J. Mol. Biol. (1995) **249**, 743–753

JMB

The *Bacillus subtilis* Flagellar Regulatory Protein σ^D: Overproduction, Domain Analysis and DNA-binding Properties

Ya-Fen Chen and John D. Helmann*

Section of Microbiology
Cornell University, Ithaca
New York 14853, USA

**Corresponding author*

Flagellar biosynthesis requires an alternative sigma (σ) subunit of RNA polymerase to allow recognition of the promoters for flagellin and other late genes of the flagellar regulon. We have now overproduced and characterized *Bacillus subtilis* σ^D: the prototype of the σ^{28} family of flagellar σ factors. Limited protease digestion studies indicate that σ^D contains an amino-terminal domain, comprising conserved regions 1.2 and 2, and a carboxyl-terminal domain containing conserved regions 3.2 and 4. The protease-sensitive region between these two domains correlates with a region of very low sequence conservation among bacterial σ factors. Unlike the primary σ factor, σ^D binds to DNA. In non-denaturing polyacrylamide gel electrophoresis the σ^D–DNA complex has an apparent equilibrium dissociation constant of 1 μM. Binding of σ^D to the promoter for flagellin, $P_{D\text{-}6}$, appears to lead to an altered DNA structure near the –35 and –10 recognition elements as detected by DNase I footprinting and by the enhanced reactivity of several bases to dimethylsulfate.

Keywords: sigma; RNA polymerase; transcription; regulation

Introduction

Promoter recognition in bacteria requires the association of RNA polymerase with one of a family of σ specificity factors (Gross *et al*., 1992; Helmann & Chamberlin, 1988; Lonetto *et al*., 1992; Helmann, 1994). The resulting holoenzyme participates in the initial steps of transcription up to and including the formation of short RNA transcripts. Then a large conformational changes ensues which ejects σ factor and establishes RNA polymerase as a stable ternary complex with the DNA template and nascent RNA transcript (Krummel & Chamberlin, 1989; Leirmo & Record, 1990).

The requirement for a σ factor for specific binding of RNA polymerase to DNA, together with the observation that different σ factors confer different promoter selectivity, suggests that σ factors bind directly to DNA (Losick & Pero, 1981). This hypothesis is supported by genetic studies which have demonstrated allele-specific suppression of promoter mutations by specific changes in the corresponding σ factor (Daniels *et al*., 1990; Gardella *et al*., 1989; Siegele *et al*., 1989; Waldburger *et al*., 1990; Zuber *et al*., 1989). These mutations identified two conserved regions, designated 2.4 and 4.2, which specify recognition of the –10 and –35 promoter elements, respectively. In addition, truncated derivatives of the primary σ^{70} factor of *Escherichia coli* bind specifically to promoter DNA *in vitro* (Dombroski *et al*., 1993, 1992). This has led to the hypothesis that the latent DNA-binding activity of σ^{70} (and related family members) is sterically blocked by interaction of the DNA-binding surface with conserved region 1.1. Finally, σ factors of the σ^{54} (*rpoN*) family, which are not detectably related to the σ^{70} family proteins, can bind to some promoter sites in the absence of core RNA polymerase (Buck & Cannon, 1992; Merrick, 1993).

The *Bacillus subtilis* σ^D (previously σ^{28}) protein was originally detected as a novel transcriptional activity in some preparations of purified *B. subtilis* RNA polymerase which could activate an uncharacterized promoter site on phage T7 DNA (Jaehning *et al*., 1979). Subsequent studies revealed that this transcription required a 28 kDa RNA polymerase subunit (σ^D) and was independent of the primary σ factor (Helmann *et al*., 1988b; Wiggs *et al*., 1981). RNA polymerase containing σ^D ($E\sigma^D$) specifically transcribes promoters with a unique consensus se-

Abbreviations used: DMS, dimethylsulfate; GST, glutathione-*S*-transferase; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; HPLC, high pressure liquid chromatography.

0022–2836/95/240743–11 $08.00/0

quence, $TAAAN_{15}GCCGATAT$, distinct from that recognized by all other *B. subtilis* σ factors (Gilman *et al*., 1981; Helmann, 1991). Mutants lacking σ^D are non-flagellate and highly filamentous (Helman *et al*., 1988*a*), a phenotype due to a lack of transcription of several flagellar genes and at least two autolysins (Chen & Helmann, 1994; Kuroda & Sekiguchi, 1993; Lazarevic *et al*., 1992; Margot *et al*., 1994; Márquez *et al*., 1990; Mirel & Chamberlin, 1989; Mirel *et al*., 1994). The presence of promoter elements resembling the σ^D consensus in front of flagellin genes from diverse organisms suggests that σ^D-like proteins (referred to as σ^{28} factors) are present in a broad range of bacteria (Helmann, 1991). In general these proteins appear to control synthesis of flagellin and other late functions of the flagellar regulon, but in some cases σ^{28} may be involved in other processes such as endospore formation in *Streptomyces coelicolor* (Chater *et al*., 1989).

The *B. subtilis* σ^D protein has now been overproduced and purified from *E. coli* in a form active for recognition of appropriate promoter sequences. Limited protease cleavage studies indicate that this protein contains two relatively stable domains which correspond to the most highly conserved regions of bacterial σ factors. In this paper, we demonstrate that σ^D binds specifically to promoter DNA in the absence of core enzyme, as evidenced by formation of a discrete complex in a gel mobility shift assay. This binding leads to specific DNase I protections and enhancements and alters the pattern of dimethylsulfate reactivity in the –35 and –10 recognition elements.

Results

Overproduction and purification of σ^D

In a previous study, we expressed σ^D in *E. coli* using plasmid pYFC-6 (Chen & Helmann, 1992). A low level of expression was achieved as judged by immunoblot analysis (data not shown) and by the ability of σ^D to partially complement a mutation in the homologous regulatory protein of *E. coli*, σ^F (Chen & Helmann, 1992). We have now subcloned *sigD* into pET11c to generate pYFC-11. This plasmid allows tightly regulated production of σ^D since transcription of both the T7 RNA polymerase gene (present in the chromosome on the λ DE3 prophage) and the *sigD* gene (on pYFC-11) is blocked by the *lac* repressor protein (Studier *et al*., 1990).

To purify the overproduced σ^D we have modified the procedure developed initially for *E. coli* σ^{70} (Gribskov & Burgess, 1983) and later adapted for the major σ factor, σ^A (Chang & Doi, 1990; Juang & Helmann, 1994). In this procedure, σ^D was denatured from inclusion bodies using 6 M guanidine hydrochloride and renatured by dilution. The renatured protein was recovered by adsorption to QAE-Sepharose and elution with a salt gradient. Final purification was achieved by FPLC Superdex-75 sizing chromatography (Figure 1). In some cases, heparin-Sepharose chromatography was also used. The yield after the first QAE-Sepharose column was >20 mg/l, but subsequent steps were limited by the capacity of the Superdex-75 sizing column. During the course of these experiments it was noted that purified σ^D slowly precipitated at concentrations greater than 1 mg/ml.

