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Biochemistry

The sigma subunit of *Escherichia coli* RNA polymerase senses promoter spacing

(transcription initiation)

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ABSTRACT **The promoters recognized by σ^{70}, the primary sigma of *Escherichia coli*, consist of two highly conserved hexamers located at −10 and −35 bp from the start point of transcription, separated by a preferred spacing of 17 bp. σ factors have two distinct DNA binding domains that recognize the two hexamer sequences. However, the component of RNA polymerase recognizing the length of the spacing between hexamers has not been determined. Using an equilibrium DNA binding competition assay, we demonstrate that a polypeptide of σ^{70} carrying both DNA binding domains is very sensitive to promoter spacing, whereas a σ^{70} polypeptide with only one DNA binding domain is not. Furthermore, a mutant σ, selected for increasing transcription of the minimal *lac* promoter (18-bp spacer), has an altered response to promoter spacing *in vivo* and *in vitro*. Our data support the idea that σ makes simultaneous, productive contacts at both the −10 and the −35 regions of the promoter and discerns the spacing between these conserved regions.**

The sigma (σ) subunits of prokaryotic RNA polymerase provide specificity for transcription initiation by directing RNA polymerase to promoter DNA sequences (1, 2). Every cell contains a primary σ factor, which is essential for expression of genes required for normal growth, and several alternative σ factors, which are used to respond to altered environmental conditions and to program developmental pathways (3). The majority of primary and alternative σ factors are related in amino acid sequence to σ^{70}, the *Escherichia coli* primary σ, and exhibit four major regions of homology (ref. 3; Fig. 1). Mutational analysis has implicated conserved region 2.4 of σ in recognition of the −10 hexamer and conserved region 4.2 of σ in recognition of the −35 hexamer of the promoter (Fig. 1; for review, see ref. 3). Biochemical characterization supports these conclusions. Partial polypeptides of σ^{70} containing either region 2 or region 4 interact specifically with the −10 or −35 promoter elements, respectively, provided that the N-terminal inhibitory domain of region 1 is removed. Moreover, a σ^{70} fragment carrying both DNA binding domains interacts specifically with DNA that retains both promoter elements. DNA binding by σ^{70} fragments accurately reflects holoenzyme interactions at the promoter because (*i*) σ fragments can distinguish promoter from nonpromoter DNA and (*ii*) σ mutations that alter the promoter recognition selectivity of holoenzyme similarly alter the binding selectivity of σ fragments (4).

The promoter spacer separates the −10 and −35 consensus hexamers and contributes to promoter function. The length of the spacer, but not its sequence, is an important determinant of promoter strength, with the consensus 17-bp spacing usually conferring optimal promoter activity (5–9). One exception is the "extended −10" promoters where TG at positions −15 and −16 is required in the spacer (10, 11). Protection studies of promoters with 16-, 17-, or 18-bp spacers have shown that RNA polymerase forms a similar pattern of contacts at all three promoters (12, 13), suggesting that the enzyme can accommodate rotational and longitudinal variation. One model proposes that RNA polymerase rotates the −10 and −35 sequences with respect to one another to make sufficient contacts with both concomitantly, thus putting torque on the spacer DNA (6). The "molecular ruler" in RNA polymerase that measures promoter spacer length is not known. DNA binding by a σ fragment containing both DNA binding domains can be disrupted by a single mutation in either the −10 or the −35 region of the promoter (4). This observation suggested to us that σ itself may interact simultaneously with the −10 and −35 promoter elements and, thus, directly sense the spacing between these elements. The experiments described in this report provide support for this idea.

