested by the faster mobility of the mutant complexes (compare *lanes 3–5* and *6–9* in Fig. 5 for wild type and mutant DNAs). Furthermore, all the early stages preceding closed complex formation at the promoter involve nonspecific binding events mediated by electrostatic interactions. Although Crl is not a DNA-binding protein, it could assist $E\sigma^S$ DNA binding by masking the negatively charged regions of the holoenzyme.

We also showed that, beyond its partially nonspecific impact in the early steps of promoter search, Crl also exhibits a direct effect on *katN* promoter melting. Permanganate footprinting experiments revealed that in the absence of Crl, the $E\sigma^S$-*katN* complexes at 20 °C are locked in an inactive state with only thymines at positions −11 and −12 single-stranded. In the presence of Crl, both the size of the transcription bubble increased (up to thymine at position −2) and productive transcription occurred, suggesting that Crl is also able to directly facilitate transcription bubble formation. This isomerization step, strictly independent of RNA polymerase concentrations, argues for a direct role of Crl in the process of transcription initiation.

Our results show that Crl exerts two widely different positive roles on σ^S-dependent transcription. Initially, at the onset of stationary phase, the fast binding of Crl to σ^S may alter the dynamic equilibrium between σ^S conformations, thereby facilitating the association of σ^S with E. This response is the most

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likely to be physiologically relevant when σ^S levels are low. It ensures significant $E\sigma^S$ levels under conditions when σ^S has to compete with the major σ factor, σ^{70}, for binding to E. As stationary phase proceeds and $E\sigma^S$ levels become more abundant, the ternary $E\sigma^S$-Crl complex could become predominant, and Crl may play a direct role in the pathway from promoter recognition to transcription initiation at some promoters. Different reports have already suggested the existence and functionality of a ternary $E\sigma^S$-Crl complex; Crl is indeed specifically co-isolated with E or $E\sigma^S$ in extracts from cells grown in Dulbecco's modified Eagle's medium (67), and cell extracts containing Crl increase the recruitment of $E\sigma^S$ at the *csgBA* promoter (24).

In vivo, Crl levels exceed σ^S levels throughout the growth phase (29), and in stationary phase, the intracellular concentration of Crl is at least 2–3-fold higher than that of σ^S (68), implying that it could reach up to 10–15 μM. Thus, although Crl predominantly associates to σ^S and $E\sigma^S$, significant amounts of excess Crl remain available for binding to other molecules. In this study, we could not detect any binding of Crl to σ^{70}, but we were able to show a transient binding of Crl to E and to $E\sigma^{70}$ at Crl concentrations higher than 1 μM. The nature of the Crl binding site on E and the physiological significance of these results are still unclear. Indeed, we were unable to detect any positive effect of Crl on *in vitro* σ^{70}-dependent transcription at the *katN* and *lac*UV5 promoters, irrespective of temperature, buffer conditions, and the order of the addition of proteins. Besides the stimulatory role of Crl on a fraction of σ^S-dependent genes (27–29), Crl is known to inhibit the expression of some members of the σ^{70}- and σ^{54}-regulons in the presence of σ^S (25, 27, 28, 69). This phenomenon most likely results from the Crl-dependent increase in $E\sigma^S$ formation. Interestingly, Crl also appears able to regulate a small number of genes in the absence of σ^S (70, 71). These regulatory effects might result from the interactions of Crl with E (this study) and/or other proteins (70, 72). Whatever the significance of these side effects, the major regulatory functions of Crl are to facilitate E-σ^S association and to promote the expression of σ^S-dependent genes.

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