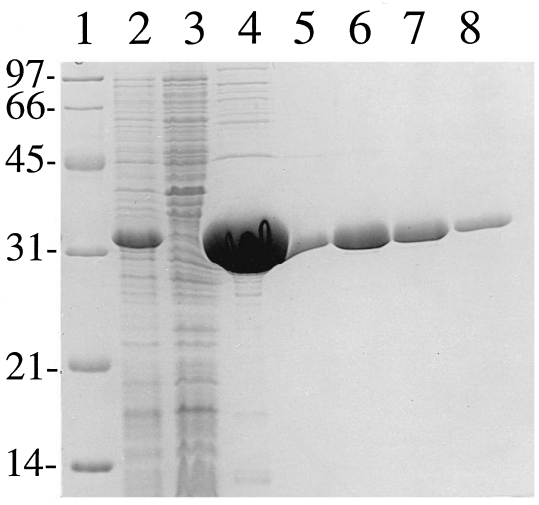

Figure 1. Purification of σ^D. SDS/PAGE 12.5% analysis of fractions from the purification of σ^D. Lane 1, molecular weight standards ($M_r \times 10^{-3}$). Lanes 2 and 3, total proteins from *E. coli* BL21/DE3 (pYFC-11) two hours after (lane 2) and at the time of (lane 3) induction with 0.4 mM IPTG. Lane 4, 8 μl of σ^D peak fraction after chromatography on Q-Sepharose. Lanes 5 to 8, 8 μl of σ^D fractions from FPLC-Superdex-75 sizing chromatography.

Physical characterization of σ^D

The identity of the overproduced σ^D was confirmed by amino-terminal sequencing of the first 20 amino acid residues which matched exactly those predicted from the DNA sequence (Helmann *et al*., 1988a). The molecular mass of σ^D was measured as 29,533 daltons by mass spectrometry which is within experimental error (approximately 1%) of the theoretical value of 29,449 daltons. The amino acid composition was also consistent with that derived from the revised gene sequence (Márquez-Magaña & Chamberlin, 1994): 12 of 17 amino acid residues determined following complete acid hydrolysis (which destroys Trp and hydrolyzes Gln to Glu and Asn to Asp) were measured within an error of ± 1.

We have used gel exclusion chromatography to determine if σ^D is a monomer in solution. σ^D eluted from an FPLC Superdex-75 column with an apparent molecular mass of 36 kDa (Figure 2), which is slightly larger than the monomer mass (29.5 kDa) but much less than that predicted for a dimer (59 kDa). Since the observed peak was quite sharp, and was not affected by protein dilution, we believe that σ^D is a monomer rather than a rapidly equilibrating mixture of monomers and dimers. Consistent with this hypothesis, both *E. coli* σ^{70} (Gill *et al*., 1991) and *B.*

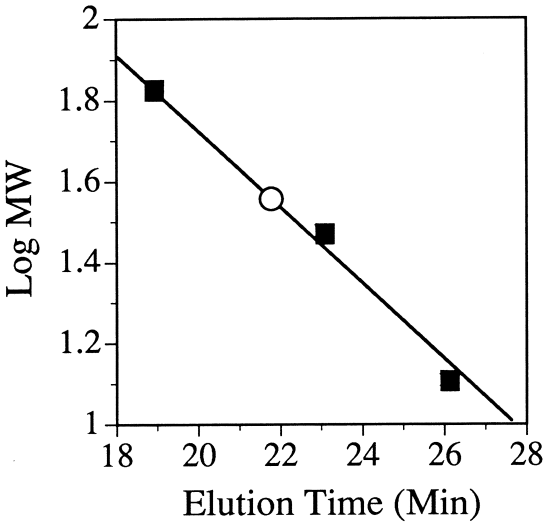

Figure 2. σ^D is a monomer in solution. An FPLC HR 10/30 Superdex-75 column (25 ml) was run at 30 ml/h in TED buffer with 0.15 M NaCl. Molecular weight standards (Sigma Chemical Co., St. Louis, MO) were: bovine serum albumin (M_r = 66,000); bovine erythrocyte carbonic anhydrase (M_r = 29,000); cytochrome *C* (M_r = 12,400) (indicated by (■)). σ^D (1.87×10^{-4} M) eluted with M_{app} = 36,300 (○).

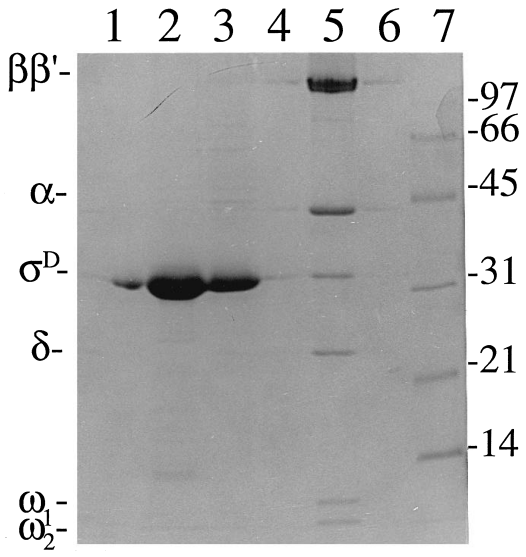

Figure 3. Purified σ^D binds core RNA polymerase. Purified core RNA polymerase (from a *sigD* mutant strain; see Materials and Methods) and σ^D were incubated and then fractionated on a FPLC-Superdex-75 gel filtration column as for Figure 2. Each fraction was analyzed by SDS/PAGE 12.5% and staining with Coomassie blue. Lanes 1 to 3, σ^D peak fractions (21 to 23); Lanes 4 to 6, peak fractions (15 to 17) of RNA polymerase illustrating the co-elution of σ^D protein. Lane 7, molecular weight standards ($M_r = 10^{-3}$). The positions of the RNA polymerase subunits are shown to the left of the Figure.

subtilis σ^A (F. López de Saro & J. D. H., unpublished results) eluted relatively early during gel exclusion chromatography. In the case of σ^{70}, it has been confirmed that the protein is a monomer in solution using independent physical techniques (Gill *et al*., 1991). The slightly aberrant elution of σ^D during gel exclusion chromatography could be due to an asymmetric shape, a high level of hydration, or to partial unfolding of the protein under these conditions.

Binding of σ^D protein to core RNA polymerase

We used gel exclusion chromatography to verify that the overproduced σ^D protein could bind to core RNA polymerase (Figure 3). For this and subsequent experiments, core enzyme was purified from a *sigD* disruption strain and, as expected, lacked detectable σ^D protein. However, this core preparation does contain the δ and ω polypeptides. Even when σ^D protein is added in large molar excess relative to core enzyme, the stoichiometry of binding appears to be less than one σ^D monomer per core (0.4 to 0.5) as judged by scanning densitometry of Coomassie-stained gels similar to those of Figure 3. This may be due to a fraction of core enzyme which is inactive or to differences in the affinities of the various proteins for Coomassie blue. Since σ^D containing RNA polymerase purified from *B. subtilis* is typically only 1 to 2% saturated with σ (Helmann *et al*., 1988b), holoenzyme prepared using the overproduced σ^D will greatly facilitate structural studies of RNA polymerase–promoter complexes.