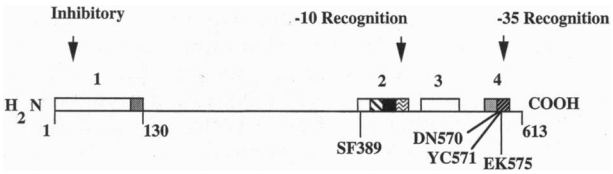

FIG. 1. Linear diagram of σ^{70}. The four most highly conserved segments are designated as regions 1, 2, 3, and 4. Subdivisions within these regions are indicated by changes in shading and correspond to regions 1.1, 1.2, 2.1, 2.2, 2.3, 2.4, 4.1, and 4.2. Region 1.1 contains a domain that inhibits DNA binding by the DNA binding domains of regions 2 and 4. Region 2.4 interacts with the −10 promoter element, and region 4.2 interacts with the −35 promoter element, as indicated by the arrows. Mutants that were selected for increased expression of the *lac* operon in the absence of cAMP activating protein (CAP)-cAMP are shown below the linear representation. The nomenclature for these mutants consists of the original amino acid followed by the substituted amino acid, in single-letter amino acid code. The position within the polypeptide is indicated by the number following the amino acid substitutions.

MATERIALS AND METHODS

Materials. Enzymes were from Promega, New England Biolabs, or Boehringer Mannheim; nucleotides were from Pharmacia; and Amplitaq DNA polymerase was from Perkin–Elmer. [γ-^{32}P]ATP [3000 Ci/mmol (1 Ci = 37 GBq)] and Sequenase were from Amersham, nitrocellulose filter disks (BA85) were from Schleicher & Schuell, isopropyl β-D-thiogalactopyranoside (IPTG) was from Fisher, and nitrocefin was from BBL Microbiology Systems.

Oligonucleotide Site-Directed Mutagenesis, Plasmid Constructions, and Fusion Proteins. Synthetic oligonucleotides carrying deletions or insertions in the spacer region of the *tac*

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Abbreviations: HTH, helix–turn–helix; CAP, cAMP activating protein.

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                -35                 -10
ptac:     5'-TTGACAATTAATCATCGGCTCGTATAATGTGTGGA-3'

ptac+1:   5'-TTGACAATTAATCTATCGGCTCGTATAATGTGTGGA-3'

ptac+3:   5'-TTGACAATTAATCTAGATCGGCTCGTATAATGTGTGGA-3'

ptac+5:   5'-TTGACAATTAATCTAGACATCGGCTCGTATAATGTGTGGA-3'

ptac+10:  5'-TTGACAATTAATCTAGACTGCAGATCGGCTCGTATAATGTGT-3'

ptac-1:   5'-TTGACAATTAATC_TCGGCTCGTATAATGTGTGGA-3'

ptac-3:   5'-TTGACAATTAAT__CGGCTCGTATAATGTGTGGA-3'

ptac-5:   5'-TTGACAATTAA____GGCTCGTATAATGTGTGGA-3'
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FIG. 2. Promoter spacing changes. Alterations in the sequence of the *tac* promoter are shown. Underlined letters designate the −35 and −10 elements of the promoter. Bold letters indicate bases inserted into the sequence. Underscores without letters indicate the positions where bases have been removed.

promoter were used to generate altered promoters (see Fig. 2) in M13 mp19ptac (4), using the method of Kunkel and coworkers (14). Expression plasmids, carrying altered spacing derivatives of p*tac* upstream of *lacZ*, were constructed by digesting the replicative form of M13 mp19ptac with *Bam*HI. The 269-bp DNA fragment was ligated into the *Bam*HI site of pJR1. pJR1 is a derivative of pRS415 (15) where a *Hin*dIII site was added to the polylinker cloning region (gift from R. Gourse, University of Wisconsin, Madison, WI). The resulting vectors, pJR1ptac, pJR1-ptac+1, pJR1-ptac+3, pJR1ptac+5, and pJR1ptac-1, were sequenced to verify the mutations. DNA encoding amino acids 360–613 of the *rpoD911* mutation was used to construct GSTσ[360–613]EK575. Purification of GSTσ fusions was as previously reported (4, 16).

Nitrocellulose Filter Binding Assays. ^{32}P-labeled DNA and competitor DNA were synthesized as described (4). The templates were M13 mp19ptac derivatives (replicative form), and the primers were the M13 universal 17-mer and a primer corresponding to the −70 region of the *tac* promoter. Following PCR, the 100-bp DNA products were purified using the Qiaquick PCR DNA purification kit (Qiagen) and were visualized on 3% Metaphor (FMC) agarose gels. Equilibrium competition nitrocelluose filter retention assays were conducted as reported (4). The reduction in filter retention as a function of increasing competitor DNA concentration was monitored. The error of the measurement is the standard deviation from several independent experiments. The nonpromoter DNA (ΔP) corresponded to DNA within the M13 mp19 polylinker region.

