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Crl Facilitates RNA Polymerase Holoenzyme Formation▽

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The *Escherichia coli* Crl protein has been described as a transcriptional coactivator for the stationary-phase sigma factor σ^S. In a transcription system with highly purified components, we demonstrate that Crl affects transcription not only by the $E\sigma^S$ RNA polymerase holoenzyme but also by $E\sigma^{70}$ and $E\sigma^{32}$. Crl increased transcription dramatically but only when the σ concentration was low and when Crl was added to σ prior to assembly with the core enzyme. Our results suggest that Crl facilitates holoenzyme formation, the first positive regulator identified with this mechanism of action.

Escherichia coli σ^S (also called σ^{38} or RpoS) has been referred to as the stationary-phase or general stress response σ factor (16). $E\sigma^S$, the RNA polymerase (RNAP) holoenzyme containing this σ factor, recognizes promoters for genes related to stress resistance. σ^S function is exquisitely regulated, with components that act at the transcription, translation, protein stability, and activity levels (16, 21, 23, 27, 28).

The concentration of σ^S is extremely low in log phase and is lower than σ^{70}, the cell's primary σ factor, even during stationary phase, when σ^S is most abundant (17). Inhibition of $E\sigma^{70}$-dependent transcription results in an increase in $E\sigma^S$-dependent transcription (9, 12, 23), most likely because σ^{70} and σ^S compete for free core RNAP.

Cells contain a variety of factors that favor utilization of $E\sigma^S$ promoters in stationary phase. These include Rsd, an anti-σ factor specific for σ^{70} (18, 19); the 6S RNA, a factor that sequesters $E\sigma^{70}$ by mimicking a promoter at the open complex stage of transcription (30); and ppGpp, a factor that inhibits $E\sigma^{70}$ utilization at the strongest cellular promoters (those for rRNAs and most tRNAs), reducing transcription of those operons and thereby freeing up core RNAP to bind to σ^S and other σ factors (4, 7, 11, 20).

Another protein, Crl, has also been implicated in $E\sigma^S$-dependent transcription. The *crl* gene was named as such because, initially, it was thought to encode curli surface structures. However, Crl is actually a cytoplasmic protein required for transcription of the true curli subunits from the *csgBA* promoter as well as from some other $E\sigma^S$-dependent promoters (1, 13, 24, 28). Strains harboring a null mutation in *crl* exhibit reduced activity at $E\sigma^S$-dependent promoters, even though σ^S protein levels do not decrease in *crl* mutants. Overexpression of Crl increases transcription from $E\sigma^S$-dependent promoters (28). Unlike Rsd, 6S RNA, and ppGpp, recent studies have suggested that Crl stimulates $E\sigma^S$-dependent promoter activity directly rather than by inhibiting $E\sigma^{70}$-dependent transcription (5, 29). Furthermore, unlike classical transcription activators, Crl does not bind to DNA but rather binds directly to σ^S in vivo and in vitro (5, 28).

As part of our continuing efforts to understand the mechanisms of control of transcription initiation by factors that do not bind DNA (2, 3, 25, 26) and control of $E\sigma^S$-dependent promoters (10), we investigated the activation of promoters by Crl in vitro. Our studies indicate that Crl activates not only $E\sigma^S$-dependent promoters in vitro but also $E\sigma^{70}$- and $E\sigma^{32}$-dependent promoters as well, that activation depends on the addition of Crl to σ prior to holoenzyme formation, and that the effects of Crl are most pronounced when σ concentrations are low. Our results therefore strongly support the model that Crl acts by facilitating the assembly of σ with core RNAP.

Crl promotes RNAP holoenzyme formation. To assess the effect of Crl in vitro, multiple-round transcription with $E\sigma^S$ was performed on a supercoiled plasmid template containing the previously characterized synthetic $E\sigma^S$-dependent promoter cc-35con driving an ~80-nucleotide transcript (10). The template also contained the RNA-I promoter, which makes an ~110-nucleotide transcript involved in copy number control of the plasmid, as well as the rRNA promoters *rrnB* P1 and *rrnB* P2. A schematic diagram of the template is shown in Fig. 1A.

