The role of the pro sequence of Bacillus subtilis $\sigma^K$ in controlling activity in transcription initiation

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The $\sigma$ (σ) subunit of prokaryotic RNA polymerase is required for specific recognition of promoter DNA sequences and transcription initiation. Regulation of gene expression can therefore be achieved by modulating the activity of the $\sigma$ subunit. In Bacillus subtilis the mother cell-specific sporulation sigma factor, $\sigma^K$, is synthesized as a precursor protein, pro-$\sigma^K$, with a 20-amino acid pro sequence. This pro sequence renders $\sigma^K$ inactive for directing transcription of $\sigma^K$-dependent genes in vivo until the pro sequence is proteolytically removed. To understand the role of the pro sequence in controlling $\sigma^K$ activity, we have constructed NH$_2$-terminal truncations of pro-$\sigma^K$ and characterized their behavior in vitro at the gerE promoter. In this report we show that the pro sequence inactivates $\sigma^K$ by interfering with the ability of $\sigma^K$ to associate with the core subunits of polymerase and also influences the interactions between holoenzyme and promoter DNA. Additionally, removal of as few as 6 amino acids (pro-$\sigma^K$Δ6) is sufficient to activate pro-$\sigma^K$ for DNA binding and transcription initiation. Surprisingly, pro-$\sigma^K$Δ6 binds to DNA with higher affinity and stimulates transcription 30-fold more efficiently than $\sigma^K$, under certain conditions.

The $\sigma$ subunit of bacterial RNA polymerase is a DNA-binding protein that confers promoter specificity to the core subunits ($\alpha$,$\beta$$\beta'$) (1, 2). $\sigma$ factors are classified into two main groups: the $\sigma^70$ family and the $\sigma^{34}$ family (3). The $\sigma^70$ family can be further divided into two major groups, the primary and alternative $\sigma$ factors. Primary $\sigma$ factors are essential proteins that are responsible for directing transcription of genes important for vegetative growth and the housekeeping functions of the cell, whereas alternative $\sigma$ factors are responsible for development and adaptive functions (3–5).

$\sigma^K$ is an alternative $\sigma$ factor from Bacillus subtilis and is a mother cell-specific member of the $\sigma$ factor cascade that controls sporulation (6–8). $\sigma^K$ is synthesized as an inactive precursor protein that is processed by proteolysis about 4 h after the onset of sporulation. Removal of the 20-amino acid pro sequence (Fig. 1) is necessary to observe $\sigma^K$-dependent transcription activity in vivo as well as in vitro (9–12). In an analysis of the effects of the amino terminus of various $\sigma$ factors on DNA binding in the absence of the core subunits, pro-$\sigma^K$ was found to bind to DNA with 10-fold lower affinity than $\sigma^K$ in vitro (13). To understand better the role of the pro sequence in controlling $\sigma^K$ activity in DNA binding and transcription initiation, we have created several NH$_2$-terminally truncated pro-$\sigma^K$ derivatives.

In this report, we describe the behavior of these $\sigma^K$ derivatives in DNA binding and holoenzyme containing the $\sigma^K$ derivatives in transcription initiation, both at the $\sigma^K$-dependent gerE promoter in vitro. We show that the pro sequence affects transcriptional activity of holoenzyme by interfering with core association by pro-$\sigma^K$ and by influencing DNA interactions, including initial binding and promoter DNA melting. Surprisingly, we discovered that a 6-amino acid deletion of the pro sequence is sufficient to activate pro-$\sigma^K$ completely, to an even greater extent than removal of the entire pro sequence, primarily through altered interactions of holoenzyme with the DNA.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England Biolabs. Calf intestinal alkaline phosphatase was from Boehringer Mannheim, and Taq DNA polymerase was from Fisher Scientific. Thrombin (T8759) was from Sigma. NTPs were from Pharmacia Biotech Inc., and [a-32P]GTP (3,000 Ci/mmol), [a-32P]CTP (3,000 Ci/mmol), and [γ-32P]ATP (3,000 Ci/mmol) were from Amersham. Purified Escherichia coli core RNA polymerase was from Epicentre Technologies (Madison, WI). Nitrocellulose filters were from Millipore. Buffer and gel components were from Sigma or Fisher Scientific. Oligonucleotides were synthesized by Bioserve Biotechnologies (Laurel, MD) or Life Technologies, Inc. Primer sequences are available upon request.

