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plified by polymerase chain reaction (PCR) with primers containing 5′-homology arms corresponding to sequences flanking the regions to be deleted. Primers used to create LSR10 (Δ*csgA*) were 5′-gttaatttccattcgacttttaaatcaatccgatgggggttttacGTGTAGGCTGGAGCTGCTTC and 5′-agggcttgcgccctgtttctgtaatacaaatgatgtATTCCGGGGATCCGTCGACC (lower-case letters correspond to *csg* sequences). The primers used to generate LSR5 (Δ*csgDEFG*;Δ*csgBA*), were 5′-agggcttgcgccctgtttctgtaatacaaatgatgtATTCCGGGGATCCGTCGACC and 5′-gccgacatcaggcacagcataacaggttcgttcgagGTGTAGGCTGGAGCTGCTTC. PCR products were electroporated into MC4100-expressing Red recombinase proteins from pKD46 (*22*). The resulting Kan^r strains were confirmed by PCR and failed to bind CR when grown on YESCA plates. The mutation from LSR5 was transferred into C600 by standard P1 transduction, creating LSR6.

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A Role for Interaction of the RNA Polymerase Flap Domain with the σ Subunit in Promoter Recognition

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In bacteria, promoter recognition depends on the RNA polymerase σ subunit, which combines with the catalytically proficient RNA polymerase core to form the holoenzyme. The major class of bacterial promoters is defined by two conserved elements (the –10 and –35 elements, which are 10 and 35 nucleotides upstream of the initiation point, respectively) that are contacted by σ in the holoenzyme. We show that recognition of promoters of this class depends on the "flexible flap" domain of the RNA polymerase β subunit. The flap interacts with conserved region 4 of σ and triggers a conformational change that moves region 4 into the correct position for interaction with the –35 element. Because the flexible flap is evolutionarily conserved, this domain may facilitate promoter recognition by specificity factors in eukaryotes as well.

At most bacterial promoters, RNA polymerase (RNAP) holoenzyme ($\alpha_2\beta\beta'\omega\sigma$) recognizes sequence elements centered ~10 and ~35 nucleotides upstream of the initiation point, with the σ subunit specifically contacting both promoter elements [reviewed in (*1*)]. Different sigmas share four evolutionarily conserved regions, which can be further subdivided (*1*). Centrally located region 2.4 interacts with the –10 promoter element, and COOH-terminal region 4.2 interacts with the –35 element (*1*). Because most free σ subunits cannot recognize promoters, conformational changes in core RNAP, σ, or both must occur during holoenzyme formation. Indeed, luminescence resonance energy transfer (LRET) measurements show that the *Escherichia coli* RNAP core induces a change in σ^{70}, the principal σ (*2*). As a result, the distance between σ^{70} regions 2.4 and 4.2 increases dramatically, to match the distance between the promoter elements (*2*). The mechanism by which the conformation of σ is altered upon holoenzyme formation has not been defined, nor have the core interaction sites that bring about this change been identified.

A structure of core RNAP from eubacterium *Thermus aquaticus* has been determined (*3*). One structural element, the "flexible flap" (comprising conserved segment G of the RNAP β subunit), protrudes away from the body of the enzyme (Fig. 1). An *E. coli* RNAP mutant lacking β amino acids 900 through 909 at the tip of the flap was previously found to be defective in transcription initiation unless the initiation region was premelted (*4*). To further examine this defect, we deleted the entire flap from *E. coli* RNAP (*5*). Inspection suggests that the RNAP structure should be minimally perturbed by the deletion (Fig. 1).

Mutant RNAP was purified (*6*), and the ability of mutant holoenzyme ($E\sigma^{70}$) to initiate transcription from T7 A2, a strong –10/–35 promoter, was tested (*7*). Wild-type $E\sigma^{70}$ was active at T7 A2; in contrast, mutant $E\sigma^{70}$ was inactive (Fig. 2A). Transcription from the *gal*P1 promoter was also tested. This promoter belongs to a class of promoters whose –10 elements are extended by an upstream dinucleotide TG (*8*). σ region 4.2 is not required for recognition of extended –10 promoters, due to additional RNAP contacts with the TG motif (*8*). $E\sigma^{70}$ lacking the β-flap was active at *gal*P1 (Fig. 2A). These results suggest that the β-flap is important for transcription from –10/–35 promoters, but is dispensable for transcription from extended –10 promoters.