σ^S and σ^{70} were overproduced using T7 polymerase, RNAP core enzyme was isolated by standard procedures, and core RNAP and σ^S or σ^{70} were incubated together to form holoenzyme (6, 10). Crl was purified with an N-terminal glutathione *S*-transferase tag by expression from a derivative of pGEX-4T-3 (Amersham Biosciences). Glutathione-Sepharose-bound glutathione *S*-transferase–Crl was cleaved with thrombin, and the thrombin was removed with a benzamidine column. Crl produced in this manner contains three additional N-terminal residues (Gly-Ser-Met) followed by a Thr residue, the N-terminal residue in the wild-type 132-amino-acid mature protein (24). It was demonstrated previously that Crl containing an N-terminal hexahistidine tag is competent for binding to σ^S (5), suggesting that modifications at the N terminus do not affect Crl function. The purified protein had the expected molecular mass (15.8 kDa) and was the only band observed on an overloaded Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel under reducing

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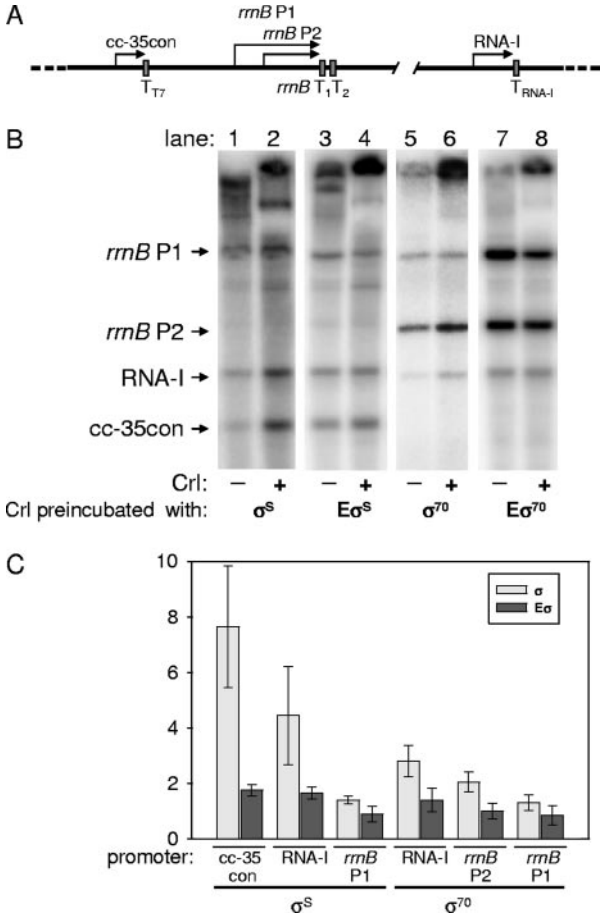