Plasmid Constructions—All $\sigma^K$ derivatives were constructed by placing portions of the sigK gene downstream of the gene encoding glutathione S-transferase (GST) in pGEX-2T (4). Oligonucleotides that incorporated EcoRI and BamHI restriction sites at the 5′- and 3′-end, respectively, were used to amplify fragments of the sigK gene from pSK5 (gift of L. Kroos) using the polymerase chain reaction (PCR). Fragments containing full-length sigK (pro-$\sigma^K$) and three successive NH$_2$-terminal deletions of sigK, removing 6 (pro-$\sigma^K$Δ6), 10 (pro-$\sigma^K$Δ10), and 20 (pro-$\sigma^K$) amino acids, were generated. PCR methods were described previously (13). Amplified DNA was inserted in-frame into the pGEX-2T plasmid using EcoRI and BamHI restriction sites. All plasmid constructs were transformed into E. coli BL21 (Novagen, Inc.) for overproduction of the GST fusion proteins.

Overproduction and Purification of Proteins—GST-$\sigma$ fusions were induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.2 mM to a liter of cells growing exponentially at 37 °C in LB plus 100 μg/ml ampicillin. Cells were harvested after 3 h of induction by centrifugation and the resulting pellet stored at −20 °C. Purification of GST-$\sigma$ fusions was performed as described by Dombroski et al. (15) except that the GST tag was removed as follows. The fusion proteins, while bound to glutathione agarose beads, were incubated with thrombin at 0.4–1.2 units/0.5 ml of beads for 2 h at 25 °C. This removes the GST tag by cleaving at a thrombin site between GST and $\sigma$ factor. Two additional amino acids, Gly and Ser, remained on the NH$_2$-terminus of the fusion protein. The abbreviations used are: GST, glutathione S-transferase; PCR, polymerase chain reaction.
The Pro Sequence of $\sigma^K$

**A**

![Diagram showing the pro-$\sigma^K$ sequence with labeled components] (Image 230x553 to 558x729)

**B**

MTGVFAALGFVVKEVFVLYSVYKNNAF

### RESULTS

The Pro Sequence Modulates DNA Binding by $\sigma^K$—Previous work revealed that pro-$\sigma^K$, in the absence of the core subunits of RNA polymerase, binds the $\sigma^K$-dependent promoter, gerE, with 10-fold lower affinity than $\alpha^K$ (13). Here we generated two successive deletions from the amino terminus of the pro sequence to determine more precisely the effect of the pro sequence on DNA binding by $\sigma^K$. Deletions of the first 6 (pro-$\sigma^K_{\Delta6}$) and 10 (pro-$\sigma^K_{\Delta10}$) amino acids of pro-$\sigma^K$ (Fig. 1B) were constructed as GST fusion proteins and purified as described under "Experimental Procedures." In this work, unlike in previous studies, we removed the GST affinity tag to eliminate any influence that GST might have on $\sigma^K$ activity.

All four pro-$\sigma^K$ derivatives were tested for DNA binding ability using nitrocellulose filter retention assays (Fig. 2) (13, 15). Pro-$\sigma^K$ bound to the gerE promoter in a manner similar to that observed in previous work (13). Pro-$\sigma^K_{\Delta6}$ and pro-$\sigma^K_{\Delta10}$ displayed higher affinity for and pro-$\sigma^K$ by approximately 100-fold and 10-fold, respectively (Fig. 2). This suggests that
the first 6 amino acids of the pro sequence are important for modulating DNA binding. Surprisingly, we observed that $\sigma^K$ retained only 20% of the input gerE promoter DNA even at the lowest salt concentrations used (3.5 mM sodium acetate). The discrepancy between this observation and data obtained previously using GST-$\sigma^K$ (13) may be attributed to the removal of the GST tag in the current experiments. We do not understand how the GST tag might have improved filter retention for $\sigma^K$ in the past, but it is most likely due to increased nonspecific interaction between the protein and the nitrocellulose filter rather than any change in the interactions between the protein and the DNA. As shown below, $\sigma^K$ is capable of effectively directing promoter binding and transcription initiation by holoenzyme.