Wild-type $E\sigma^{70}$ protected T7 A2 promoter DNA from deoxyribonuclease I (DNase I) digestion (Fig. 2B) (*7*). In contrast, the pattern of DNase I digestion in reactions containing mutant $E\sigma^{70}$ was similar to the naked DNA pattern, suggesting that $E\sigma^{70}$ lacking the β-flap is unable to form complexes with –10/–35 promoters.

The restricted promoter specificity caused by the β-flap deletion could be direct (i.e., the flap contributes directly to promoter recognition) or indirect (i.e., the flap positions σ region 4.2 for interaction with the –35 element). The following experiments support the second possibility. We studied σ^{70} region 4.2-DNA interactions in *gal*P1 complexes, where region 4.2 makes favorable, but nonessential DNA interactions ~35 base pairs (bp) upstream of the initiation point (*8*). Overall, the *gal*P1 complexes formed by mutant $E\sigma^{70}$ appeared similar to the wild-type complexes (Fig. 2C) (*8*). However, DNA between positions –34 and –39 was protected in the wild-type, but not in the mutant complexes (Fig. 2C, arrowheads), suggesting that in the absence of the β-flap, interactions between σ region 4.2 and *gal*P1 upstream DNA do not occur.

To show directly that the β-flap is required for the conformational change in σ that occurs upon holoenzyme formation, we used LRET, which uses energy transfer between a luminescent donor and fluorescent acceptor to determine atomic-scale distances between the probes (*9*). LRET donor and acceptor probes were incorporated into different σ domains, and inter-

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domain distances were determined in free σ, wild-type $E\sigma^{70}$, and mutant $E\sigma^{70}$ (*9, 10*). The calculated distances between regions 1.1 and 4.2, and regions 2.4 and 4.2, were much greater for wild-type $E\sigma^{70}$ compared to free σ^{70} (Fig. 3) (*2*). In contrast, the distance between regions 2.4 and 4.2 changed little (Fig. 3A) and the distance between regions 1.1 and 4.2 was unchanged (Fig. 3B) in mutant $E\sigma^{70}$. However, the distance between σ regions 1.1 and 2.4 increased in both wild-type and mutant $E\sigma^{70}$, proving that both holoenzymes were formed under the conditions of the experiment. Thus, the β-flap is required for correct positioning of σ region 4.2 in the holoenzyme. In the absence of the flap, regions 2.4 and 4.2 fail to move away from each other, preventing simultaneous recognition of the –10 and –35 promoter elements by $E\sigma^{70}$.

To test directly whether the β-flap and σ region 4 interact, we used a bacterial two-hybrid system (*11*). Transcription from the test promoter depicted in Fig. 4A can be activated by interaction between a protein domain fused to the bacteriophage λ cI protein (λcI) and a partner domain fused to the α subunit of RNAP. Accordingly, we fused the β-flap (residues 858 through 946) to the COOH-terminus of λcI (*12*), and we made use of two previously constructed α-σ chimeras which contain region 4 of either σ^{70} or σ^{38} (the second major σ in *E. coli*) in place of the COOH-terminal domain of α (*13*). We then investigated whether the λcI–β-flap fusion protein could activate transcription from the test promoter in cells containing either the α-σ^{70} or the α-σ^{38} chimera. Plasmids expressing λcI–β-flap and the α-σ chimeras were introduced into *E. coli* strain KS1 (*11*) harboring the test promoter (p*lac*O_R2-62) linked to a *lacZ* reporter gene. The λcI–β-flap fusion protein activated transcription strongly (up to ~17-fold) in cells containing the α-σ^{38} chimera (Fig. 4B), but we detected only a marginal stimulatory effect of the λcI–β-flap fusion protein in the presence of the α-σ^{70} chimera (*12*). However, in support of the idea that region 4 of σ^{70} can interact directly with the β-flap, the λcI–β-flap fusion protein stimulated transcription ~sixfold in the presence of a mutant form of the α-σ^{70} chimera bearing amino acid substitution $Asp^{581} \rightarrow Gly^{581}$ (D581G) in the σ^{70} moiety (Fig. 4B) (*14*). Control assays indicated that λcI by itself did not activate transcription from the test promoter in the presence of either the α-σ^{38} or the α-σ^{70} chimera (*12*).

Our in vivo results suggest that region 4 of σ^{38} and region 4 of σ^{70} can interact directly with the β-flap. It remains to be seen whether the apparent difference in the strengths of the interactions between the β-flap and regions 4 of σ^{70} and σ^{38} is biologically significant; it is possible that the strength of the interaction between the β-flap and different σ factors contributes to the specificity of promoter recognition and/or the strength of promoter binding by holoenzymes containing different sigmas. At least one other σ, a minor σ factor from *Helicobacter pylori* (σ^{28}), has been found to interact with the β-flap region (*15*).