FIG. 1. Crl activates transcription when preincubated with σ^S or σ^{70} before the addition of core RNAP. (A) Schematic representation of the promoters and transcription terminators on plasmid pBP5. The cc-35con transcript is terminated by the T7 Te terminator, the *rrnB* P1 and *rrnB* P2 transcripts are terminated by the *rrnB* T_1T_2 terminators, and the RNA-I transcript is terminated by the RNA-I terminator. (B) Transcription in the presence or absence of Crl. Lanes 1, 2, 5, and 6, 16 μM Crl, or buffer as a control, was incubated with 32 nM σ^S or σ^{70} in transcription buffer (40 mM Tris-Cl, pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml BSA) for 10 min. This mixture was added to an equal volume of 333 nM core RNAP for 10 min, and then 2 μl of this reaction mixture was added to 8 μl transcription buffer containing 200 μM ATP, GTP, CTP, and 10 μM UTP with 1 μCi [α-^{32}P]CTP and the plasmid template. All steps were performed at 22°C. The final concentrations of σ, core RNAP, and Crl in the transcription reaction mixture were therefore 3.2 nM, 33.4 nM, and 1.6 μM, respectively. The reactions were terminated after 15 min and examined on 5.5% polyacrylamide denaturing gels (10). Lanes 3, 4, 7, and 8, holoenzyme was formed by mixing core RNAP with σ^{70} or σ^S for 20 min, and then the reaction mixture was diluted into transcription buffer with or without 8 μM Crl for 10 min. Two microliters of this reaction mixture was then used for each 10-μl transcription reaction mixture (as indicated above). The final concentrations of σ, core RNAP, and Crl per reaction were therefore the same as in the above-described reactions. Care was always taken to keep reaction conditions and times of incubation exactly the same when samples were compared in the order-of-addition experiments. High σ and/or Crl concentrations resulted in radioactivity that remained in the sample wells. This may derive from protein aggregation, since it was partially eliminated by heat treatment (5 to 10 min at 95°C). In addition, the responses of additional RNA species migrating more slowly than the test transcripts also suggest that there are effects of Crl on the plasmid-derived RNA-II promoter, on the *bla* promoter, and/or on termination readthrough products (Fig. 2E and 3A; data not shown). (C) Transcripts were quantified by phosphorimaging and are presented as ratios with or without Crl. Data from two experiments are averaged, and the ranges are shown.

conditions (data not shown). Mass spectrometric analysis further confirmed the identity of this band as Crl.

Figure 1B illustrates the effects of Crl on transcription in vitro in a typical experiment, and Fig. 1C quantifies the effects on each promoter as a ratio (±Crl). Transcription from the cc-35con promoter increased dramatically when σ^S was preincubated with Crl before the addition of core RNAP (~8-fold) (Fig. 1B, lanes 1 and 2), but when Crl was added to preformed $E\sigma^S$ (lanes 3 and 4), transcription increased <2-fold, consistent with the ≤2-fold effects on transcription reported previously (29).

Likewise, Crl stimulated $E\sigma^S$-dependent transcription from the RNA-I promoter (~4-fold) when incubated with σ^S prior to the addition of core RNAP (Fig. 1B, lanes 1 and 2), and Crl had only a small effect (<2-fold) on the RNA-I promoter when added to the $E\sigma^S$ holoenzyme (lanes 3 and 4). The effects of Crl on $E\sigma^S$-dependent transcription of *rrnB* P1 were smaller but reproducible. Transcription from *rrnB* P2 by $E\sigma^S$ under these conditions was too weak for us to evaluate whether Crl affected its activity.

The effects of Crl on transcription by $E\sigma^S$ reported here are much greater than those reported previously (29). We suggest that this discrepancy is attributable to a positive role of Crl on RNAP assembly, which is minimized when Crl is added to the assembled holoenzyme.

We also tested whether Crl affected transcription by $E\sigma^{70}$. As shown previously (10), the cc-35con promoter was not transcribed by $E\sigma^{70}$ (Fig. 1B, lanes 5 to 8). However, $E\sigma^{70}$-dependent transcription from each of the other three promoters was stimulated by Crl. Although the overall effects of Crl on transcription by $E\sigma^{70}$ were smaller than those for $E\sigma^S$, stimulation by Crl again depended on incubation with σ prior to core RNAP addition.