Deletion of Only 6 Amino Acids of the Pro Sequence Activates Pro-$\sigma^K$—Transcription from $\sigma^K$-dependent promoters by $\sigma^K$-holoenzyme (E$\sigma^K$) requires the removal of the pro sequence, since it has been shown that pro-$\sigma^K$ is unable to direct transcription (10, 11). Because pro-$\sigma^K$ alone can interact with promoter DNA as shown and because deleting as few as 6 amino acids of the pro sequence increases binding affinity, we hypothesized that Epro-$\sigma^K\Delta6$ and Epro-$\sigma^K\Delta10$ may be active in transcription from the $\sigma^K$-dependent promoter gerE.

Holoenzymes containing each of the $\sigma^K$ derivatives were reconstituted in vitro using E. coli core RNA polymerase and were tested for their ability to synthesize runoff transcripts. The core subunits are very highly conserved, and others have used such heterologous holoenzymes successfully to examine transcription properties in vitro (19–21). The synthesis of a 131-nucleotide transcript from the $\sigma^K$-dependent gerE promoter on a linear DNA fragment, was monitored. Initially, we analyzed runoff transcription for all four E$\sigma^K$ derivatives at low salt (100 mM KCl). We found that pro-$\sigma^K\Delta6$ was active in directing transcription by holoenzyme and that the level of transcription was similar to E$\sigma^K$ (data not shown).

The same analysis was performed at high salt (250 mM KCl), where Epro-$\sigma^K$ synthesized an undetectable level of runoff transcript (Fig. 3A). This is consistent with the lack of significant activity reported for this enzyme in the past (10). Surprisingly, we found that Epro-$\sigma^K\Delta6$ activity was optimal at high salt concentrations, resulting in an approximately 34-fold higher level of product than that observed for E$\sigma^K$ under the same conditions (Fig. 3A). Epro-$\sigma^K\Delta10$ was also active, generating transcripts at a level similar to E$\sigma^K$.

Transcription initiation has been described as a multistep process. Initially, $\sigma$ factor associates with the core subunits of RNA polymerase to form holoenzyme (R) and then directs holoenzyme binding to the promoter (P) to form a closed complex (RP$_r$). DNA promoter melting near the $-10$ recognition site, presumably by isomerization of holoenzyme, forms the open complex (RP$_o$). Polymerase then incorporates nucleotide triphosphates to form an initiated complex (RP$_{in}$), which is capable of abortive RNA synthesis, followed by promoter escape and elongation of the transcript (22).

During initiation, RNA polymerase can remain at the promoter and generate small (2–10 nucleotides) abortive transcripts. An abortive transcription assay has been developed which exploits this ability and allows assessment of events that precede promoter clearance (23, 24). We used this assay to determine if the differences observed for the $\sigma^K$ derivatives at 250 mM KCl in the runoff assay occurred before or after promoter clearance. The conditions were identical to the runoff assays except that a dinucleotide (GpA) primer and [$\alpha$-32P]CTP were used, rather than all four NTPs, to limit transcription to a three-nucleotide product (GpApC). All of the holoenzymes were capable of synthesizing abortive transcripts even at 250 mM KCl (Fig. 3B). The fact that Epro-$\sigma^K$ and Epro-$\sigma^K\Delta10$ generated abortive transcripts more efficiently than runoff transcripts (compare Fig. 3A with Fig. 3B) implies a defect in promoter clearance under the conditions used.

