Amino-terminal amino acids modulate σ -factor DNA-binding activity

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Prokaryotic transcription initiation factor σ is required for sequence-specific promoter recognition by RNA polymerase. Genetic studies have indicated that σ itself interacts with DNA at the -10 and -35 promoter consensus sequences. Binding of *Escherichia coli* σ^{70} to DNA in vitro, however, can only be observed for truncated polypeptides lacking the amino-terminal amino acids. We have investigated the role of the amino terminus of *E. coli* σ^{70} in controlling DNA-binding ability. Deletion analysis indicates that amino acids within amino-terminal region 1.1 of σ^{70} inhibit DNA binding by the carboxy-terminal DNA-binding domains. Furthermore, inhibition of binding by the amino-terminal inhibitory domain of σ^{70} can be observed in *trans*. Likewise, the amino-terminal extensions of two alternative σ -factors, *E. coli* σ^{32} and *Bacillus subtilis* σ^K , negatively affect the DNA binding activity of their carboxy-terminal domains. We propose that initiation of transcription is subject to modulation as a result of the composition and/or structure of the amino terminus of the σ -subunit and that the σ family of proteins belong to a larger class of intramolecularly regulated transcriptional effectors.

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Initiation of transcription at prokaryotic promoter DNA sequences requires one of the σ -subunits of bacterial RNA polymerase (Burgess et al. 1969; Burgess and Travers 1970). There are two distinct families of σ -factors. One family includes σ factors that are related to σ^{54} , the *Escherichia coli* σ -factor required for nitrogen-regulated gene expression. The majority of known σ -factors, however, belong to the other family whose members are evolutionarily related to σ^{70} , the *E. coli* primary σ -factor. The σ^{70} family is comprised of highly homologous proteins that have been subdivided into two groups: (1) the primary σ -factors, which are required for vegetative growth of the bacterial cell, and (2) the alternative σ -factors, which participate in the transcriptional response to changing environmental conditions or physiological states (Stragier et al. 1985; Gribskov and Burgess 1986; Helmann and Chamberlin 1988; Gross et al. 1992; Lonetto et al. 1992). Both primary and alternative σ -factors from the σ^{70} family are subjects of this report.

The particular σ -factor associated with RNA polymerase holoenzyme provides specificity for promoter sequence recognition by the enzyme, leading to the proposal that σ -factors are sequence-specific DNA-binding proteins (Losick and Pero 1981). Genetic studies have implicated two highly conserved regions of σ in pro-

motor recognition. Mutations in region 2.4 of σ affect the ability of the holoenzyme to discriminate between base pairs in the -10 hexamer of the promoter consensus sequence, and mutations in conserved region 4.2 affect recognition of the -35 hexamer (Fig. 1A) (Gardella et al. 1989; Siegle et al. 1989; Zuber et al. 1989; Daniels et al. 1990; Waldburger et al. 1990). Recently, a biochemical analysis of σ -promoter interactions showed that partial polypeptides carrying conserved region 2 or 4 of σ^{70} bind independently to promoter DNA with appropriate specificity, providing further support for the idea that σ itself can recognize and interact with specific DNA sequences (Dombroski et al. 1992).

Although the isolated DNA-binding domains of σ^{70} specifically recognize promoter DNA, full-length σ , in the absence of the core subunits of RNA polymerase ($\alpha_2\beta\beta'$), does not interact specifically or nonspecifically with DNA (Burgess et al. 1969; Zillig et al. 1970; Wu et al. 1975; Kudo and Doi 1981; Kudo et al. 1981; Ramesh and Meares 1988; Wellman and Meares 1991). At least for σ^{70} , the amino-terminal region of σ appears to block the DNA-binding capacity of the more carboxy-terminal portion of the protein (Dombroski et al. 1992). In this report we further characterize the amino-terminal inhibitory domain of σ^{70} and its mechanism of inhibition. Additionally, we have analyzed the DNA-binding properties of several alternative σ -factors. Our results indicate that the amino-terminal regions of both the primary and alternative σ -factors participate in modulation of DNA-binding activity, thus revealing a powerful mech-

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anism for transcriptional regulation through manipulation of the σ -subunit of RNA polymerase. Implications for the mechanism of transcription initiation by holoenzyme ($\alpha_2\beta\beta'\sigma$) are discussed.