These results suggested that Crl might affect the equilibrium between core RNAP and the holoenzyme, increasing RNAP activity when core RNAP is not saturated with σ. This model predicts that the effects of Crl would be greatest at the lowest σ concentrations. We tested this prediction by adding a constant amount of Crl to decreasing amounts of σ^S and then incubating the σ^S-Crl complex with a constant amount of core RNAP before addition to the template (Fig. 2A). (The concentrations of the reactants were 5.5 to 350 nM σ and 333 nM core RNAP at the time of addition of σ to core RNAP, before dilution with the remainder of the transcription reaction mix [see Fig. 2 legend for further details].) Not surprisingly, transcription in the absence of Crl increased with increasing σ^S. However, the effect (*n*-fold) of Crl decreased substantially as the σ^S concentration increased, as predicted by the model that Crl facilitates the assembly of σ^S with core RNAP. Transcription from cc-35con, RNA-I, and *rrnB* P1 increased ~8-fold, 5-fold, and ~3-fold, respectively, at the lowest σ^S concentration (Fig. 2B), consistent with the model that Crl affects the core-holoenzyme equilibrium. In support of this interpretation, the magnitude of transcription stimulation by Crl was also inversely proportional to the core RNAP concentration (data not shown).

The effect of σ concentration on stimulation by Crl was also assessed using σ^{70} (Fig. 2C). At the lowest σ^{70} concentration, Crl increased transcription from RNA-I and *rrnB* P2 almost fivefold and threefold, respectively, and had a smaller effect on