Transcription activity of σ^D protein

We have analyzed the ability of reconstituted σ^D holoenzyme to initiate transcription from several cognate promoter sites. Using plasmid pYFC-12, which contains the *B. subtilis* flagellin promoter ($P_{D\text{-}6}$), addition of σ^D to core enzyme stimulated transcription between 60-fold (on linear DNA) and 150-fold (on supercoiled plasmid) compared to core alone (data not shown). In run-off transcription assays, core programmed with σ^D correctly recognized the σ^D-dependent promoters of the *flgM* operon ($P_{D\text{-}1}$; Mirel *et al*., 1994), *hag* ($P_{D\text{-}6}$; Mirel & Chamberlin, 1989), the *fliD* operon ($P_{D\text{-}8}$; Chen & Helmann, 1994) and *cheV* ($P_{D\text{-}cheV}$; Fredrick & Helmann, 1994) (Figure 4). Qualitatively, the hierarchy of *in vitro* promoter strength corresponded well with the hierarchy of *in vivo* strength: $P_{D\text{-}6}$ is the strongest and $P_{D\text{-}cheV}$ is the weakest of these four promoters. RNA polymerase reconstituted with overproduced and renatured σ^D protein has been demonstrated to accurately initiate transcription from the *cheV* promoter (Fredrick & Helmann, 1994). In addition, reconstituted σ^D holoenzyme bound specifically at several promoter sites as judged by DNase I protection studies (Fredrick *et al*., 1995; and see below).

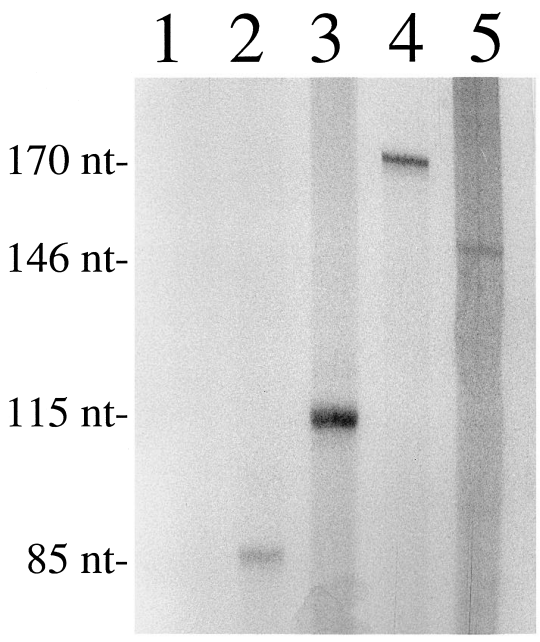

Figure 4. Reconstituted Eσ^D RNA polymerase specifically recognizes cognate promoters. Reconstituted Eσ^D RNA polymerase was used to transcribe four different promoters in a run-off transcription assay as described in Materials and Methods. The templates were: lane 1, pUC18 linearized with *Eco*RI (negative control); lane 2, pJH102T (P_{D-1}) linearized with *Dra*I; lane 3, pYFC-12 (P_{D-6}) linearized with *Ssp*I; lane 4, pYFC-13 (P_{D-8}) linearized with *Hin*dIII; and lane 5, pKF1 (P_{cheV}) linearized with *Xmn*I. The sizes of the transcripts in lanes 2 to 5 are 85, 115, 170 and 146 nucleotides (nt), respectively, as indicated on the left.

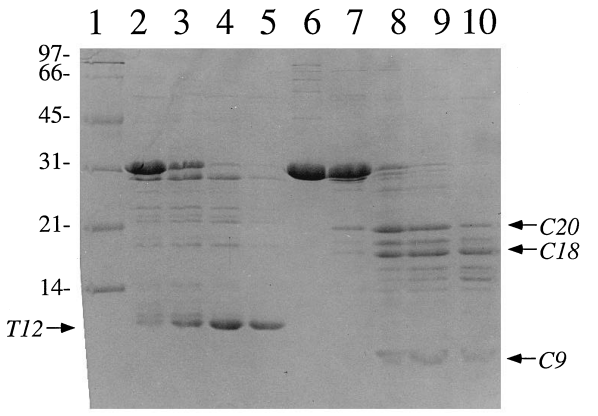

Figure 5. Partial proteolysis of σ^D. SDS/PAGE 20% analysis of tryptic (lanes 2 to 5) and chymotryptic (lanes 7 to 10) digestions of σ^D. Reactions contained 10 μg of σ^D and protease as indicated. Lane 1, molecular weight standards ($M_r \times 10^{-3}$). Lanes 2 to 5, digestion with 10, 25, 50, or 100 ng of trypsin, respectively. Lane 6, 10 μg of intact σ^D (QAE fraction; Figure 1). Lanes 6 to 9, digestion with 20, 50, 80, or 120 ng of chymotrypsin, respectively.

Domain structure as judged by partial proteolysis

We incubated purified σ^D with trypsin or chymotrypsin to analyze the kinetics of protease attack. In the presence of 20% glycerol, several discrete intermediates appeared which suggested that some sites were cleaved much more rapidly than others (Figure 5). In contrast, if glycerol was omitted, the protein was rapidly cleaved into small fragments, which suggests that glycerol may be required to maintain a compact folded state.

We determined the positions of protease attack by amino acid sequencing and molecular mass determination (by MALDI-TOF MS) for several of the proteolytic products (Table 1). These data identify two regions of preferential protease attack: one at the end of conserved region 2 and the other in the relatively non-conserved region 3.1-3.2 boundary (Lonetto *et al*., 1992, 1994). Polypeptides containing region 2 (T12 and C18) and region 4 (C9) were the most stable products of the digestion reactions, indicating that these two regions, involved in –10 and –35 recognition, respectively, are indeed stable domains. These data suggest that trypsin preferentially degrades σ^D from the carboxyl terminus to leave a stable 12 kDa product while chymotrypsin degradation follows pathways leading to a relatively stable amino-terminal product (C18), a carboxyl-terminal product (C9), or both (Figure 6).

Specific DNA-binding by σ^D

It has been reported that truncated derivatives of *E. coli* σ^{70} lacking region 1.1 are able to bind DNA specifically, as judged by nitrocellulose filtration in the presence of specific and non-specific competitor DNA (Dombroski *et al*., 1992). DNA binding has also been observed with truncated derivatives of σ^{32} (Dombroski *et al*., 1993) and *B. subtilis* σ^A (our unpublished results). Since σ^D lacks conserved region 1.1, it is predicted to have intrinsic DNA-binding ability. To test this prediction, the ability of σ^D to form stable complexes with a DNA fragment spanning the flagellin promoter region was investigated. The addition of increasing concentrations of σ^D led to the formation of a discrete band (complex I) in an electrophoretic mobility shift assay with an apparent equilibrium dissociation constant of 1 μM (Figure 7). At higher protein concentrations, complex I disappeared and more slowly migrating species appeared (complexes II and III). These presumably represent the presence of additional σ^D monomers binding to the DNA fragment.