Our principal finding is that the ability of σ region 4.2 to interact with the –35 promoter element is dramatically reduced in the absence of the β flexible flap. Moreover, we find that the conformational change within σ, which occurs upon holoenzyme formation and is required for promoter recognition (*2*), does not occur in the absence of the β-flap. Finally, we demonstrate an interaction between the β-flap and region 4 of σ^{38}, a σ factor that is closely related to σ^{70} (*1*). These results, taken together with other evidence on σ-core interactions and bacterial promoter recognition, allow us to propose the following succession of allosteric changes required for promoter recognition by bacterial RNAP holoenzyme. The primary interaction between σ and RNAP core occurs through strong contacts between sigma region 2.2 and the coiled-coil element of the β′ subunit (*16*). This interaction enables σ region 2.4 to recognize the –10 promoter element (*17*). Protein-protein interaction between the β-flap and σ region 4 activates an additional allosteric switch that

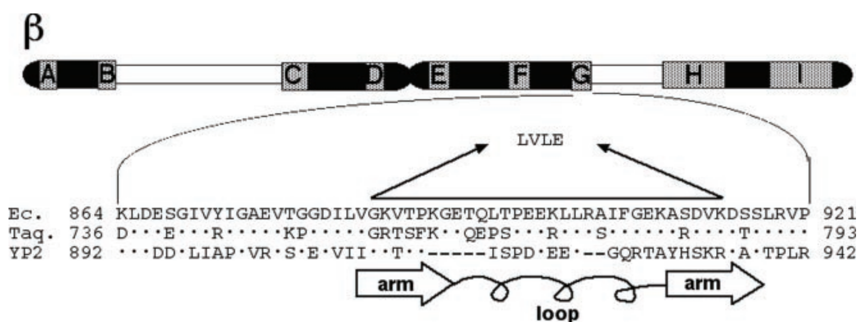

Fig. 1. Structural context of RNAP flap. The black bar at the top represents the *E. coli* RNAP β subunit. The lettered boxes indicate evolutionarily conserved segments; white boxes denote dispensable regions (*21*). The *E. coli* segment G sequence (Ec) is expanded and aligned with the corresponding segments from *T. aquaticus* (Taq) and yeast RNAP II (Yp2). Dots and hyphens show identical and missing amino acids, respectively. The secondary structure of the β-flap from *T. aquaticus* is schematically illustrated. The deletion studied in this work is shown above the *E. coli* sequence. Below, a view of *T. aquaticus* RNAP core structure (*3*) is presented. β′ is in pink, β in cyan, α in green, and ω in white. The view is roughly perpendicular to the axis of the DNA-binding channel of the enzyme. The active-center Mg^{2+} is in blue. The portion of the β-flap corresponding to the deletion studied here is shown in yellow.

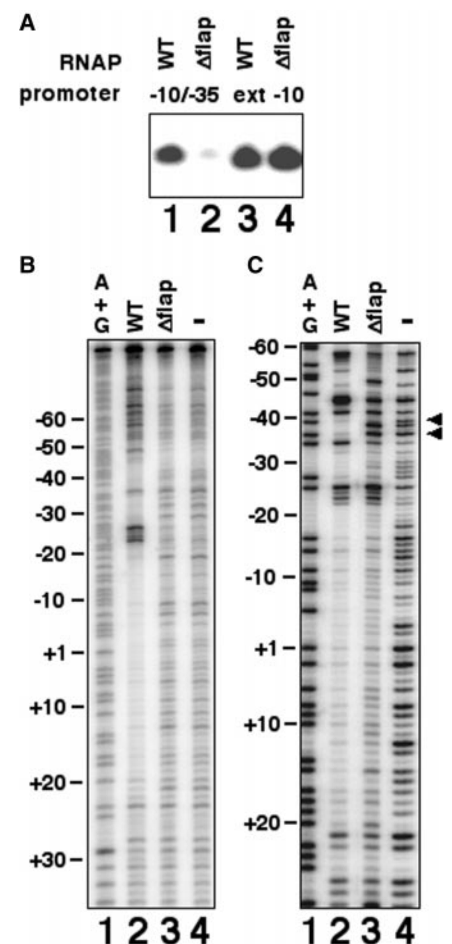

Fig. 2. Deletion of β-flap restricts RNAP to one class of bacterial promoters. (**A**) Results of abortive transcription initiation reactions performed on the –10/–35 class T7 A2 promoter and the extended –10 class *gal*P1 promoter using wild-type $E\sigma^{70}$ or mutant $E\sigma^{70}$ lacking the β-flap. (**B** and **C**) Promoter complexes formed by wild-type or mutant $E\sigma^{70}$ on the T7 A2 (B) or the *gal*P1 (C) promoter were footprinted with DNase I. Lanes 4 are controls (no RNAP added to footprinting reactions); lanes 1 are marker lanes.