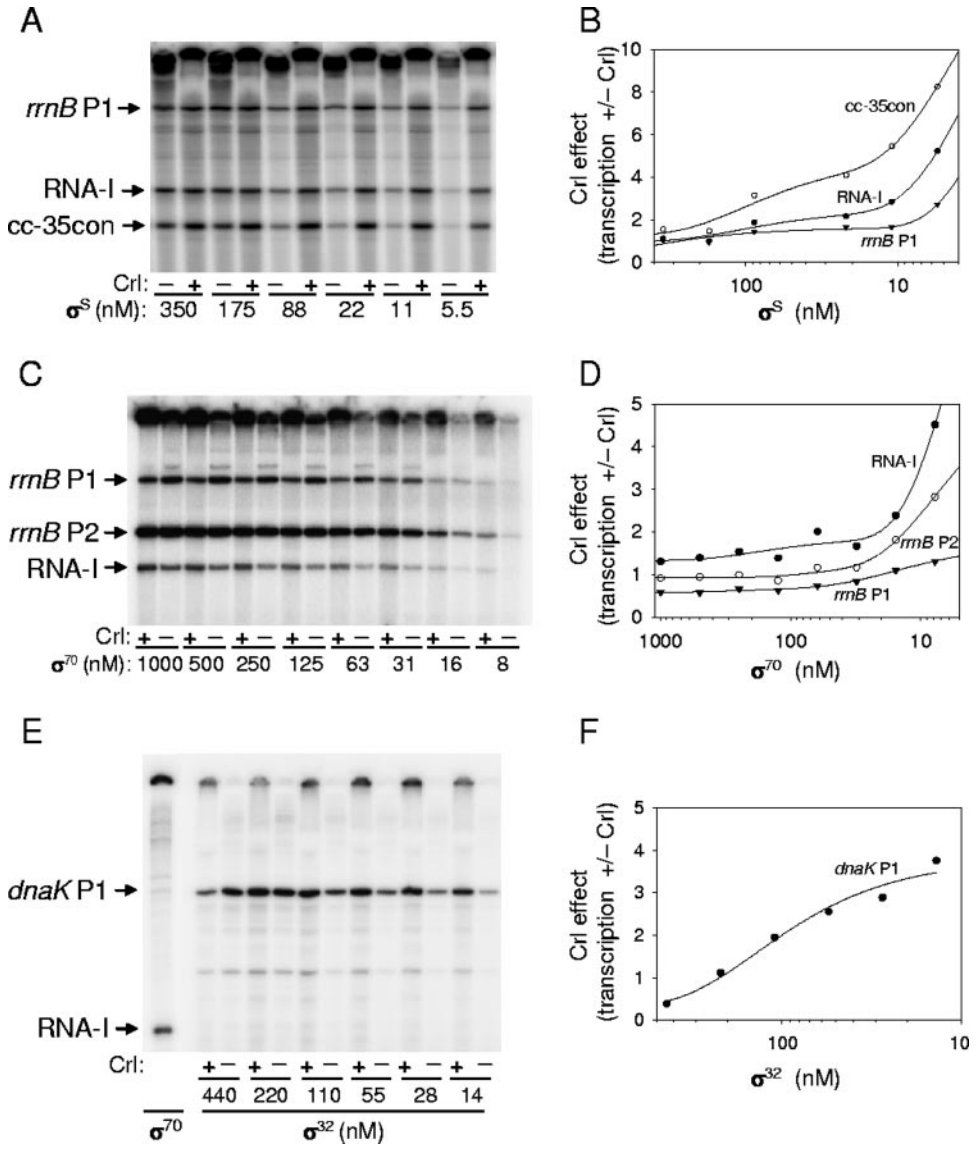

FIG. 2. Crl activates transcription only at low σ concentrations. Representative gels are shown for illustrative purposes, but each experiment was performed at least three times with very similar results. (A) σ^S-dependent transcription. Transcription was performed by incubation of Crl (8 μM) with the indicated concentrations of σ^S before addition to core RNAP (333 nM) for 10 min. Two microliters of this mixture was used in each 10-μl transcription reaction mixture, as for Fig. 1. (B) Quantitation of the data from panel A. (C) σ^{70}-dependent transcription. Transcription was performed by incubation of Crl (8 μM) with the indicated concentrations of σ^{70} before addition to core RNAP (88 nM) for 10 min. Two microliters of this mixture was used in each 10-μl transcription reaction mixture, as for Fig. 1. (D) Quantitation of the data from panel C. (E) σ^{32}-dependent transcription. Transcription was performed by incubation of Crl (8 μM) with the indicated concentrations of σ^{32} before addition to core RNAP (333 nM) for 10 min. Two microliters of this mixture was used in each 10-μl transcription reaction mixture. The template was plasmid pJET40, a pUC19 derivative encoding the *dnaK* P1 promoter as well as the RNA-I promoter (22). A control reaction was performed with $E\sigma^{70}$ (left lane). This holoenzyme produced little or no transcript from the *dnaK* P1 promoter, and the $E\sigma^{32}$ preparation did not make a transcript corresponding to RNA-I, confirming that Crl affected $E\sigma^{32}$-dependent transcription. (F) Quantitation of the data from panel E.

rrnB P1 (Fig. 2D). These results indicate that the mechanism of Crl action does not depend on determinants in σ that are limited to σ^S. However, the physiological significance of the effects of Crl on the assembly of $E\sigma^{70}$ is unclear, since σ^{70} concentrations are normally quite high (12).

As a control, we also tested whether an unrelated protein, bovine serum albumin (BSA), would also increase transcription. BSA (NE Biolabs) was added to σ^S or σ^{70} before the addition of core RNAP at the same concentrations (on a molar or weight basis) as Crl and under the same solution conditions where, in parallel, Crl increased transcription as much as ~10-fold. BSA had absolutely no effect on transcription (data not shown).

A general role for Crl in the assembly of RNAP holoenzymes? We next tested the effects of Crl on another σ factor, the heat shock σ, σ^{32}. Crl increased $E\sigma^{32}$-dependent transcription as much as ~4-fold from the *dnaK* P1 promoter on plasmid pJET40 (Fig. 2E) (22), and as observed for the other two