Enzyme Assays and Primer Extension Analysis of 5′ Ends. Strains 19269, 19270, 19271, 19272, and 19273 (Table 1) were transformed with pJR1 derivatives containing various spacing alterations in the *tac* promoter. Overnight cultures were diluted 1:100 in fresh Luria–Bertani plus ampicillin and incubated at 37°C until the OD_{600} was 0.15–0.25. IPTG was added to 1 mM and incubation continued for 2 hr. The cultures were placed on ice for 30 min, the OD_{600} was measured, and 0.1 ml was used in the β-galactosidase assay (17). To correct for plasmid copy number, β-galactosidase activity was divided by β-lactamase activity (18). For determinations of the 5′ end of the mRNA, cells were grown in M9 glucose minimal medium and induced as above. Preparation of total RNA, hybridization, and extension procedures were done according to Tsui and coworkers (19). The probe was a 15-nt-long primer, labeled with [γ-^{32}P]ATP (19), which produced a primer extension product of 240 nt.

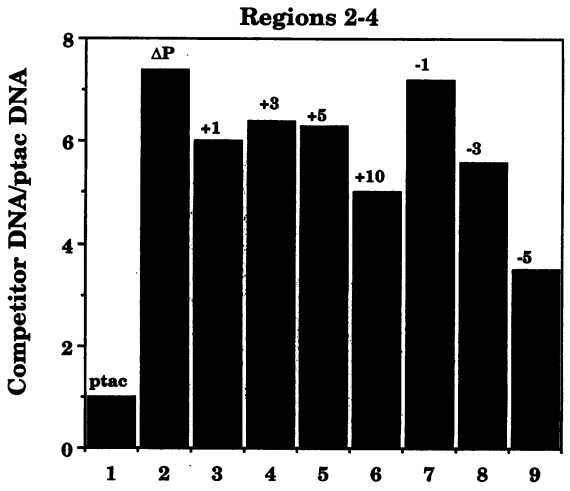

FIG. 3. Equilibrium competition DNA binding for GSTσ[360–613]. The ratio of unlabeled competitor DNA relative to labeled p*tac* required to reduce DNA filter retention to one-half its original level is indicated by the bars. ΔP is a nonspecific competitor containing no promoter-like sequences. The numbers above the bars indicate the base pairs added or deleted from the spacer of the *tac* promoter. The data are the average from the results of two or more independent experiments. The error in the raw DNA binding data was less than 20%.

RESULTS

A Polypeptide of σ^{70} Carrying Both DNA Binding Domains Is Sensitive to Promoter Spacing. The strong *tac* promoter (p*tac*) consists of a consensus −35 hexamer from the *trp* promoter, a consensus −10 hexamer from the *lac*UV5 promoter, and a 16-bp spacing (21). To test the idea that σ itself

Table 1. Bacterial strains

Strain	Relevant genotype	Sigma	Source
MH7295	F′[*lac*I^q (kan)]		W. Margolin
XL1-Blue	*recA, lac*, F′ [*proAB, lac*I^q, ZΔM15, Tn10 (tet)]	wt	Stratagene
CAG7189	MG1655 *gal-3 Δcya*	wt	Ref. 20
CAG7354	CAG7189 *rpoD*$^+$ *zgh*::Tn10 (tet)	wt	D. Siegele
CAG7355	CAG7189 *rpo901 zgh*::Tn10 (tet)	SF389	D. Siegele
CAG7356	CAG7189 *rpo904 zgh*::Tn10 (tet)	YC571	D. Siegele
CAG7357	CAG7189 *rpo911 zgh*::Tn10 (tet)	EK575	D. Siegele
CAG7358	CAG7189 *rpo912 zgh*::Tn10 (tet)	DN570	D. Siegele
19269	CAG7354 F′[*lac*I^q (kan)]	wt	This study
19270	CAG7355 F′[*lac*I^q (kan)]	SF389	This study
19271	CAG7356 F′[*lac*I^q (kan)]	YC571	This study
19272	CAG7357 F′[*lac*I^q (kan)]	EK575	This study
19273	CAG7358 F′[*lac*I^q (kan)]	DN570	This study

wt, Wild type.