Results

Amino acids within conserved region 1.1 of σ^{70} inhibit DNA binding

The amino-terminal portion of σ , called region 1, is divided into two conserved segments, termed 1.1 and 1.2 (Fig. 1A). Region 1.1 is conserved only among the primary σ -factors, whereas region 1.2 is conserved among both primary and alternative σ -factors (except for σ^F and σ^S). Our previous experiments indicated that removing all of region 1 from σ^{70} (amino acids 1–130) was sufficient to permit specific and nonspecific binding of σ^{70} to DNA. To further define the role of the amino-terminal region of σ^{70} in controlling DNA-binding activity, we made deletions into both region 1 and the "spacer," a region unique to σ^{70} that follows region 1 (Fig. 1B). The relative DNA-binding activity of each deleted protein [purified as glutathione *S*-transferase (GST) fusion proteins] was assessed using nitrocellulose filter-binding assays (see Materials and methods), and the results are

shown in Figure 1B. Removal of the first 50 amino acids of σ^{70} was sufficient to permit binding to DNA, indicating that the amino-terminal half of region 1.1 is necessary for inhibition of binding. Removing 180–273 amino acids from the amino terminus allowed truncated derivatives to bind DNA an order of magnitude more tightly, suggesting that amino acids in this region also play a role in modulating DNA binding.

The activity of the altered σ^{70} fusion proteins was also tested *in vivo*. With the exception of GST σ^{70} [8–613], none of the amino-terminal truncations or internal deletions was able to complement the temperature sensitive defect of a mutant σ^{70} strain (*rpoD285*). We assume that these deleted proteins fold properly because of their DNA-binding activity. This suggests that each deletion removes an essential portion of σ^{70} . One of the noncomplementing deletions (GST σ^{70} [Δ 100–130]) removes only region 1.2, providing evidence that this region is necessary for σ^{70} function. Interestingly, fusions lacking either the first 50 or 72 amino acids of σ^{70} were detrimental to cell growth under noninducing conditions and lethal upon overexpression (Fig. 1B). These two derivatives may interfere with normal σ^{70} function *in vivo*, possibly by competing for binding to core RNA polymerase or by carrying out inappropriate transcription.

Figure 1. Characterization of GST σ^{70} fusion proteins. (A) Linear diagram of σ^{70} . The most highly conserved blocks of amino acid sequence are numbered 1–4 (Lonetto et al. 1992). Subregions are indicated by different patterns within each main region. Region 2 is implicated in recognition of the -10 promoter consensus. Region 4 contains a helix–turn–helix motif and is implicated in recognition of the -35 promoter consensus. (B) Function of amino-terminally altered GST σ^{70} fusion proteins. GST was fused to the amino terminus of several truncated σ^{70} derivatives. The amino acid sequences included are designated by the schematic diagram at right. The first column indicates the amino acids that remain, except for the bottom line where region 1.2 (amino acids 100–130) was deleted. The second column indicates the ability of the fusion, encoded by the pGEX plasmid, to complement the temperature-sensitive growth defect of the *rpoD285* (*rpoD800*) allele at 42°C in the absence of IPTG. The third column displays the effect of overexpression of each fusion protein on viability. The strain used for these experiments was XL-1. The fusions were encoded by the pGEX plasmid under the control of the *ptac* promoter. The *lacI^q* allele is present on the plasmid. Growth was at 37°C to mid-log phase, where IPTG was added to a final concentration of 0.1 mM. The fourth column summarizes the DNA-binding ability for each fusion. DNA-binding titrations, using 32 P-labeled *ptac* promoter DNA, were conducted as described previously (Dombroski et al. 1992). The amount of fusion protein required for half-maximal retention of protein–DNA complexes on nitrocellulose filters was in the range of 1–100 nM. Each plus sign (+) roughly represents an order of magnitude difference in binding affinity, where ++ is the strongest binding.

The amino-terminal inhibitory domain of σ^{70} interacts with carboxy-terminal amino acids

We determined whether the amino-terminal region of σ^{70} could inhibit DNA binding in *trans*. Several GST fusion proteins were constructed to carry only the amino-terminal sequences of *E. coli* σ^{70} (Fig. 2A). Each of these potential "inhibitors" was assayed for its ability to inhibit the DNA binding of GST σ^{70} [360–613]. GST σ^{70} [360–613] is a σ^{70} fusion protein containing both the region 2 and region 4 DNA-binding domains and was shown previously to bind to DNA (Dombroski et al. 1992). The three amino-terminal fusions containing 100 or more amino acids inhibited DNA binding in *trans* (Fig. 2B; data not shown). An excess of the inhibitory protein was required for inhibition, to approximate the high local concentration of the amino terminus with respect to the carboxyl terminus in the intact protein. Although deletion of the first 50 amino acids is sufficient to relieve inhibition of DNA binding, an amino-terminal fusion carrying only the first 50 amino acids of σ^{70} did not inhibit DNA binding in *trans*, even at a 50-fold molar excess. There are several plausible explanations. First, this small fusion protein may not fold correctly in vitro. Second, it may not be able to associate properly with the binding domain. Finally, the presence of GST may potentially interfere with normal function of this inhibitor.