Analysis of Holoenzyme-Promoter Complexes Using DNase I Footprinting—The ability of holoenzyme to bind to DNA, and the boundaries of the complexes on the DNA, can be assessed using DNase I footprinting (25, 26). We used this technique to analyze the E$\sigma^K$ derivatives for interactions with the gerE promoter.

All four E$\sigma^K$ derivatives were able to produce specific protein-promoter DNA complexes (Fig. 4A). Protection of the gerE promoter extended from +20 to −60 relative to the +1 start site. The length of the footprint suggested that these complexes
were similar to either the extended closed (RPc) or the open (RPo) complexes, characterized for EoK (22). Epro-αKΔ6 showed a stronger affinity for the DNA than either Epro-αK or EoK, at 100 mM KCl, given the overall better protection of the gerE promoter. Also, Epro-αKΔ6 created hypersensitive bands between the −10 and −35 promoter recognition sites (−21 and −22), which were not visible in the Epro-αK and EoK footprint, indicating that pro-αKΔ6 may be distorting the DNA in that region. Epro-αKΔ10 protected the gerE promoter similarly to Epro-αKΔ6, but with a reduction in the intensity of the hypersensitive sites.

Many EoK-DNA open (RPo) complexes are stable to challenge by heparin, a polyanionic competitor, whereas closed (RPc) complexes are not (18, 27–29). We used heparin as a tool to assess the stability of the complexes to KMnO4 treatment, which modifies pyrimidines (particularly thymines) on single-stranded DNA. Modification of single-stranded pyrimidines by KMnO4 and cleavage of the DNA by piperidine. We were unable to detect any DNA cleavage due to KMnO4 sensitivity for Epro-αK-gerE complexes at either salt concentration (100 mM or 250 mM), suggesting that the Epro-αK complex observed in DNase I footprinting at low salt are primarily extended closed complexes (Fig. 5). For EoK, Epro-αKΔ6, and Epro-αKΔ10, we observed that approximately equal amounts of DNA were susceptible (50%) at low salt, indicating that these three proteins form RP, equivalently (Fig. 5A). Three bands were resolved in these experiments, corresponding to thymines −9, −6, and −4 of the gerE promoter. A band appearing at cytosine −1 was visible but at much lower levels. As the salt concentration was increased, EoK open complexes decreased (8% cleaved) (Fig. 5B) in agreement with the loss of DNase I protection as a function of salt concentration. Epro-αKΔ6 and Epro-αKΔ10 maintained formation of RPc at high salt conditions.

FIG. 4. DNase I footprinting. Panel A. EoK derivatives were analyzed for gerE promoter DNA interaction using DNase I footprinting at 100 mM KCl. The −10 and −35 promoter elements are indicated by brackets, the hypersensitive bands observed for mutants and the extent of protection, relative to the start point of transcription, are indicated by arrows. Panel B. Epro-αKΔ6 was analyzed for gerE promoter DNA interaction using DNase I footprinting at 60–200 mM KCl, as indicated.

FIG. 5. KMnO4 footprinting. EoK derivatives were analyzed for the formation of open complexes using modification of single-stranded pyrimidines by KMnO4, and cleavage of the DNA by piperidine. Panel A, open complexes were analyzed at 100 mM KCl. The positions of cleavage within the promoter region are indicated by arrows. The bar plots indicate the fraction of DNA cleaved for each derivative normalized to EoK. Panel B, same as panel A but at 250 mM KCl.
because cleavage under high salt conditions was similar to that observed at low salt (60 and 40%). We found that for both Epro-αKΔ6 and Epro-αKΔ10, another cleavage site appeared at cytosine −21. This band corresponds to the hypersensitive site found in the DNase I footprint experiments and indicates that base pairing in this region has been disturbed.