We have performed several experiments to assess the specificity of the σ^D–DNA interaction. The same specific complex was observed in a mobility-shift experiment performed using 20 μM σ^D and a >100-fold excess (by weight) of non-specific (plasmid) DNA as a competitor (data not shown). In control experiments with a DNA fragment lacking a σ^D promoter, σ^D failed to form a discrete complex and the apparent binding constant for DNA was reduced at least several-fold. These results are consistent with studies of DNA-binding by GST-σ^{70} fusion proteins lacking region 1.1 which demonstrate a fivefold difference in the ability of specific and non-specific

Table 1. Characterization of σ^D proteolytic fragments

Protein	Observed N-terminal sequence	Size (Da) by MALDI-TOF MS	Assignment[a] (calc. size, Da)
σ^D	MQSLNYEDQVLWTRWKEWKD	29,533	1-254 (29,449)
T27	MQSLN	(ND)[b]	1-240 (27,811)
			1-233 (27,017)
T22	MQSLN	(ND)	1-188 (21,880)
			1-198 (22,990)
T19	MQSLN	(ND)	1-175 (20,356)
			1-160 (18,592)
T12[c]	MQSLN	12,123	1-102 (12,056)
C25	(ND)	25,054	1-214 (24,905)
			1-213 (24,737)
C20	MQSLN	20,300	1-173 (20,087)
C19	TRWKE	(ND)	13-173[d] (18,580)
C18[c]	MQSLN	(ND)	1-155[d] (18,020)
C12	SIDEKLHDQDDGENIQVMIR	11,561	156-254 (11,447)
C9[c]	IRDDK	9508	174-254 (9380)

[a] When an unambiguous assignment was not possible the two most likely fragments are presented.
[b] ND, not determined.
[c] The most stable products of each digestion reaction.
[d] These endpoints are assigned based on the apparent size by SDS / PAGE and the detection of the corresponding carboxyl-terminal cleavage product.

DNA to compete for limiting GST-σ^A protein (Dombroski *et al*., 1992). Since non-promoter binding sites are present in a large molar excess over promoter sites, this may indicate a difference in affinity of as much as 100-fold for promoter over non-promoter DNA sites.

σ^D alters the conformation of promoter DNA

To further explore the specificity of the interaction of σ^D with the flagellin promoter we determined the sensitivity of end-labeled DNA fragments to DNase I digestion in the presence and absence of σ^D. For these experiments, concentrations of σ^D were chosen which led to the formation of large amounts of complex I. Even under these conditions, a variety of DNA complexes were present in the reaction including a fraction of molecules with no bound protein. Despite these technical problems, several changes in the DNase I digestion pattern were reproducibly observed (Figures 8 and 9). New DNase I cleavage sites (hypersensitive sites) were induced by σ^D and several of these correlate with the conserved –35 (–33 and –34, Figure 8A, lane 1; –32 Figure 8B, lane 5) and –10 (–8, Figure 8A, lane 1; –9 Figure 8B, lane 5) regions of the flagellin promoter, suggesting that these changes were induced by a specifically interacting σ^D. In addition, σ^D repro-

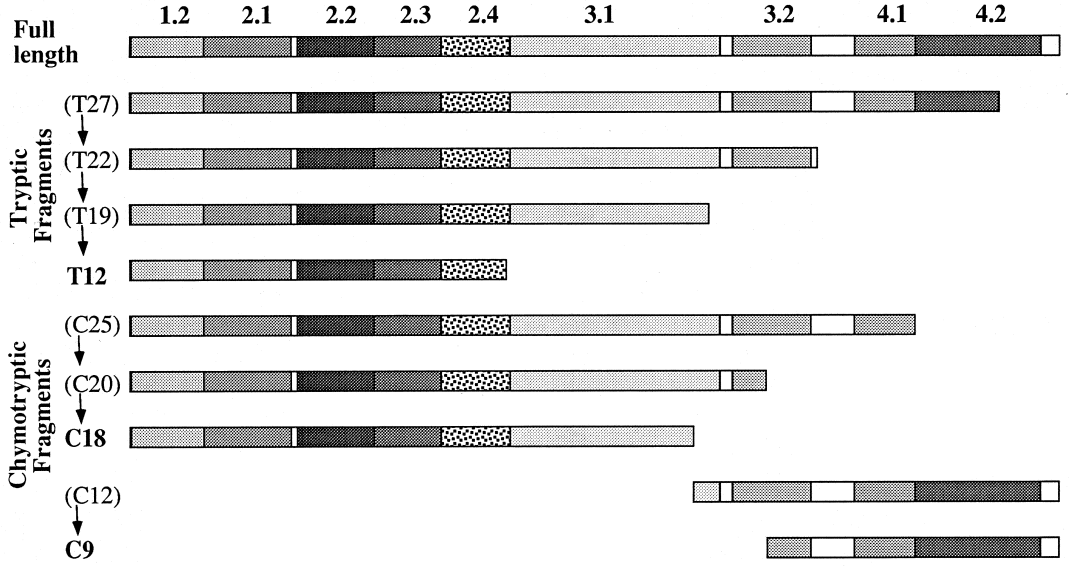

Figure 6. Domain structure of σ^D. Schematic illustrating the digestion products shown in Figure 5. The conserved regions of σ^D (Helmann & Chamberlin, 1988; Lonetto *et al*. 1992) are shown across the top of the Figure. The fragments illustrated were judged to be relatively unstable (shown in parentheses) or relatively stable during the 20 minute protease digestion. Plausible precursor to product relationships are indicated based on Figure 5 and the data of Table 1.

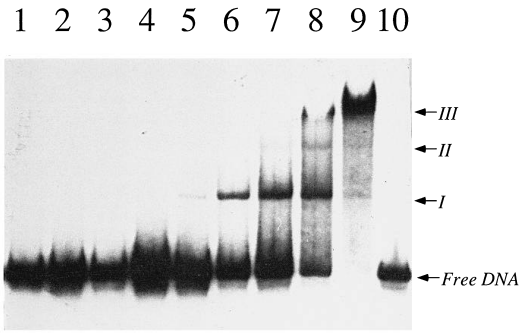

Figure 7. σ^D binds DNA. Increasing amounts of σ^D protein (lanes 1 to 9 contained 0.01, 0.06, 0.19, 0.52, 1.0, 4.2, 8.3, 10, 17 pmol, respectively) were allowed to equilibrate for 20 minutes at 37°C with 0.1 pmol of ^{32}P-labeled P_{D-6} promoter fragment. Free DNA and σ^D–DNA complexes (complexes I, II and III) were separated by 4% native PAGE. Lane 10 is a probe alone control (0.05 pmol). The apparent equilibrium dissociation constant (K_d) for σ^D binding to P_{D-6} is about 1 μM.

ducibly reduced the extent of DNase I cleavage at –32, and between –6 and –3 relative to the start of transcription (Figure 8A, lane 1). Finally, we also noted several alterations in DNase I cleavage flanking the promoter region. For example, at the σ^D concentration used in these experiments positions –49 and –50 become sensitive to DNase I (Figure 8A). It seems unlikely that all these changes are due to a single specifically bound monomer. Therefore, we postulate that these changes in flanking regions are due to non-specifically interacting σ^D.