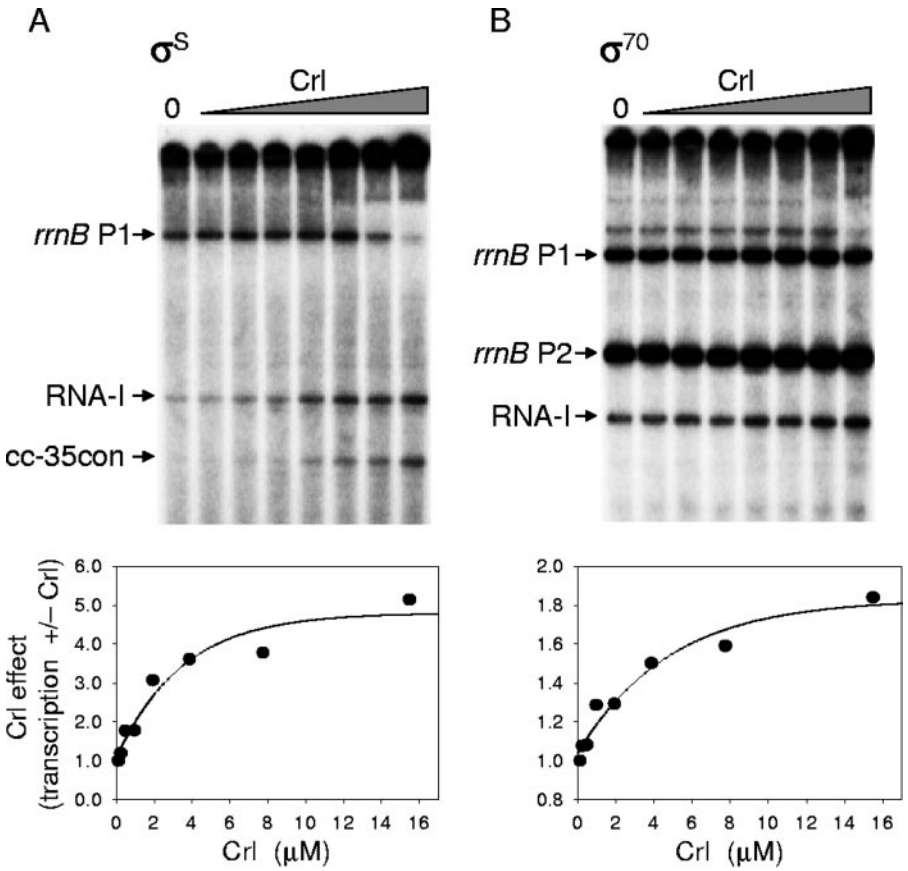

FIG. 3. Concentration dependence of activation of transcription by Crl. σ (8 nM) and Crl (0 to 16 μM) were incubated for 10 min and added to an equal volume of core RNAP (167 nM). The plasmid template and other components of the transcription reaction mixture were added, and transcripts were measured as described above. A representative gel is shown for each form of the holoenzyme, and the ratio of transcription from the RNA-I promoter in these reactions with or without Crl at different Crl concentrations is quantified below for each gel. (A) σ^S. (B) σ^{70}. At very high concentrations of Crl, we sometimes observed inhibition of the *rrnB* P1 promoter. However, we did not observe effects of inactivation of the *crl* gene on rRNA promoter activity in vivo (data not shown), suggesting that this phenomenon is unlikely to be physiologically relevant.

holoenzymes, stimulation was inversely proportional to the concentration of the σ factor (Fig. 2F). The σ^{32} concentration under non-heat shock conditions in vivo is very low (12), like the concentration of σ^S in log phase (17). The free concentrations of several of the other alternative σ factors from *E. coli* are also very low in vivo under noninducing conditions. We speculate that Crl might have a general role in increasing the rates at which alternative holoenzymes are assembled following induction and, as a result, the rates at which the promoters that use these enzymes are turned on.

We also measured the concentration of Crl required for stimulation of holoenzyme activity. The increase in the activity of the RNA-I promoter (which is transcribed by both $E\sigma^S$ and $E\sigma^{70}$) was half-maximal when the concentration of Crl incubated with σ^S and σ^{70} before the addition of core RNAP was 2 to 3 μM (Fig. 3). Although the Crl preparation was free of contaminants, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown), we do not know the specific activity of the Crl preparation. Therefore, further studies will be needed to rule out the possibility that the effects on RNAP assembly attributed here to Crl do not result from some very minor but highly active contaminant in the Crl preparation.