The site of inhibition within σ^{70} was investigated by determining whether the amino-terminal inhibitory domain affects the DNA binding of region 2 or region 4 in *trans*. GST σ^{70} [506–613], carrying a portion of region 3 (14 amino acids) and the region 4 DNA-binding domain, was negatively affected by GST σ^{70} [1–100], whereas GST σ^{70} [372–466], comprised solely of the region 2 DNA-binding domain, was not (Fig. 2B). These experiments suggest that the target of the amino-terminal regulatory region of σ^{70} lies within the carboxy-terminal 107 amino acids and directly affects the DNA-binding domain of region 4.

If the amino-terminal inhibitory domain interacts solely with region 4, then GST σ^{70} [8–506], which maintains DNA-binding domain 2.4 in *cis* to the amino-terminal inhibitory domain, should still bind DNA. However, this simple prediction is not borne out completely. This fusion still binds DNA but with about a 10-fold reduction in affinity (data not shown). We consider this observation further in the discussion.

Binding of alternative σ -factors to DNA is controlled by their amino termini

The alternative σ -factors are required for transcription of specific sets of genes during various physiological or de-

Figure 2. Inhibition of DNA binding in *trans*. (A) GST σ^{70} constructions. (1) Carboxy-terminal polypeptides of σ^{70} fused to GST are referred to as binders. Fusions were constructed such that GST was present on the amino-terminal side of the amino acid stretch indicated. Binders contained both DNA-binding domains of regions 2 and 4, or one DNA-binding domain from either region 2 or region 4. (2) Amino-terminal polypeptides of σ^{70} fused to GST are referred to as inhibitors. Fusions were constructed where GST was present on the amino-terminal side of the amino acid stretch indicated. Inhibitors consisted of subsets of regions 1.1, 1.2, and the nonconserved spacer region of σ^{70} . (B) Region 1.1 inhibits DNA binding by region 4. End-labeled ptac DNA, which contains a perfect –10 and –35 consensus at 100 pM, was mixed with 25 nM GST σ [506–613] (▲), 5 nM GST σ [360–613] (□), or 5 nM GST σ [372–466] (●) and increasing amounts of GST σ [1–100] in binding buffer containing 25 mM Tris (pH 7.5), 14 mM potassium acetate, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.01% Triton X-100, and 100 μ g/ml of BSA. After 20 min at 37°C, complexes were trapped on nitrocellulose filters and washed with a 10 $\times$ volume of 25 mM Tris (pH 7.5), 0.1 mM EDTA, and 1 mM DTT. Fractional retention of labeled promoter DNA as a function of increasing ratio of inhibitor to binder is plotted. Data for GST σ [372–466] is labeled Reg 2, data for GST σ [360–613] is labeled Reg 2-4, and data for GST σ [506–613] is labeled Reg 4 to designate the DNA-binding domains present on each binder fusion. The data shown represent the average of at least two independent experiments.

developmental states of the prokaryotic cell. Each alternative σ has a distinct amino terminus that is not conserved among σ -factors, raising the possibility that amino-terminal modulation of σ function may not be ubiquitous. As a preliminary assessment of the generality of such regulation, we examined the DNA-binding properties of three alternative σ -factors with and without their amino termini. These σ -factors lack any homology to the putative inhibitory domain of region 1.1 of σ^{70} (Lonetto et al. 1992), and each is distinctly different with respect to its amino-terminal composition (Fig. 3).

σ^F from *Salmonella typhimurium*, has a short 9-amino-acid block of nonconserved sequence at its amino terminus immediately preceding the first DNA-binding domain. It is rare among the bacterial σ -factors because it is devoid of sequences corresponding to region 1.2 as well as 1.1. Full-length σ^F fused to GST bound to pflIC promoter DNA (data not shown), but the magnitude of binding was ~10-fold lower than for the analogous σ^{70} fusion. Deletion of the first 9 amino acids of nonconserved sequence did not improve binding ability. Thus, σ^F is not amino-terminally controlled for DNA binding.

σ^K from *Bacillus subtilis* has a 20-amino-acid amino-terminal extension immediately preceding conserved regions 1.2–4. This extension comprises a "pro" sequence that is cleaved in a developmentally regulated manner to generate active σ^K (Lu et al. 1990). Two derivatives of *B. subtilis* σ^K were characterized for DNA binding and amino-terminal regulation: GST σ^K [Δ pro] deleted for the 20-amino-acid pro sequence and GST σ^K [28–221] deleted for both the pro sequence and the first 27 amino acids, including region 1.2 (Fig. 4A). While full-length σ^K bound to DNA, deletions of either the pro sequence alone or the pro sequence plus region 1.2 resulted in an order of magnitude increase in tightness of binding (Fig. 4B). In this case, it appears that the amino-terminal pro sequence is acting to modulate the DNA-binding activity of σ^K but places less severe restrictions on binding than does the amino-terminal inhibitory domain of σ^{70} .