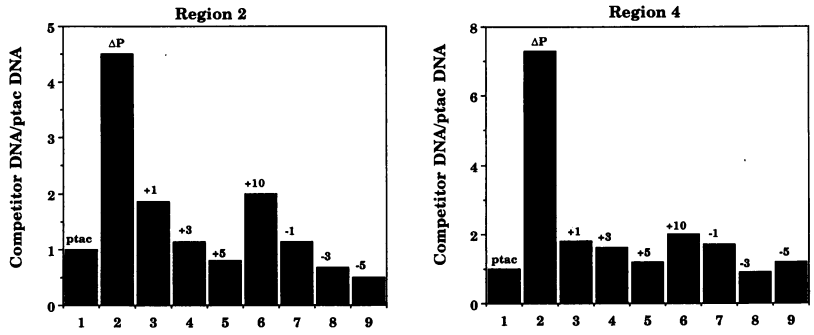

FIG. 4. Equilibrium competition DNA binding for GSTσ[372–466] and GSTσ[506–613]. (*Left*) GSTσ[372–466] carrying only the region 2 DNA binding domain. (*Right*) GSTσ[506–613] carrying only the region 4 DNA binding domain. The ratio of unlabeled competitor DNA relative to labeled p*tac* required to reduce DNA filter retention to one-half its original level is indicated by the bars. ΔP is a nonspecific competitor containing no promoter-like sequences. The numbers above the bars indicate the base pairs added or deleted from the spacer of the *tac* promoter. The data are the average from the results of two or more independent experiments. The error in the raw DNA binding data was less than 20%.

recognizes the length of the spacer, we constructed spacing variants of p*tac* and used them to examine DNA binding by σ *in vitro*.

We previously used equilibrium competition binding experiments to show that partial polypeptides of σ^{70}, lacking the N-terminal inhibitory domain, interact preferentially with promoter DNA (4). In those experiments, a 5′-end-labeled 100-bp DNA fragment carrying the *tac* promoter was mixed with increasing amounts of unlabeled competitor DNA, and a fixed amount of σ polypeptide was allowed to bind. Retention of σ:DNA complexes on nitrocellulose filters was used to measure DNA binding. A reduction in filter retention of the labeled promoter fragment to 50% required addition of either an equivalent amount of unlabeled promoter DNA or about a 5-fold molar excess of unlabeled nonpromoter DNA. Thus, on a per site basis, these σ fragments exhibited a 500-fold selectivity for promoter over nonpromoter DNA.

We used this same competition binding assay to examine the ability of fusion protein GSTσ[360–613], carrying regions 2–4 of σ, to interact with the p*tac* promoter spacing mutants. We monitored the reduction in DNA binding as the amount of competitor DNA was increased (data not shown) and determined the ratio of competitor DNA to labeled p*tac* DNA required to reduce binding by one-half (Fig. 3). In this set of experiments, specific competitor (unlabeled p*tac*) reduced binding by one-half at a 1:1 ratio, whereas the nonspecific competitor (which contains no promoter sequences) required a 7- to 8-fold higher concentration to achieve an equivalent reduction in binding. Like the nonspecific competitor, each of the p*tac* spacing variants requires a 5- to 10-fold molar excess of fragment to achieve a 50% reduction in binding. Thus, perturbing spacer length interferes with the ability of the σ fragment to bind this promoter effectively. These results suggest that σ must span the distance between the −10 and −35 sequences.