The appearance of DNase I hypersensitive sites at –33, –34, and –8 (non-template strand) and –9 (templates trend) is reminiscent of changes induced in these same regions by the σ^D RNA polymerase when bound in an open complex. In the case of bound holoenzyme (Figure 8, lane 3), DNase I sensitivity at –8 was retained although the rest of this region was protected against cleavage. In other experiments, σ^D holoenzyme leads to strong hypersensitivity at both –8 and –31 (but not –33, –34) at the flagellin promoter (Fredrick *et al*., 1995), indicating that σ^D alone and σ^D-containing RNAP may induce similar structural alterations in these regions.

Binary complexes between the flagellin promoter and σ^D have also been probed by dimethylsulfate (DMS; Figure 8C) and potassium permanganate footprinting (data not shown). As observed with DNase I, DMS treatment detects distortion of the –35 and –10 regions of the promoter. In the presence of σ^D, strong DMS reactivity is observed on the non-template strand at –35A, –34T and –9A, –8T (Figure 8C). DMS reacts with adenine residues by methylation of the N-3 position in the minor groove. Since DNase I makes contacts with the minor groove of duplex DNA (Lahm & Suck, 1991), these data may indicate a local increase in minor groove width which enhances accessibility or reactivity of these two reagents. Reactivity of DMS with thymine residues is rare, but has been reported (Sasse-Dwight & Gralla, 1988). The nature of the DMS reactivity with thymine has not been determined, but both the N-3 and O-4 positions are susceptible to alkylation (Blackburn, 1990). In general, DMS is more reactive with nitrogen than with oxygen, but the N-3 position is not available for reaction in duplex DNA (Blackburn, 1990). It is unlikely that σ^D has locally melted the DNA in these regions since reactivity with the single-strand selective reagent, potassium permanganate, is not increased by the presence of σ^D alone (data not shown).

Discussion

We have developed an efficient method for the overproduction and purification of *B. subtilis* σ^D, the prototype of the flagellar subgroup of the σ^{70} protein family (Lonetto *et al*., 1992). The identity of the overproduced protein has been verified by determination of the amino-terminal sequence, amino acid composition, and molecular mass. When added to purified core RNA polymerase, σ^D allows the specific recognition of at least four promoter sites known to be σ^D-dependent *in vivo* and *in vitro* (Fredrick & Helmann, 1994; Gilman & Chamberlin, 1983; Mirel & Chamberlin, 1989; Mirel *et al*., 1994).

Like the *E. coli* σ^{70} and σ^{54} proteins (Gill *et al*., 1991) the purified σ^D protein is a monomer in solution. σ^D elutes during gel exclusion chromatography with an apparent molecular mass of 36 kDa (Figure 2), which suggests that it may be a somewhat asymmetric protein. Several lines of evidence, including gel exclusion chromatography (Gill *et al*., 1991) and low angle X-ray scattering studies of *E. coli* σ^{70} (Meisenberger *et al*., 1980), indicate that σ factors may have an asymmetric shape in solution. In addition, the rotational correlation time of dansylated σ^{70} protein, observed by fluorescence depolarization spectroscopy, was anomalously long even when corrected for protein hydration (Wu *et al*., 1975). It has been argued that σ factors, particularly the smaller alternative σ factors, must assume an elongated structure if they simultaneously contact the –35 and –10 regions (Losick & Pero, 1981).

Domain structure of σ^D

We have used partial proteolysis to investigate the domain structure of σ^D (Figure 5). The appearance of stable fragments upon incubation with either trypsin or chymotrypsin indicates that the protein adopts a compact folded structure. Control experiments suggested that this structure may require stabilization by glycerol. The locations of the sites of preferential cleavage indicate that σ^D contains two protease-resistant domains spanning highly conserved regions 2 and 4, respectively (Table 1 and Figure 6). The sites most rapidly cleaved by trypsin and chymotrypsin fall at the carboxyl terminus of region 2, just after the putative –10 recognition helix, and in the non-conserved spacer between regions 3.1 and 3.2. These are the least conserved regions of σ