Deleting the amino-terminal amino acid sequences of σ^K also had profound effects on the stability of the poly-

Figure 3. Comparison of amino termini for σ^{70} , σ^{32} , σ^K , and σ^F . The composition of the amino terminus of the alternative σ -factors, σ^{32} , σ^K , and σ^F is shown in relation to the amino terminus of the primary σ -factor, σ^{70} . σ^{32} lacks region 1.1 but has an 18-amino-acid amino-terminal extension of nonconserved sequence. σ^K also lacks region 1.1 but carries a 20-amino-acid pro sequence at its amino terminus. σ^F has neither regions 1.1 nor 1.2.

Figure 4. Binding of GST σ^K derivatives to DNA. (A) GST σ^K fusion proteins. The amino termini of several truncated σ^K derivatives are shown in comparison to σ^{70} . Each derivative was fused at its amino terminus to GST. Full-length σ^K is 221 amino acids; σ^K [Δ pro] is a deletion of the 20-amino-acid pro sequence; σ^K [28–221] is a deletion of the pro sequence as well as the 27 amino acids of region 1.2. (B) GST σ^K fusion peptides bind to pgerE DNA. Equilibrium binding analysis was performed under the same buffer conditions as in Fig. 2B. Increasing concentrations of GST σ^K ($\bullet$), GST σ^K [Δ pro] ($\blacktriangle$), or GST σ^K [28–221] ($\square$) were added. Complexes were filtered as described in Fig. 2B. The fraction of DNA retained as a function of protein concentration is plotted. The data shown represent the average from two or more independent experiments. Error was typically $\pm 15\%$.

peptides. GST σ^K was detrimental to *E. coli* growth and was less stable than its σ^{70} counterparts. GST σ^K [Δ pro] and GST σ^K [28–221] were progressively less stable in vivo, in agreement with studies of *B. subtilis* σ^E , for which deletion of the pro sequence and other amino-terminal amino acids alters protein stability in *B. subtilis* (Peters et al. 1992).

σ^{32} , the heat shock σ -factor in *E. coli*, has 18 amino acids of nonconserved sequence at its amino terminus, which is followed by homology to σ^{70} in conserved regions 1.2, 2, 3, and 4. This σ is more closely related to σ^{70} than the other alternative σ -factors tested (Lonetto et al. 1992). Full-length σ^{32} , free or fused to GST, was unable to bind to pgroE promoter DNA. However, deletion of the first 46 amino acids, which removes both the nonconserved amino-terminal sequences and region 1.2, allowed σ^{32} to bind DNA (Fig. 5B). Thus, in analogy to σ^{70} , the ability of σ^{32} to bind to DNA is tightly controlled by

Figure 5. Binding of $\text{GST}\sigma^{32}$ derivatives to DNA. (A) $\text{GST}\sigma^{32}$ fusion proteins. The amino termini of several truncated σ^{32} derivatives are shown in comparison to σ^{70} . Each derivative was fused at its amino terminus to GST. Full-length σ^{32} is 284 amino acids. $\sigma^{32}[19-284]$ is a deletion of the 18 amino acids of nonconserved amino-terminal sequence. $\sigma^{32}[47-284]$ is a deletion of the amino-terminal 46 amino acids, including region 1.2. (B) $\text{GST}\sigma^{32}$ fusion peptides bind to pgroE DNA. Equilibrium binding analysis was performed under the same buffer conditions as in Fig. 2B. Increasing concentrations of $\text{GST}\sigma^{32}$ ($\blacktriangle$) or $\text{GST}\sigma^{32}[47-284]$ ($\bullet$) were added. Complexes were filtered as described in Fig. 2B. The fraction of DNA retained as a function of protein concentration is plotted. The data shown represent the average from two or more independent experiments. Error was typically $\pm 10\%$.

an amino-terminal inhibitory region. We attempted to further define the inhibitory region by constructing $\text{GST}\sigma^{32}[19-284]$, which leaves region 1.2 intact. However, this fusion was so unstable that we were unable to recover any intact protein, even from protease-deficient strains of *E. coli*.

All three $\text{GST}\sigma^{32}$ fusions were tested for their ability to complement *rpoH165*, an allele encoding a temperature-sensitive σ^{32} . Both $\text{GST}\sigma^{32}$ and $\text{GST}\sigma^{32}[19-284]$ restored growth at high temperature, whereas $\text{GST}\sigma^{32}[47-284]$ did not. In addition, $\text{GST}\sigma^{32}$ and $\text{GST}\sigma^{32}[19-284]$ both restored transcription from σ^{32} promoters in a strain deleted for σ^{32} . These results suggest that the first 18 amino acids of σ^{32} are dispensable but that region 1.2, contained between amino acids 18 and 46, is necessary for some aspect of σ^{32} function in vivo.