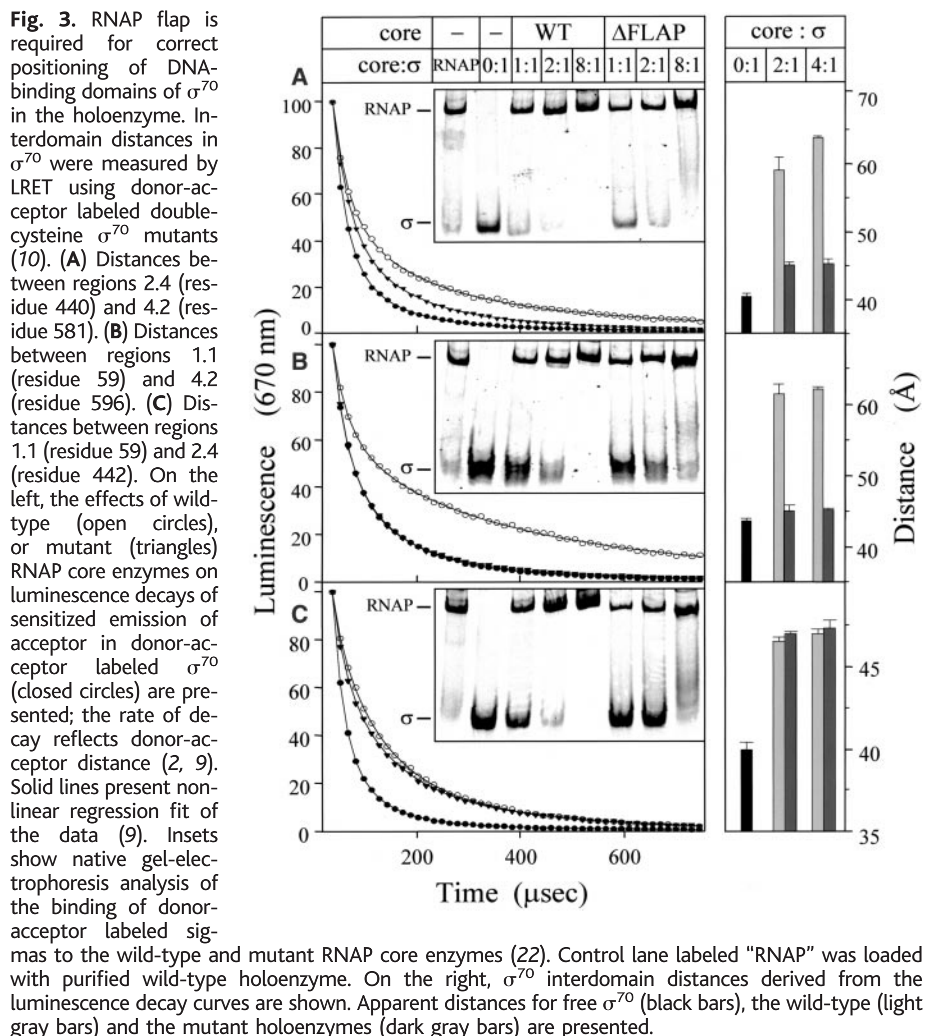

Fig. 3. RNAP flap is required for correct positioning of DNA-binding domains of σ^{70} in the holoenzyme. Interdomain distances in σ^{70} were measured by LRET using donor-acceptor labeled double-cysteine σ^{70} mutants (*10*). (**A**) Distances between regions 2.4 (residue 440) and 4.2 (residue 581). (**B**) Distances between regions 1.1 (residue 59) and 4.2 (residue 596). (**C**) Distances between regions 1.1 (residue 59) and 2.4 (residue 442). On the left, the effects of wild-type (open circles), or mutant (triangles) RNAP core enzymes on luminescence decays of sensitized emission of acceptor in donor-acceptor labeled σ^{70} (closed circles) are presented; the rate of decay reflects donor-acceptor distance (*2, 9*). Solid lines present nonlinear regression fit of the data (*9*). Insets show native gel-electrophoresis analysis of the binding of donor-acceptor labeled sigmas to the wild-type and mutant RNAP core enzymes (*22*). Control lane labeled "RNAP" was loaded with purified wild-type holoenzyme. On the right, σ^{70} interdomain distances derived from the luminescence decay curves are shown. Apparent distances for free σ^{70} (black bars), the wild-type (light gray bars) and the mutant holoenzymes (dark gray bars) are presented.