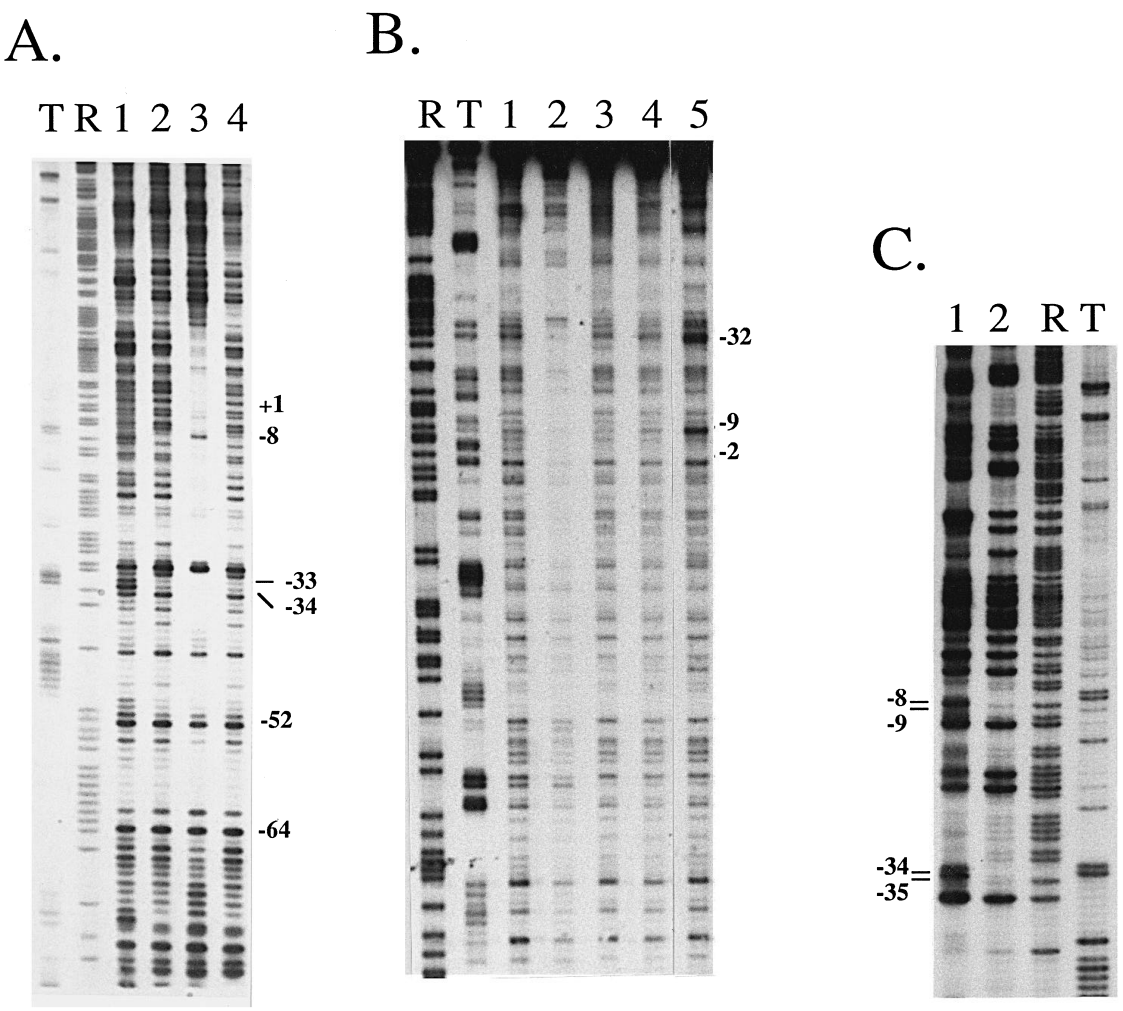

Figure 8. σ^D interacts with –10 and –35 regions in solution. A, DNase I cleavage of P_{D-6} DNA (non-template strand). Lane T, thymine-specific chemical sequencing ladder. Lane R, A and G chemical sequencing ladder. lane 1, 6 pmol σ^D; lane 2, no protein control; lane 3, $E\sigma^D$ holoenzyme, lane 4, no protein control. Binding reactions were in binding buffer containing 150 mM NaCl (lanes 1 and 2) or 50 mM NaCl (lanes 3 and 4). B, DNase I cleavage of P_{D-6} DNA (template strand). Lanes T and R as above; lane 1, Eδ (9 pmol core and 47 pmol δ); lane 2, $E\sigma^D$ holoenzyme; lane 3, no protein control; lane 4, 8 pmol σ^D; lane 5, 33 pmol σ^D. $E\sigma^D$ holoenzyme was prepared by incubating 9 pmol of core RNA polymerase with 6 pmol of σ^D and 47 pmol of δ. C, DMS protection of P_{D-6} DNA (template strand). Lanes T and R as above; lane 1, 20 μM σ^D; lane 2, no protein control.

factors as judged by amino acid alignment of 31 proteins of the σ^{70} family (Lonetto *et al*., 1992). The *B. subtilis* σ^A protein is also cleaved quite rapidly in region 2.4 and between regions 3 and 4 (Chang & Doi, 1990).

The division of σ factors into two relatively stable

Figure 9. Summary of DNase I footprinting data for P_{D-6}. The sequence of the flagellin promoter (P_{D-6}) is shown. The boundaries of DNase I protection by holoenzyme are indicated by brackets ([,]) and the hypersensitive sites induced by σ^D are indicated by large arrows for changes in the –35 and –10 regions (enclosed in boxes) and smaller arrows for the generally weaker effects noted elsewhere. Enhanced reactivity of adenine residues to DMS is indicated by the open arrows (⇓).

domains, represented by conserved regions 2 and 4, is supported by several other lines of evidence. In σ^{70}, both of these domains bind DNA when fused to glutathione-*S*-transferase (GST; Dombroski *et al*., 1992), indicating that they can assume a stable folded structure. In addition, a 13 amino acid residue deletion in the non-conserved spacer between conserved regions 3.1 and 3.2 of the *E. coli* σ^{32} protein leads to production of functional protein (Calendar *et al*., 1988), albeit decreased in its core binding affinity (Zhou *et al*., 1992). In fact, several alternative σ factors, including the recently described ECF sub-family, lack conserved region 3.2 completely and instead have region 2 and region 4 linked by a spacer distantly related to region 3.1 (Lonetto *et al*., 1992, 1994).

DNA-binding by σ^D

We have demonstrated that σ^D binds to DNA as judged by the formation of a discrete complex in a mobility shift assay and by footprinting with enzymatic (DNase I) and chemical (DMS) probes. Both σ^{70}-GST and *B. subtilis* σ^K–GST fusion proteins bind DNA with equilibrium dissociation constants (K_d) in the range of 10 nM in nitrocellulose filtration assays (Dombroski *et al*., 1993). In contrast, the DNA-binding we detect for σ^D is relatively weak (K_d = 1 μM), although it is difficult to compare our results with earlier studies due to differences in the assays and the incubation conditions. *Salmonella typhimurium* σ^F, a member of the σ^{28} sub-family of σ factors, has also been reported to bind DNA weakly (Dombroski *et al*., 1993). Finally, we note that several studies have demonstrated specific DNA binding by σ^{54} (Buck & Cannon, 1992; Merrick, 1993) an alternative σ factor not detectably related to the σ^{70} family of proteins.

Treatment of σ^D–DNA complexes with DNase I reveals a partial protection at several positions, and the appearance of hypersensitive sites at –33, –34, and –8 relative to the transcription start site. The hypersensitive sites correspond to the conserved –35 and –10 regions, suggesting that σ^D directly contacts these regions of DNA. Similarly, hypersensitive sites are observed at –31 and –8 in the presence of σ^D holoenzyme (Fredrick *et al*., 1995). In the presence of σ^D, the –35 and –10 regions also display an altered reactivity to DMS, which methylates the N-7 of guanine (in the major groove) and the N-3 of adenine (in the minor groove). Specifically, σ^D induces strong reactivity of adenine residues at positions –35 and –9 on the non-template strand and in both cases reaction with a downstream thymine (–34 and –8) is also apparent. Although the chemical nature of the thymine modification is not known, these data, together with the DNase I cleavage study, suggest that σ^D binding alters the local structure of the DNA.

There is now strong genetic and biochemical evidence to support the proposition that promoter recognition is mediated by direct contacts between σ factors and DNA (Gross *et al*., 1992; Dombroski *et al*., 1992). The σ^{54} protein can bind to some promoters in the absence of core enzyme (Buck & Cannon, 1992), and removal of conserved region 1 from several σ^{70} family members exposes a latent DNA binding activity (Dombroski *et al*., 1993). Despite the ability of some σ^{70} family proteins to bind DNA *in vitro*, the specificity observed is not sufficient to suggest that promoter-specific binary complexes of σ factors with DNA form *in vivo*. Therefore, *in vivo* promoter recognition is almost certainly mediated by the various holoenzyme species, rather than free σ factors.

Materials and Methods

Overexpression and purification of σ^D

The *sigD* gene was cloned into a pBlueScript (Stratagene) derivative and modified by the PCR to introduce an *Nde*I site overlapping the start codon (Chen & Helmann, 1992). The resulting plasmid, pYFC-6, contains the *sigD* coding sequence on a 877 bp *Nde*I to *Bam*HI fragment. This plasmid produces active σ^D protein as judged by complementation of suitable *E. coli* mutants (Chen & Helmann, 1992). To construct an overproducing plasmid, this 877 bp *Nde*I-*Bam*HI fragment was cloned into *p*ET11c (Studier *et al*., 1990) after digestion with *Nde*I and *Bam*HI. The resulting plasmid, pYFC-11, allows high level overproduction of σ^D in *E. coli* BL21/DE3 (Studier *et al*., 1990).