Equilibrium competition experiments were also conducted using GSTσ[506–613], carrying region 4 alone, and GSTσ[372–466], carrying region 2 alone (4, 16). In both cases, the promoter spacing variants behave like specific competitors, requiring less than a 2-fold excess over the labeled promoter DNA to reduce binding by one-half (Fig. 4). Thus, alterations in spacing have no effect on the ability of the individual DNA binding domains to bind to their preferred segment of the promoter. This observation is consistent with previous studies showing that each DNA binding domain can interact with its preferred half of the promoter independently (4) and provides further support for the idea that to sense spacing, σ must

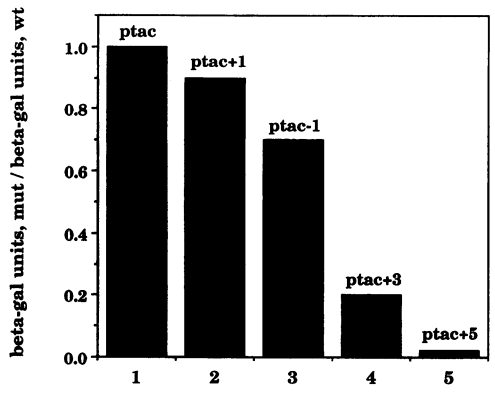

FIG. 5. Activity of promoter spacing mutants *in vivo*. Promoters cloned into pJR1, upstream of the *lacZ* gene, were tested for transcriptional activity in *E. coli* strain XL1-Blue. The β-galactosidase activity from the indicated mutated promoters is expressed relative to the β-galactosidase activity from the wild-type (wt) p*tac* promoter. The Miller units, corrected for plasmid copy number, for each polymerase at the wild-type ptac promoter were *rpoD* (56036), *rpoD901* (147707), *rpoD904* (78739), *rpoD911* (53070), and *rpoD912* (38913). The data are the average from the results of two or more independent experiments. The error in the raw β-galactosidase data was 7% or less.

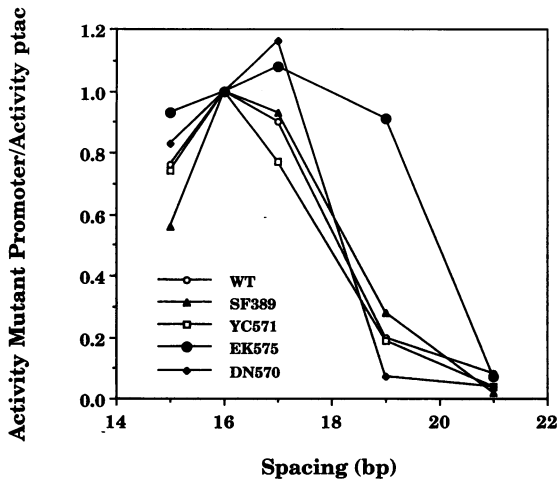

FIG. 6. β-galactosidase activity for σ mutants and p*tac* promoter spacing variants. The β-galactosidase activity is expressed as the ratio of activity for a particular σ with an altered promoter, relative to activity of the same σ with the unaltered *tac* promoter. Two or more independent determinations were averaged for each value shown. The β-galactosidase activity ranged from 819 to 58,696 units with an average error of ±11.5%. The actual values for β-galactosidase activity were corrected for plasmid copy number by dividing by the units of β-lactamase produced from the same plasmid.

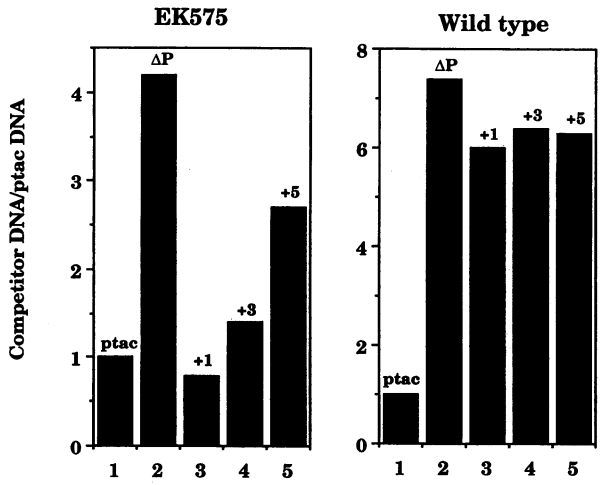

FIG. 7. Equilibrium competition DNA binding for GSTσ[360–613]EK575 and GSTσ[360–613]. The ratio of unlabeled competitor DNA relative to labeled p*tac* required to reduce DNA filter retention to one-half its original level is indicated by the bars. ΔP is a nonspecific competitor containing no promoter-like sequences. The numbers above the bars indicate the base pairs added or deleted from the spacer of the *tac* promoter. The data are the average from the results of two or more independent experiments. The error in the raw DNA binding data was less than 20%.

interact simultaneously with the −10 and −35 regions of the promoter.