All of the various derivatives of the three alternative

σ -factors were characterized for DNA-binding specificity using equilibrium competition analysis as described previously (Dombroski et al. 1992). In all cases, specificity for promoter over nonpromoter DNA was in the range of two- to fivefold (data not shown). Thus, as seen for σ^{70} , the majority of binding that we observe is attributable to nonspecific interactions between σ and DNA.

The amino-terminal inhibitory domain of σ^{70} inhibits σ^{32}

The specificity of inhibition by region 1.1 of σ^{70} was addressed by assessing the effect of $\text{GST}\sigma^{70}[1-100]$ in *trans* on the binding of σ^{32} , σ^K , and σ^F to DNA (Fig. 6A). No inhibition was observed for $\text{GST}\sigma^F$ or $\text{GST}\sigma^K[28-221]$, as shown in Figure 6B, even at 50-fold molar excess of inhibitor over binding protein. However, a striking effect was observed for $\text{GST}\sigma^{32}[47-284]$. The magnitude of inhibition for this σ^{32} derivative was very similar to that seen with the analogous σ^{70} fusion, $\text{GST}\sigma^{70}[360-613]$ (see Fig. 2B). These results indicate that the amino-terminal amino acids of σ^{70} are able to associate with the carboxy-terminal amino acids of either σ^{70} or σ^{32} and act to mask or inhibit DNA binding. The mechanism for amino-terminal modulation of σ^{32} may be quite similar to σ^{70} despite the lack of amino acid homology in their respective inhibitory domains.

Several mutations that occur in the helix-turn-helix of region 4 alter the promoter recognition phenotypes of holoenzyme. An arginine to phenylalanine substitution at position 588 of σ^{70} affects recognition of a particular base pair in the -35 consensus (Dombroski et al. 1992). Arg-588 is conserved in σ^{70} and σ^{32} , both of which are inhibited by the amino terminus of σ^{70} , whereas neither σ^K nor σ^F carry an Arg at the equivalent position (Lonetto et al. 1992), and neither is inhibited. Thus, we tested whether Arg-588 was involved in the inhibition of binding seen for σ^{70} and σ^{32} . DNA-binding fusions were constructed corresponding to $\text{GST}\sigma^{70}[420]$, carrying amino acids 420–613 (Dombroski et al. 1992), with either wild type region 4 or the RF588 change in region 4. Both were inhibited to an equivalent extent by the amino-terminal 100 amino acids of σ^{70} (data not shown). It appears that the occurrence or absence of inhibition cannot be explained simply by this single amino acid difference.

Discussion

σ -Factors have long been recognized as the specificity subunit for interactions between RNA polymerase and promoter DNA, but the mechanism by which σ confers promoter specificity has only begun to be understood. Genetic experiments initially provided evidence for interaction between σ and the -10 and -35 consensus hexamers of promoters (Gardella et al. 1989; Siegle et al. 1989; Zuber et al. 1989; Daniels et al. 1990; Waldburger et al. 1990). Direct physical contact between σ and promoter DNA, however, could not be observed. This paradox was resolved by the demonstration that

Figure 6. Specificity of inhibition in *trans*. (A) GSTσ constructions. (1) Carboxy-terminal polypeptides fused to GST are referred to as binders. Fusions were constructed such that GST was present at the amino-terminal side of the amino acid stretch indicated. Each binder contained both DNA-binding domains from regions 2 and 4. GSTσ[360–613] is derived from σ⁷⁰, GSTσ^F from σ^F, GSTσ^K[28–221] from σ^K, and GSTσ³²[47–284] from σ³². (2) The amino-terminal 100 amino acids from σ⁷⁰ (region 1.1) were fused to GST; this construction is referred to as the inhibitor. (B) Region 1.1 of σ⁷⁰ inhibits DNA binding by GSTσ³²[47–284]. End-labeled ptac, pgroE, pfliC, or pgerE DNA at 100 pM was mixed with 5 nM GSTσ[360–613] (●), 10 nM GSTσ³²[47–284] (□), 25 nM GSTσ^F (○), or 25 nM GSTσ^K[28–221] (■), respectively. Increasing amounts of GSTσ[1–100] were used as inhibitor as described in Materials and methods. The binding buffer and assay conditions were the same as Fig. 2B. Fractional retention of labeled promoter DNA as a function of increasing ratio of inhibitor to binder is plotted. The data shown represent the average of at least two independent experiments.