For purification of σ^D, BL21/DE3 (pYFC-11) was grown to mid-logarithmic phase at 37°C in 8 l of 2 × YT medium (Sambrook *et al*., 1990) supplemented with 0.4% (w/v) glucose and 100 μg/ml ampicillin. IPTG was added to 0.4 mM and the culture incubated for three hours. After centrifugation, 23 g of cell paste was recovered and suspended in 60 ml of disruption buffer (Juang & Helmann, 1994). Cells were lysed and σ^D was renatured after extraction from the washed cell pellet with 6 M guanidine hydrochloride as described for purification of σ^D (Juang & Helmann, 1994), except that all volumes were increased proportionally. After gradual dilution of the extracted protein with 1 l of TED buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM DTT) to allow renaturation, the soluble portion was batch-adsorbed to 80 ml of QAE-Sepharose which was then poured into a column. During step elution, σ^D remained bound while washing the column in TED buffer with 0.15 M NaCl but eluted with 0.40 M NaCl. Approximately 160 mg of σ^D was recovered with peak fractions containing 8 mg/ml protein. At this high concentration, σ^D tends to slowly aggregate. To avoid this problem, the protein was stored in aliquots at –80°C and samples were removed for further purification as needed. Typically, 1 to 2 mg of σ^D were further purified by chromatography on an FPLC Superdex-75 column in TED buffer containing 0.15 M NaCl.

Physical characterization of σ^D

The molecular mass of σ^D was determined at the Cornell Biotechnology Facility by matrix-assisted laser-desorption and ionization time-of-flight mass spectroscopy (MALDI-TOF MS) using a matrix of α-cyano-4-hydroxycinnamic acid or cinnamic acid. The amino-terminal amino acid sequence was determined by automated Edman degradation using an Applied Biosystems 470A gas phase protein sequencer equipped with a 120A PTH analyzer. The amino acid composition was determined by HPLC

analysis of a complete acid hydrolysate. The oligomeric state of σ^D was determined by gel exclusion chromatography on an FPLC (Pharmacia) Superdex-G75 column equilibrated in TED buffer containing 0.15 M NaCl.

Core enzyme preparation

CB100 (*sigD*::pLM5) cells (Márquez *et al*., 1990) were grown at 37°C in 8 l of 2 × YT medium with 5 μg / ml chloramphenicol. Cells were harvested, suspended in 60 ml of buffer A (10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 0.165 mM DTT, 7.5% (v / v) glycerol, 50 mM KCl), and lysed by a French pressure cell in the presence of 0.1 mM PMSF to inhibit protease activity. After centrifugation at 12,000 ***g*** for 20 minutes the low speed supernatant fraction was loaded onto an 80 ml heparin-Sepharose (Pharmacia) column and RNA polymerase was eluted using a salt gradient from 0.05 to 1.05 M KCl. Fractions containing polymerase were pooled, precipitated with ammonium sulfate (0.42 g / ml), and the resulting precipitate was dissolved in TEDG buffer (50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 0.5 M NaCl, 10% glycerol) prior to loading on a sizing column (S-300 or A1.5M). The pooled fractions were concentrated by Centriprep-10 (Amicon), dialyzed in TMGED buffer (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, 50% glycerol), and applied to a FPLC MonoQ anion exchanger. For core enzyme containing δ (Eδ), the pooled fractions from MonoQ were dialyzed into HMGED buffer (50 mM Na-Hepes (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, 50% glycerol) and applied to an FPLC MonoS cationic ion exchange column. The flow-through fractions, containing core enzyme (Juang & Helmann, 1994), were pooled and dialyzed into TMGED buffer. For some experiments (e.g. Figure 8), core was supplemented with a stoichiometric excess of δ purified from an *E. coli* overproducing strain (F. López de Saro & J.D.H., unpublished results).

Limited proteolytic digestion

SigD (50 μg) was incubated at 22°C for 30 minutes in reactions containing 20% glycerol and varying amounts of TPCK-treated trypsin (Worthington) or TLCK-treated chymotrypsin (Sigma Chemical Co., St. Louis, MO). Reactions were stopped by addition of 10 mM PMSF and visualized by SDS / PAGE 20%. Samples were blotted to polyvinylidenedifluoride membrane, stained with 0.1% (w / v) Coomassie blue in 50% (v / v) methanol, and analyzed by automated amino acid sequencing. Analysis by MALDI-TOF MS was performed as described above after dialysis to remove glycerol and salts.

Core-binding assay

Purified core RNA polymerase (20 μg) was incubated with 20 μg of purified σ^D at room temperature for ten minutes and the mixture was fractionated by chromatography on an FPLC Superdex-75 gel exclusion column in TED buffer containing 0.15 M NaCl. RNA polymerase and associated proteins are excluded from this column and elute in the void volume. Fractions were precipitated with 10% (w / v) trichloracetic acid at 0°C for ten minutes and the precipitate was collected by centrifugation at 10,000 ***g***. The pellets were washed with ice-cold acetone, air-dried, and dissolved in 10 μl of SDS-sample buffer (Sambrook *et al*., 1990) before fractionation by SDS / PAGE 10%.

In vitro transcription assay

Reactions contained 0.3 pmol of plasmid DNA, 0.9 pmol of core enzyme, and 9 pmol of σ^D in 25 μl of transcription buffer (19.2 mM Tris-HCl (pH 8.0), 9.6 mM $MgCl_2$, 9.6 mM β-mercaptoethanol, 8 mM EDTA, 40% glycerol, 960 μM of ATP, GTP, and CTP, 120 μM [α-^{32}P]UTP at about 1000 cts / min per pmol). DNA and RNA polymerase were preincubated for eight minutes at 37°C to allow promoter binding, NTPs were added for an additional eight minutes, and 6 μl of urea / stop solution (20 μg / ml xylene cyanol, 20 μg / ml bromophenol blue, 60 mg / ml urea in 0.1 × TBE: Sambrook *et al*. (1990)) was added to stop the reaction. RNA was visualized by heat denaturation, separation by 8 M urea / 6% PAGE, and autoradiography.

Purified plasmid DNA was used as the template for transcription. P_{D-1}, the promoter of the *flgK-flgM* operon (Mirel *et al*., 1994), is present on pJH102T (Helmann *et al*., 1988*b*). P_{D-6}, the *hag* promoter, was cloned as a 683 bp *Hin*dIII-*Eco*RI fragment from pDM67 (Mirel *et al*., 1992) into pBSK$^+$ to generate pYFC-12. The 267 bp *Sau*3AI-*Ssp*I fragment from pYFC-12 containing P_{D-6} was subcloned into pBKSII$^+$ to generate pYFC-16. P_{D-8}, the promoter for the *fliDST* operon (Chen & Helmann, 1994), was cloned as a 470 bp *Eco*RI-*Hin*dII fragment from pDM67 (Mirel & Chamberlin, 1989) into pBSK$^+$ to generate pYFC-13. For P_{cheV}, pKF1 (Fredrick & Helmann, 1994) was used. All plasmid DNA was purified from 1 l of *E. coli* cultures using an alkaline lysis protocol (Sambrook *et al*., 1990) and linearized at a unique downstream restriction site for use in run-off assays. The concentration of DNA was determined by A_{260} measurement.