Activity of Promoter Spacing Variants *in Vivo*. The activity of several *tac* promoters with altered spacing was assayed *in vivo* by measuring β-galactosidase activity. The highest activity was attained for the wild-type *tac* promoter. Increasing or decreasing the spacer by 1 bp had a minimal effect on promoter strength; however, more drastic alterations of the spacer length decreased activity more severely (Table 1; Fig. 5). We note that spacing alterations affect binding of σ fragments *in vitro* more severely than holoenzyme transcription *in vivo*. We return to this point in the *Discussion*.

A Mutation in *rpoD* Can Suppress the Transcriptional Defects of Promoter Spacing Variants *in Vivo*. Siegele and coworkers (20) isolated several mutations in σ^{70} that increased expression of the *lac* operon 5- to 7-fold in the absence of CAP-cAMP (Table 1). One of the reasons that p*lac* is a weak promoter in the absence of activators is that it has an 18-bp spacer (21). Thus, one way for the mutants to increase expression of the *lac* promoter is to permit better utilization of promoters with nonoptimal spacer lengths. To determine whether any of these mutant σ factors exhibited this phenotype, we tested whether they altered expression of the spacing variants of p*tac*. One of the mutants, EK575, increased transcription of p*tac* variants having a 19-bp spacing (Fig. 6). Primer extension analysis of the 5′ ends of RNA from the wild-type and EK575 strains showed no difference in the start site for the *lacZ* transcript (data not shown). We argue in the *Discussion* that this phenotype most likely results from a change in the way that σ senses spacing.

The EK575 Mutant Binds to a 19-bp Spacing Promoter *in Vitro*. Equilibrium competition binding experiments using GSTσ[360–613]EK575 showed that both ptac+1 and ptac+3 promoter variants competed with p*tac* for binding at nearly equimolar amounts (Fig. 7). In contrast, for wild-type GSTσ[360–613], neither spacing variant was an effective binding competitor for the p*tac* competitor (Fig. 3). Thus, as expected from its behavior *in vivo*, EK575 is better able that wild-type σ to bind to promoters with longer spacing.

DISCUSSION

Numerous studies indicate that RNA polymerase holoenzyme is sensitive to the spacing between the −10 and −35 recognition sites of the prokaryotic promoter, with a 17-bp spacing considered optimal for activity (5–9). In this report, we present two lines of evidence consistent with the idea that σ itself discriminates the spacer length of promoters.

Interactions Between σ^{70} and Spacing Variants of the *tac* Promoter. When the σ^{70} fragment carries both DNA binding domains, spacing between the −10 and −35 hexamers is a critical parameter in the interaction between the σ^{70} fragment and the promoter. In the *tac* promoter, a 16-bp spacing is optimal. A change of as little as 1 bp in either direction abolishes the ability of the σ fragment to discriminate promoter from nonpromoter DNA. In contrast, spacing is not critical for the DNA–protein interaction that occurs for σ^{70} fragments with only a single DNA binding domain. These results suggest that the functional interdependence of the two DNA binding domains of σ^{70} underlies its ability to sense spacing between the −10 and −35 recognition elements of the promoter.

The fact that isolated σ^{70} senses spacing suggests that it also performs this function in the context of RNA polymerase holoenzyme. However, although qualitatively similar, there are distinct differences in the response of σ^{70} and holoenzyme to altered promoter spacing. Whereas changes of 1 bp in spacing abolish the ability of σ^{70} fragments to discriminate promoter from nonpromoter DNA, spacing changes of 3 bp or more are required for large effects on holoenzyme at the *tac* promoter (Fig. 5). Whereas holoenzyme may compensate for nonoptimal promoter spacing by bending, twisting, or other DNA deformations before strand opening, there is no evidence that σ alone can perform these manipulations. By aligning the promoter recognition elements, the DNA transactions of holoenzyme may increase the range of promoter spacing variants successfully recognized by σ.