truncation of σ⁷⁰ from the amino terminus generates polypeptides that are now capable of promoter-specific DNA recognition and binding, suggesting that the amino-terminal amino acids act to inhibit DNA binding (Dombroski et al. 1992). A model was proposed whereby σ⁷⁰ undergoes an allosteric interaction with core RNA polymerase, which exposes its DNA-binding domains and allows holoenzyme to engage in specific transcriptional activity (Fig. 7). Here, we report that the minimal amino-terminal inhibitory domain of σ⁷⁰ resides within conserved region 1.1. Inhibition of binding can also be

observed in *trans* and requires between 50 and 100 amino acids from the amino terminus. The fact that the isolated region 4 DNA-binding domain is sensitive to inhibition, whereas the isolated region 2 domain is not, suggests that region 4 may serve as the primary site for interaction with the amino-terminal inhibitory domain. Additionally, we show that several alternative σ-factors are able to bind to DNA and that, in some cases, binding is affected by the amino-terminal domains of these σ-factors. From these results we propose a general mechanism for modulation of σ-factor activity, where the amino-

Figure 7. Model for σ⁷⁰ function. Free full-length σ⁷⁰ is shown folded into a conformation, where the amino-terminal inhibitory domain is positioned such that it blocks the carboxy-terminal DNA-binding domains. We propose that upon interaction with the core subunits to generate holoenzyme, σ⁷⁰ undergoes a conformational rearrangement that exposes the DNA-binding domains and permits sequence-specific promoter recognition and transcription initiation.

terminal domain is responsible for determining ability to recognize and bind to promoter DNA and can contribute to the stability of certain σ -factors in vivo.

Amino-terminal inhibition of DNA binding

The primary σ -factors possess a novel region of homology from amino acids 1–100 called region 1.1. The DNA-binding characteristics of a progressive deletion series from the amino terminus of σ^{70} indicate that within the first 50 amino acids lies an inhibitory domain, which prevents DNA binding by the carboxy-terminal DNA-binding domains. The alternative σ -factors, which are required for more specialized transcriptional control, have no amino-terminal homology to region 1.1 of σ^{70} , prompting us to ask whether these σ -factors can bind to DNA. Three distinct alternative σ -factors, from three different organisms were included in this study. Each of these σ -factors controls a specific response or developmental pathway. The heat shock σ from *E. coli*, σ^{32} , is responsible for transcription of the heat shock genes upon temperature upshift. σ^{32} shares a great degree of similarity to σ^{70} , with the exception of its amino terminus. It lacks region 1.1 but has an 18-amino-acid extension of nonconserved sequence. Surprisingly, our results show that full-length σ^{32} cannot bind to DNA even though it lacks a σ^{70} -like inhibitory domain. Deletion of the first 46 amino acids allows DNA binding at a level equivalent to the σ^{70} homolog GST σ^{70} [360–613], suggesting that σ^{32} contains its own amino-terminal inhibitory domain. The amino terminus of σ^{32} is unrelated in primary sequence to the inhibitory domain of σ^{70} , but binding of σ^{32} to DNA appears to be controlled by a qualitatively similar mechanism.

σ^K from *B. subtilis* is required for expression of mother-cell-specific genes during the developmental cascade of events leading to sporulation. σ^K is unusual because it belongs to a small group of σ -factors that is synthesized with an amino-terminal pro sequence and must be processed to activate transcription (LaBell et al. 1987; Kroos et al. 1989; Stragier et al. 1989). Pro- σ^K possesses a 20-amino-acid amino-terminal extension that lacks homology to any other σ proteins. Although pro- σ^K is inactive in vivo, its fusion protein derivative GST σ^K binds to DNA in vitro. Removal of the pro sequence improves binding by an order of magnitude. Thus, the ability of σ^K to bind DNA is modulated, but not tightly controlled, by its amino-terminal domain. This increase in affinity for DNA may reflect the requirement for removal of the pro sequence in vivo to activate σ^K . The discovery that the pro sequence of σ^K governs DNA binding fits with studies conducted with the related σ^E . LaBell et al. (1987) found that pro- σ^E associates with core RNA polymerase but is unable to initiate transcription until the pro sequence is removed.

In *S. typhimurium*, the product of the *fliA* gene, σ^F , is utilized by RNA polymerase for the transcription of flagellin and all other late genes in the complex transcriptional cascade of events resulting in flagellar biosynthesis (Helmann 1991). σ^F completely lacks se-

quences corresponding to regions 1.1 or 1.2 in the σ^{70} family. As expected, GST σ^F was able to bind to DNA because it is devoid of a significant amino-terminal extension. Although the binding was weaker than for most other σ -factors, tightness of binding to promoter DNA may be an element of the mechanism for control of the flagellar operons.

In summary, both the primary and alternative σ -factors possess an inherent DNA-binding activity. This activity, however, cannot be observed under all conditions or states of the protein. Our results argue that the interaction between several different σ -factors and DNA can be regulated at the level of protein structure and/or conformation. The amino-terminal extensions of these σ -factors provide the means for inhibition or modulation through interactions with carboxy-terminal portions of the polypeptide. The details of these interactions are likely to be specific for each different σ .