Characterization of RPinit—Previous work has shown that formation of the open complex by EραK on the APk promoter in the presence of a subset of initiating nucleotides, was stable to a 0.8 M NaCl wash (16, 18). This stabilization is caused by the formation of RPinit, which has incorporated a few nucleotides but has not left the promoter and is thus more resistant to 0.8 M NaCl than RPo. This 0.8 M NaCl challenge assay can be used to determine the relative extents of RPo and RPinit formation. The αK derivatives were incubated with 32P-labeled gerE promoter with and without NTPs (GTP, ATP, and CTP). The mixtures were filtered through nitrocellulose, and the filters were washed with a buffer containing 0.1 or 0.8 M NaCl.

All four EραK-DNA complexes were resistant to a 0.1 M NaCl wash both with and without the addition of NTPs (Table I). As expected, all of the complexes were destabilized when the filters were washed with 0.8 M NaCl. In the presence of NTPs, all four EραK-DNA complexes became more resistant to the 0.8 M NaCl wash, indicating that they are forming RPinit complexes (Table I) although with varying efficiency. Because RPo is in rapid equilibrium with RPinit, a retention of approximately 50% of the input DNA is expected for those polymerases forming RPinit efficiently. The resistance to 0.8 M NaCl was weakest for Epro-αKΔ6-DNA complexes and strongest for Epro-αKΔ6-DNA complexes. About half of the Epro-αKΔ6-gerE complexes were resistant to the high salt wash, indicating equal distribution between RPo (not resistant) and RPinit (resistant). Only 25% of the EραK and Epro-αKΔ10 complexes were resistant to the 0.8 M NaCl wash, implying that the equilibrium favors RPo over RPinit. For EραKΔ6, less than 10% of the complexes were resistant to the high salt wash. The results of the NTP stabilization assays are in good agreement with the transcriptional behavior of these enzymes.

The Pro Sequence Inhibits Core Binding by Pro-αK—One explanation for weak protein-DNA interactions by pro-αK is reduced ability to associate with the core subunits of RNA polymerase. To test this, we used the KMMo4 footprinting assay at 250 mM KCl. We found that Epro-αKΔ6 forms KMMo4-sensitive complexes very well under these conditions, whereas EραKΔ6 and EραK do not. We reasoned that if pro-αK or αK could compete with pro-αKΔ6 for core association, then the amount of cleaved single-stranded DNA in a KMMo4 footprint would decrease when either pro-αK or αK was added. On the other hand, if both failed to compete for core binding then cleavage of the DNA would be the same as when no competitor was added. Similar methodology has been used previously to assess core binding (16).

Equimolar amounts of either αK or pro-αK were mixed with pro-αKΔ6 and then incubated with the core subunits. Holoenzyme mixtures were then added to 32P-labeled gerE promoter DNA and incubated for 30 min at 37 °C. Samples were then subjected to KMMo4 footprinting as described above. αKΔ6 was able to compete efficiently with pro-αKΔ6 for core binding as shown by a reduction in the amount of cleaved DNA by half compared with the amount of cleaved DNA in Epro-αKΔ6 only samples (Fig. 6). This suggests that pro-αK and pro-αKΔ6 have a similar affinity for core. On the other hand, we found that pro-αK was unable to compete with pro-αKΔ6. Cleavage of DNA in these competition assays was the same as that observed for pro-αKΔ6 alone. A concentration series with increasing ratios of pro-αK to pro-αKΔ6 was performed to determine relative affinity of these sigmas for core. We found that pro-αK was unable to compete with pro-αKΔ6 even when the ratio of pro-αK to pro-αKΔ6 was 10:1 (data not shown).

**DISCUSSION**

Analysis of the mechanism of transcription initiation by E. coli RNA polymerase containing σ70 has identified multiple intermediate steps in the process of open complex formation (22). The σ subunit has been implicated in recognition of the −10 and −35 promoter elements (3, 5) as well as in isomerization and promoter melting (16, 30–32). Homology among both the primary and alternative σ factors in regions 2 and 4, which recognize the −10 and −35 elements, suggests that their modes of DNA interaction may be similar (3). However, the amino terminus of σ70, which inhibits DNA binding by σ factor alone and is required for efficient open complex formation during initiation (13, 16), is poorly conserved or absent in many alternative σ factors implying fundamental differences in some modes of DNA interaction.