A σ^{70} Mutant Affecting Spacing. The EK575 mutant suppresses the low transcriptional activity of the p*tac* variant with a 19-bp promoter spacer. Residue 575 of σ^{70} is located in the upstream helix of the helix–turn–helix (HTH) motif, which recognizes the −35 region of the promoter. Although an EK substitution at this position could simply compensate for nonoptimal spacing by strengthening the σ–DNA interaction, several considerations suggest that EK575 is more likely to directly alter the mechanism by which σ senses spacing. (*i*) Four mutants exhibit a comparable increase in p*lac* transcription, but only EK575 suppresses long spacing (Fig. 6). It is particularly significant that DN570 and YC571, located at the N-terminal side of the upstream helix of the HTH motif in positions known to contact DNA in other HTH-type proteins, do not alter response to spacing. (*ii*) EK575 manifests a phenotype only in the context of promoters with longer spacing. It specifically increases transcription of the 19-bp promoter variant from the correct start site but does not increase transcription of p*tac* itself. (*iii*) Regions 2–4 of EK575 bind better than regions 2–4 of wild-type σ^{70} to promoters with increased spacing, arguing strongly that EK575 directly affects the ability of σ to detect spacing changes. The amino acid substitution in EK575 may provide σ with increased flexibility, which would facilitate simultaneous recognition of −10 and −35 binding sites at longer spacing.

How σ Can Be a Molecular Ruler? The simplest model to account for the ability of σ to recognize spacing would suggest that σ spans the distance between the −10 and −35 sequences while simultaneously making productive contacts at both. If the spacing is too large, σ cannot reach across the gap. If the spacing is too small, σ may not be able to physically compress its structure enough to accommodate the reduced spacing. In these cases, σ may not be able to position the −10 and −35 sequences properly. We consider two alternative configurations of σ that would allow it to measure distance. In the first, we imagine that the two DNA binding domains, region 2.4 and region 4.2, are separated by a relatively rigid structure composed primarily of region 3. The length of the structure

separating the two DNA binding domains would measure spacing. An alternative model envisions regions 2.4 and 4.2 folding independently but then interacting with each other, possibly looping out region 3. This interaction would fix the distance between regions 2.4 and 4.2, thus setting the optimal spacer length.

Our experiments do not permit us to distinguish between these models. However, the idea that regions 2.4 and 4.2 interact is quite appealing. σ fragments containing both DNA binding domains are sensitive to the sequence and spacing of both promoter elements, whereas single-domain fragments are not. Interaction between these two DNA binding domains would explain why these σ fragments behave qualitatively differently from those containing only one domain. We note that homology arguments suggest that E575 might be well-positioned to carry out this interaction. Profile analysis indicates that the HTH of σ^{70} is most similar to that of 434 cro. In that structure, a residue in the middle of the upstream structural helix would be away from the DNA and thus in a position to interact with other regions of the protein.

The idea that an interaction between regions 2.4 and 4.2 measures spacing has implications for how promoter activity is controlled. This interaction could be a point of regulation. Effectors could alter this interaction and, hence, change optimal spacing at a particular promoter. The length and strength of the homology regions at -10 and -35 could be allosteric effectors of this interaction, thus permitting optimal spacing to vary according to promoter sequence. In addition, several regulated promoters have nonoptimal spacings. The P_{TPCAD} promoter of the mercuric ion resistance operon has a 19-bp spacer. It has been proposed that the activator Hg(II)-MerR distorts the spacer DNA to allow RNA polymerase to contact the -10 region of the promoter (22). In *Bacillus subtilis*, the *spoIIG* and *spoIIE* genes are transcribed by the σ^{70} analog, σ^{A}. These promoters have 22- and 21-bp spacers, respectively. One model for activation of these promoters is that phophorylated Spo0A modifies the RNA polymerase-promoter complexes to stimulate initiation and overcome the overlong spacing (23). Activator proteins may also function to alter the interaction between the two DNA binding domains of σ, allowing the nonconsensus spacing at a particular promoter to be optimal for σ function.

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