Mechanism of inhibition

The target site for the inhibitory action of the amino terminus of σ^{70} may be the DNA-binding domain of region 4. Inhibition of binding in *trans* by region 1.1 was observed for fusions containing either regions 2–4 (both DNA-binding domains) or region 4 (including the carboxy-terminal 14 amino acids of region 3), but not region 2 alone. Because our model predicts that full-length σ undergoes a conformational change upon association with core to expose its DNA-binding domains, it follows from these experiments that region 1.1 is directly involved in conformational adjustments that affect DNA recognition and binding by the region 4 DNA-binding domain. This proposal must be qualified by the observation that a fusion protein consisting of amino acids 8–506 (regions 1, 2, and two-thirds of region 3) does not bind to DNA as well as region 2 alone does. This introduces the possibility that region 1 can also affect region 2 when it is in the context of its normal flanking sequences. Possibly, the inhibitory effect on region 2 is mediated by an allosteric change that must be communicated in *cis*, rather than by direct binding. Inhibition of binding by the amino terminus for full-length σ may be more complicated than predicted simply by experiments that demonstrate inhibition of binding in *trans*.

At present, we do not know the target for amino-terminal inhibition in region 4. One possibility is that the amino-terminal inhibitory region recognizes amino acids involved in base-specific contacts with the promoter. The fact that the amino-terminal inhibitory domain of σ^{70} functions to effectively inhibit DNA binding in *trans* by regions 2–4 of σ^{32} would be in accord with this idea, as the recognition helices of the two σ -factors differ by only a single amino acid. Lack of inhibition of σ^K and σ^F might reflect less homology in the recognition helix. However, the fact that the arginine to phenylalanine substitution at position 588 in the recognition helix does not relieve amino-terminal inhibition argues against, but does not rule out, this idea.

It is currently unclear whether RNA polymerase rec-

ognizes the -35 and -10 regions of the promoter simultaneously or sequentially. Footprinting studies on putative intermediates in transcription obtained at low temperature favor a sequential model, with initial recognition in the -35 region of the promoter (Kovacic 1987; Cowing et al. 1989; Meccas et al. 1991). Some studies of mutant promoters are also in accord with the idea that initial recognition resides solely in -35 contacts (Hawley and McClure 1980, 1982; Shih and Gussin 1983); however, other studies contradict this idea (Stefano and Gralla 1982; Szoke et al. 1987). Our data can be interpreted to favor the sequential mechanism. Because region 4 is responsible for -35 contacts, and if -35 contacts represent the earliest stages of promoter binding, then primary inhibition of binding at the region 4 DNA-binding domain would be a straightforward approach to modulating σ -factor activity.

Many examples of amino-terminally self-regulated proteins have been cited in both prokaryotic and eukaryotic organisms. In *B. subtilis*, the activation function of the Spo0A transcription factor is inhibited by its amino-terminal domain (Ireton et al. 1993). Likewise, the amino-terminal domain of the FixJ transcriptional activator from *Rhizobium meliloti* negatively regulates its activation function (Kahn and Ditta 1991). The CheB methylesterase is regulated in the same manner (Simms et al. 1985). In the case of the *luxR* activator of *Vibrio fischeri*, the amino-terminal domain masks the activation function of the carboxy-terminal domain unless an autoinducer is present (Choi and Greenberg 1991). The masking of a functional domain by an accessory domain has also been documented for the glucocorticoid receptor where in the absence of hormone, the steroid hormone binding domain inhibits the transcriptional activation domain (Godowski et al. 1987; Hollenberg et al. 1987). In conclusion, the σ -factors likely represent another set of members for this growing family of intramolecularly regulated macromolecules, where a conformational change must be induced to relieve the effect of the inhibitory domain.

Materials and methods

Materials

Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs and were used according to manufacturer's recommendations. AmpliTaq DNA polymerase was from Perkin-Elmer. [γ - 32 P]ATP (3000 Ci/mmol) was purchased from New England Nuclear or Amersham. Nitrocellulose filter disks (BA85) were from Schleicher & Schuell. Isopropyl- β -D-thiogalactoside was obtained from Sigma. Buffer components were from Mallinkrodt, Sigma, or Fisher Scientific. Oligonucleotides were synthesized by Genosys.