Mechanism of enhancement of transcription. Regulatory proteins, the "anti-sigma factors" (15), that bind to their cognate sigmas and inhibit transcription by preventing holoenzyme formation have been identified previously. In contrast, Crl functions as an activator. Unlike typical activators that bind to DNA and interact with σ in the context of holoenzyme to increase transcription (e.g., λ cI) (8), we conclude that Crl increases transcription by binding to σ and stimulating holoenzyme formation.

Although we have not demonstrated that Crl binds directly to σ^{70} or σ^{32}, binding of Crl to σ^S has been reported previously (5). As speculated above, Crl could facilitate holoenzyme formation with many or all of the *E. coli* σ factors. Additional studies will be needed to determine whether Crl interacts with the other four σ factors, whether the interaction interface is conserved among σ factors, how Crl promotes binding of σ to the core enzyme, and whether Crl homologs help promote holoenzyme assembly in other bacterial species.

Since the σ concentration dependence of the Crl effect indicates that it affects the core-holoenzyme equilibrium, we speculate that Crl serves as a chaperone, aiding in the folding/unfolding of a section(s) of the long, multidomain σ. However, a preliminary BLAST search did not reveal similarities to

known chaperones. An alternative model is that Crl contacts free σ, but rather than serving as a σ chaperone, it stabilizes the interaction between σ and core by contacting both, once holoenzyme forms. We also point out that although the σ (and core) concentration dependence of the effect of Crl on transcription indicates that it affects the σ-core equilibrium, we have not ruled out models involving effects on additional, subsequent steps in the initiation pathway.

Crl affects different $E\sigma^S$-dependent and $E\sigma^{70}$-dependent promoters to different extents (Fig. 1 and 2) (5, 28, 29). We suggest that the distribution of the promoters affected and the magnitude of these effects may simply reflect the kinetic differences intrinsic to different promoters (i.e., the intrinsic binding constants of different promoters for RNAP). In this model, promoters with weaker RNAP binding constants would be affected more by an increase in the RNAP holoenzyme concentration caused by Crl. Tests of this hypothesis will require measurement of association rates at a range of RNAP concentrations in order to derive the kinetic constants for a range of Crl-responsive and -unresponsive promoters (3, 26). Additional (but not mutually exclusive) models are that another factor (not present in our reactions) accounts for the promoter-specific effects of Crl in vivo and that there are other promoter-specific effects beyond those deriving from differences in the promoters' intrinsic kinetic characteristics.

A previous study concluded that Crl increases transcription by recruiting $E\sigma^S$ to the promoter (5). Consistent with our model, the previous study concluded that Crl binds to free σ^S and $E\sigma^S$ and not to core RNAP. Although purified Crl did not increase $E\sigma^{70}$ activity (promoter binding) in vitro, $E\sigma^{70}$-promoter complexes did increase when extracts from cells overproducing Crl were added (compared to what was observed when extracts from wild-type or *crl* mutant cells were added). We suggest that the effects of purified Crl on $E\sigma^{70}$ activity were not detected at least in part because Crl was added after holoenzyme formation.

Crl is not the only transcription factor that binds to RNAP and modulates its activity without interacting with DNA. For example, DksA is a small protein that binds in the secondary channel of RNAP and, in conjunction with changing concentrations of small molecules (ppGpp and the initial nucleoside triphosphate), exerts either negative or positive effects on transcription, depending on the intrinsic kinetic characteristics of the promoter (14, 25, 26). We suggest that other transcription factors such as Crl and DksA that alter the rates of specific steps in the transcription cycle without binding to DNA may await discovery.

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