![Fig. 6. KMMo4 core binding competition assay.](image-url) Equal amounts of pro-αKΔ6 and either pro-αK or αK were mixed and allowed to compete for binding to a fixed amount of core RNA polymerase. Complexes formed on linear pgerE DNA and were probed for strand melting using KMMo4 modification and piperidine cleavage at 250 mM KCl. The positions of cleavage within the promoter region are indicated by arrows. The bar plots indicate the fraction of the total input DNA cleaved.

**TABLE I**

| NTP stabilization assay | pro-αKΔ6 | αKΔ6 | pro-αKΔ10 | αKΔ10 |
|-------------------------|---------|------|----------|------|
| −NTPs 0.1 M NaCl        | 0.64    | 0.62 | 0.66     | 0.63 |
| −NTPs 0.8 M NaCl        | 0.00    | 0.03 | 0.06     | 0.04 |
| +NTPs 0.1 M NaCl        | 0.62    | 0.63 | 0.62     | 0.63 |
| +NTPs 0.8 M NaCl        | 0.06    | 0.15 | 0.33     | 0.12 |
aspect of σ factor function and hinting at a role for the NH₂ terminus in specifying behavior that is characteristic of a particular σ factor. In this report, we find that the NH₂-terminal pro sequence of σK from B. subtilis controls core binding by pro-σK and affects DNA interactions by Epro-σK.

Characterization of Transcription Initiation in Vitro by EσK—DNase I footprinting of EσK on the gerE promoter demonstrated protection extending from −60 to +20 relative to the start site of transcription. The extent of this footprint is similar for polymerases carrying other members of the σ70 family (25, 26, 33). For Eσ70 on many promoters, these footprints are indicative of heparin-stable open complexes. The EσK-gerE complexes were also open complexes as demonstrated by KMnO₄ footprinting, however they were unstable to low levels of heparin even in the presence of initiating nucleotides. Additionally, only 25% of EσK-initiated complexes (RP_init) were stable to 0.8 M NaCl, whereas 50% of Eσ70-∆P₆ complexes are typically stable (16, 18).

Taken together, our evidence suggests that at least two different EσK-DNA complexes (RP₀ and RP_init) are generally less stable than their Eσ70 counterparts. Because σK lacks region 1.1, its structure may be distinct from sigma factors that possess region 1.1, and this may alter the type or strength of interactions that σK makes with the core subunits and with DNA during initiation. Because σK-dependent transcription is utilized only transiently during sporulation, perhaps stability of the complexes is not crucial for adequate levels of mRNA synthesis. Dissociable open and initiated complexes have also been observed for B. subtilis EσK at some promoters (34).

Role of the Pro Sequence of σK in Controlling Activity—B. subtilis has evolved pro sequences to regulate the activity of two different σ factors, σK and σE. Both pro-σK and pro-σE are regulated developmentally by requiring proteolytic removal of the pro sequences for activation. In the case of σK, the pro sequence is also required for accumulation of pro-σK in vivo (35). This is not the case for pro-σE, since σE can be expressed stably without pro, and it retains full activity in vivo (10, 12). This suggests that pro is not necessary for proper folding of σK (35). Previous work had indicated that the pro sequence decreases the affinity of σK for DNA (13). However, it was still unclear whether pro-σK was able to bind to core, but the resulting holoenzyme was unable to recognize the promoter or whether the pro sequence was preventing core association.

Here we observed that the pro sequence affects at least three aspects of σK activity: core binding, DNA binding, and DNA melting. Pro-σK was unable to compete effectively with pro-σKΔ6 for binding to core RNA polymerase even when present in 10-fold excess. In the control experiment, σK lacking the pro sequence was able to compete equally with pro-σKΔ6, despite slightly lower affinity of EσK for DNA. Thus, we believe that this competition assay does reflect accurately the differential affinity of the σ factors for core. This corresponds with recent analysis of the distribution of pro-σK and σK in vivo, where the majority of pro-σK fractionates with the cell membrane and not with the core subunits of polymerase, and the majority of σK is associated with core. This work proposes that the pro sequence masks the core binding activity of σK and targets pro-σK to the membrane to interact with the processing machinery. This is in agreement with the results presented here.