Plasmid constructions

The parent plasmid for all fusions of σ to GST was pGEX-2T (Smith and Johnson 1988). Expression of the fusion proteins is under the control of the IPTG-inducible *ptac* promoter. The *lacI^q* allele is present on the same plasmid. Insert DNA, carry-

ing specific segments of *rpoD*(σ^{70}), *rpoH*(σ^{32}), *fliA*(σ^F), or *sigK*(σ^K), was generated using the polymerase chain reaction (PCR). PCR reactions contained 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 50 pmol of each primer, 50 ng of template DNA, and 2.5 units of AmpliTaq DNA polymerase. A Coy TempCycler was programmed for 35 cycles employing 95°C for denaturation, 50–60°C for annealing, and 75°C for extension with a time of 1 min for each segment. Oligonucleotides (27–30 nucleotides in length) corresponding to the 5' and 3' ends of these segments were designed to amplify the region of interest and to incorporate *Bam*HI and *Eco*RI restriction sites, respectively. pH62 (Hu and Gross 1988) was the template DNA for the *rpoD* fusions, whereas pKH439 (gift of K. Hughes, University of Washington, Seattle) and pSK5 (Lu et al. 1990) were the templates for the *fliA* and *sigK* fusions. DNA fragments were purified by elution from agarose gels (Sambrook et al. 1989) and ligated (Maniatis et al. 1982) into the pGEX-2T vector to form an in-frame fusion. One internal deletion of σ^{70} was created by synthesizing two PCR fragments where the amino terminus of the protein was encoded by a fragment with a 5' *Bam*HI site and a 3' *Xba*I site while the carboxyl terminus was encoded by a fragment with a 5' *Xba*I site and a 3' *Eco*RI site. These two fragments were then ligated into the *Bam*HI and *Eco*RI sites of pGEX-2T in a single step. This construction results in insertion of a serine and arginine between amino acids 100 and 130 of σ^{70} . pGEX- σ^{70} [Δ 8, Δ 506–613] was constructed by digesting pGEX- σ^{70} [8] (Dombroski et al. 1992) with *Nco*I to drop out region 4 and religating.

Complementary oligonucleotides, corresponding to the *fliC* promoter were synthesized to a length of 50 nucleotides, annealed to form double-stranded DNA, digested with *Eco*RI and *Hind*III, and ligated into the *Eco*RI and *Hind*III sites of M13mp19. pAD100 carries the *groE* promoter, from -60 to $+10$, upstream of the *galK* gene. The *gerE* promoter is derived from pSC146 (gift of L. Kroos, Michigan State University, East Lansing). These plasmids served as PCR templates to generate DNA substrates for nitrocellulose filter binding.

Overproduction and purification of fusion proteins

Most of the fusion proteins were purified from the *E. coli* strain XL-1 (Stratagene); however GST σ^K [28–221] and GST σ^{70} [Δ 100–130] were sufficiently unstable in this strain that a protease-deficient strain, BL21 (*ompT*, *lon*) (Novagen, Inc.), was required to obtain reasonable amounts of intact protein. Expression of each GST- σ fusion protein was induced with 0.1 mM IPTG at 37°C for 3–4 hr. Affinity purification using glutathione-agarose beads (sulfur linkage, Sigma) has been described in detail elsewhere (Dombroski et al. 1992).

Nitrocellulose filter-binding assays

The 32 P-labeled DNA fragments used as substrates in binding assays were synthesized using PCR amplification as described previously (Dombroski et al. 1992). The binding of various σ derivatives was measured by determining the retention of protein-DNA complexes on nitrocellulose filters. These experiments were performed exactly as described previously (Dombroski et al. 1992).

Inhibition of binding *in trans* was executed by first mixing the inhibitor protein with the binding protein on ice and incubating for at least 5 min. Proteins were previously diluted appropriately in protein dilution buffer [10 mM Tris-HCl (pH 8), 10 mM KCl, 10 mM β -mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100, 0.4 μ g/ml of BSA]. Extended incubation periods did not affect the inhibition results. Nitrocellulose filter-binding assays

were then performed as described above. All data represent the average of two or more duplicate experiments.

Complementation of defective σ alleles

The ability of the fusion proteins to rescue σ mutant phenotypes was assessed by transforming the pGEX derivatives of σ^{70} into P90A5c [Calendar et al. 1988], which carries the temperature-sensitive *rpoD285* allele [Harris et al. 1978]. *rpoD285* is identical to *rpoD800*, as determined by sequence analysis. The transformants were grown at 30°C, 37°C, and 42°C in the absence of IPTG and scored for complementation of the temperature-sensitive growth phenotype. pGEX derivatives of σ^{32} were transformed into ML20035, an R40 derivative carrying a deletion of the *rpoH* allele [Kusukawa and Yura 1988], as well as a λ prophage carrying the σ^{32} promoter (pHS) [Cowing et al. 1985] upstream of *lacZ*. Transformants were scored for color phenotype on X-gal indicator plates. Additionally, the σ^{32} derivatives were tested in the strain CAG597 *rpoH165* carrying *supC* (temperature-sensitive), encoding a temperature-sensitive suppressor tRNA and the *rpoH165* amber mutation [Neidhardt et al. 1984]. The resulting strains were grown at 30°C, 37°C, and 42°C in the absence of IPTG and scored for complementation of the temperature-sensitive growth phenotype.

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