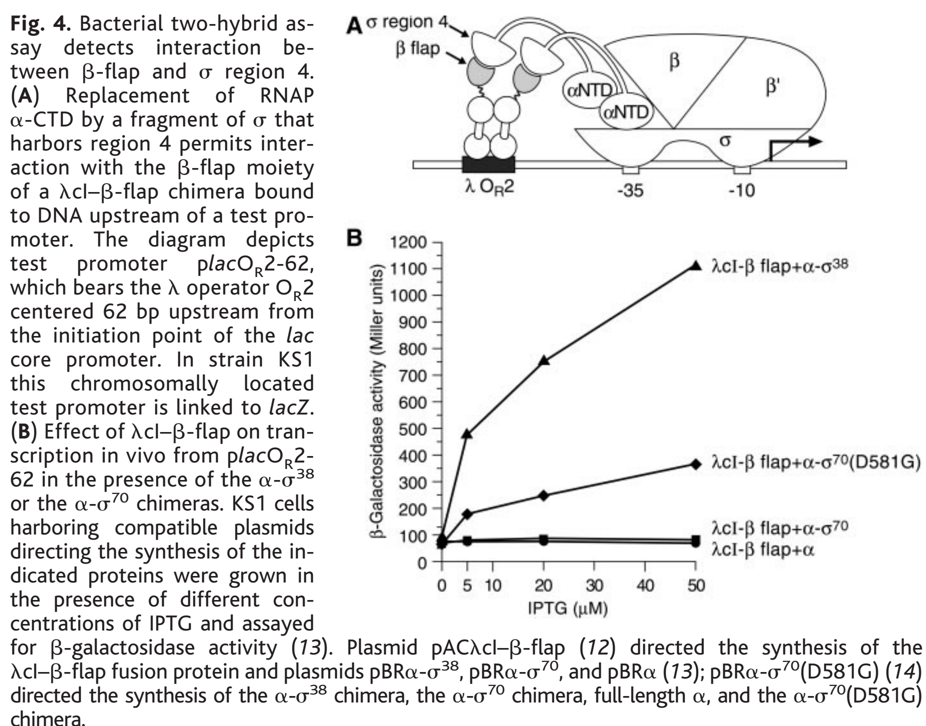

Fig. 4. Bacterial two-hybrid assay detects interaction between β-flap and σ region 4. (**A**) Replacement of RNAP α-CTD by a fragment of σ that harbors region 4 permits interaction with the β-flap moiety of a λcI–β-flap chimera bound to DNA upstream of a test promoter. The diagram depicts test promoter p*lac*O_R2-62, which bears the λ operator O_R2 centered 62 bp upstream from the initiation point of the *lac* core promoter. In strain KS1 this chromosomally located test promoter is linked to *lacZ*. (**B**) Effect of λcI–β-flap on transcription in vivo from p*lac*O_R2-62 in the presence of the α-σ^{38} or the α-σ^{70} chimeras. KS1 cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity (*13*). Plasmid pACλcI–β-flap (*12*) directed the synthesis of the λcI–β-flap fusion protein and plasmids pBRα-σ^{38}, pBRα-σ^{70}, and pBRα (*13*); pBRα-σ^{70}(D581G) (*14*) directed the synthesis of the α-σ^{38} chimera, the α-σ^{70} chimera, full-length α, and the α-σ^{70}(D581G) chimera.

brings σ regions 2 and 4 further apart and allows –10/–35 promoter complex formation through simultaneous recognition of the –10 and –35 promoter elements. The flexibility of the β-flap may be important for this second allosteric switch. According to this view, the β-flap may dictate recognition of the correct spacing (17 ± 1 bp) between promoter elements. It is possible that factors that interact with the β-flap and affect its interaction with σ might permit recognition of promoters with suboptimal spacers, thus altering the promoter specificity of RNAP. More generally, there may exist a class of regulatory factors that affect promoter recognition by either disrupting or stabilizing the interaction between the β-flap and region 4 of σ. In fact, the *H. pylori* σ^{28}–β-flap interaction is disrupted by an antisigma protein that down-regulates σ^{28}-dependent transcription (*15*). It is conceivable that other transcriptional regulators currently thought to target sigma region 4.2 may also influence the interaction of σ with the β-flap. Because eukaryotic multisubunit RNAPs also contain the flap domain (*18*), the β-flap may contribute to promoter recognition in eukaryotes as well.

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