The pro sequence is known to negatively affect DNA binding by σK in the absence of core (15). Here we see that whereas Epro-σK and EσK appear to have similar DNA binding affinity in DNase I footprinting experiments, Epro-σK is defective in melting the DNA, even in the presence of NTPs, as judged by KMnO₄ susceptibility. Thus, although pro-σK binds more weakly to core, once holoenzyme is formed it can bind to the promoter, but the equilibrium is shifted toward the closed complex.

The regions of σ factor implicated in core binding include region 2.1 of σE (36), region 2.2 of σE (37), and region 3.2 of σE (38). Because σK lacks region 3.2 (Fig. 1A), it seems likely that region 2 plays an important role in mediating core binding. One model to explain the function of the pro sequence of σK is down-regulating σK activity in vitro is that the pro sequence participates in intramolecular interactions with another region of the σ subunit. The hydrophobic nature of the pro sequence would suggest that it is not solvent-exposed and might interact with another hydrophobic region of σK, which could mask the core binding domain and/or the DNA binding domain or more generally perturb the structure of region 2 to affect both core binding and DNA interactions. The pro sequence also appears to interact peripheral with some component of the membrane in vivo, and removal of pro may cause a conformational change that permits σK to interact with core and prevents it from interacting with the membrane.

Pro-σKΔ6 and Pro-σKΔ10—We constructed two σK derivatives that retained only a portion of the pro sequence, pro-σKΔ6 and pro-σKΔ10. These deletion derivatives displayed some novel properties in DNA binding and transcription initiation. Most notably pro-σKΔ6 was hyperactive for DNA binding in the absence of the core subunits and showed transcriptional behavior similar to σK at lower salt concentrations. This suggests that the entire pro sequence is necessary for inhibition of σK activity and that removal of only a few amino acids is all that is required for activating pro-σK. Epro-σKΔ6 and Epro-σKΔ10 both bind more tightly to DNA than Epro-σK. This implies that either the lack of the first 6–10 amino acids or the presence of the remaining 14 residues of the pro sequence can modify the interactions of holoenzyme with the promoter.

The altered interactions between Epro-σKΔ6 and the gerE promoter were most evident in transcription, where at 250 mM KCl a 34-fold increase in runoff transcripts was observed compared with EσK. The nature of the interactions contributing to this effect was easily observed in DNase I footprinting. Pro-σKΔ6 appears to bind very tightly to the promoter, particularly in the upstream regions, at low concentrations of salt, and this may hinder initiation, which is consistent with the lower level of transcription observed at 100 mM KCl. At the salt concentration is increased, the Epro-σKΔ6-DNA footprint shifts forward toward +20 relative to the +1 start site, relinquishing upstream contacts and establishing downstream contacts. This repositioning of Epro-σKΔ6 on the promoter may permit more efficient transcription initiation, perhaps through an improvement in −10 interaction or an increase in the rate of promoter escape. Interestingly, Epro-σKΔ6 and Epro-σKΔ10 both caused a noticeable distortion in the promoter DNA between the −10 and −35 element, which is susceptible to KMnO₄ modification. Thus, the binding of these polymerases is strongly influencing the DNA structure in the promoter region. A similar distortion has been observed for Eσ70 at the rrnBP1 promoter (17).

In summary, B. subtilis has evolved a 20-amino acid pro sequence that functions to regulate σK activity during the cascade of events leading to spore formation. From our experiments it is clear that removal of as few as 6 amino acids can activate σK, yet the level of activity obtained for Epro-σKΔ6 may be inappropriate for the level of gene expression needed at this point in development. In the process of spore formation, the level and timing of gene expression are critical for spore maturation. From an evolutionary standpoint then, it appears...
that cleavage of pro-$\sigma^K$ to remove 20 amino acids results in an appropriately active version of $\sigma^K$.

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