DNA-binding assays

DNase I footprinting reactions (25 μl) contained 0.3 pmol of 5'-end-labeled DNA fragment and proteins (as indicated) incubated in binding buffer (40 mM Tris-HCl (pH 8.0), 0.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.25 mg / ml BSA) containing NaCl (as indicated) for ten minutes prior to addition of RQ1 RNase-free DNase I (Promega Biotech.) to a final concentration of 0.2 unit / μl for one minute at 37°C. Reactions were stopped by DNase I stop solution (30 mM EDTA, 240 μg / ml salmon testes DNA (Sigma Chemical Co., St. Louis, MO), 0.3 M sodium acetate (pH 7.0)), phenol extracted, ethanol precipitated and resuspended in 8 μl formamide loading buffer (Sambrook *et al*., 1990) prior to heat denaturation and separation by 8 M urea / PAGE. To label the DNA, pYFC-16 was digested first with *Bsp*120i or *Eag*I, depending on the strand to be labeled. The DNA was dephosphorylated with calf-intestine alkaline phosphatase (New England Biolabs), labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the promoter containing fragment (267 bp) removed by digestion with the second enzyme (either *Bsp*120i or *Eag*I) and purified by 5% PAGE and elution using 0.5 M ammonium acetate, 10 mM magnesium acetate, and 1 mM EDTA. Fragments labeled at the *Bsp*120i 5' end are labeled on the template strand of the P_{D-6} fragment. For electrophoretic mobility shift analysis, σ^D was incubated with the same 5'-end-labeled DNA fragment at 37°C in binding buffer containing 50 mM NaCl for 20 minutes prior to separation by native 4% PAGE at room temperature.

For DMS protection experiments, 20 μM σ^D was incubated at 25°C with 0.01 pmol of 5'-end-labeled P_{D-6} promoter DNA in 42.5 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.31 mg / ml BSA, 1% (v / v) Tween, in the presence of 7 μg / ml non-specific plasmid DNA and 0.24 mg / ml sonicated salmon sperm DNA. Then reactions (25 μl) were

treated with 5 µl of a 1:40 dilution of DMS (Sigma Chemical Co.) for five minutes at room temperature prior to addition of 200 µl of stop solution (10 mM EDTA, 0.24 mg / ml sonicated salmon sperm DNA, 1 M β-mercaptoethanol, 0.3 M sodium acetate (pH 7.0). Nucleic acids were recovered by ethanol precipitation and treated with piperidine as described (Sambrook *et al*., 1990), prior to heat denaturation and separation on 8 M urea / PAGE.

Acknowledgement

This work was supported by NIH grant GM-47446.

References

Blackburn, G. M. (1990). Covalent interactions of nucleic acids with small molecules. In *Nucleic Acids in Chemistry and Biology* (Blackburn, G. M. & Gait, M. J., eds), pp. 259–293, IRL Press, Oxford, England.

Buck, M. & Cannon, W. (1992). Specific binding of the transcription factor sigma-54 to promoter DNA. *Nature,* **358**, 422–424.

Calendar, R., Erickson, J. W., Halling, C. & Noles, A. (1988). Deletion and insertion mutations in the *rpoH* gene of *Escherichia coli* that produce functional σ^{32}. *J. Bacteriol.* **170**, 3479–3484.

Chang, B. Y. & Doi, R. H. (1990). Overproduction, purification, and characterization of *Bacillus subtilis* RNA polymerase σ^A factor. *J. Bacteriol.* **172**, 3257–3263.

Chater, K. F., Bruton, C. J., Plaskitt, K. A., Buttner, M. J., Mendez, C. & Helmann, J. D. (1989). The developmental fate of *Streptomyces coelicolor* hyphae depends crucially on a gene product homologous with the motility σ factor of *Bacillus subtilis. Cell,* **59**, 133–143.

Chen, L. & Helmann, J. D. (1994) The *Bacillus subtilis* σ^D-dependent-operon encoding the FliD, FliS, and FliT proteins. *J. Bacteriol.* **176**, 3093–3101.

Chen, Y. F. & Helmann, J. D. (1992). Restoration of motility to an *Escherichia coli fliA* flagellar mutant by a *Bacillus subtilis* sigma-factor. *Proc. Natl Acad. Sci. USA,* **89**, 5123–5127.

Daniels, D., Zuber, P. & Losick, R. (1990). Two amino acids in an RNA polymerase σ factor involved in the recognition of adjacent base pairs in the − 10 region of a cognate promoter. *Proc. Natl Acad. Sci. USA*, **87**, 8075–8079.

Dombroski, A. J., Walter, W. A., Record, M. T., Jr, Siegele, D. A. & Gross, C. A. (1992). Polypeptides containing highly conserved regions of transcription initiation factor σ^{70} exhibit specificity of binding to promoter DNA. *Cell,* **70**, 501–512.

Dombroski, A. J., Walter, W. A. & Gross, C. A. (1993). Amino-terminal amino acids modulate σ-factor DNA-binding activity. *Genes. Dev.* **7**, 2446–2455.

Fredrick, K. & Helmann, J. D. (1994). Dual chemotaxis signaling pathways in *Bacillus subtilis*: A σ^D-dependent gene encodes a novel protein containing both CheW and CheY homologous domains. *J. Bacteriol.* **176**, 2727–2735.

Fredrick, K., Caramori, T., Chen, Y. F., Galizzi, A. & Helmann, J. D. (1995). Promoter architecture in the flagellar regulon of *Bacillus subtilis*: high level expression of flagellin by the σ^D RNA polymerase requires an upstream promoter element. *Proc. Natl. Acad. Sci. USA*, **92**, 2582–2586.

Gardella, T., Moyle, H. & Susskind, M. M. (1989). A mutant *Escherichia coli* σ^{70} subunit of RNA polymerase with altered promoter specificity. *J. Mol. Biol.* **206**, 579–590.

Gill, S. C., Yager, T. D. & von Hippel, P. H. (1991). *Escherichia coli* sigma70 and NusA proteins. 2. Physical properties and self-association states. *J. Mol. Biol.* **220**, 325–333.

Gilman, M. Z. & Chamberlin, M. J. (1983). Developmental and genetic regulation of *Bacillus subtilis* genes transcribed by σ^{28}-RNA polymerase. *Cell,* **35**, 285–293.

Gilman, M. Z., Wiggs, J. L. & Chamberlin, M. J. (1981). Nucleotide sequences of two *Bacillus subtilis* promoters used by the *Bacillus subtilis* sigma-28 RNA polymerase. *Nucl. Acids Res.* **9**, 5991–6000.

Gribskov, M. & Burgess, R. R. (1983). Overexpression and purification of the sigma subunit of *Escherichia coli* RNA polymerase. *Gene,* **26**, 109–118.

Gross, C. A., Lonetto, M. & Losick, R. (1992). Bacterial sigma factors. In *Transcriptional Regulation*. pp. 129–176, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Helmann, J. D. (1991). Alternative sigma factors and the regulation of flagellar gene expression. *Mol. Microbiol.* **5**, 2875–2882.

Helmann, J. D. (1994). Bacterial sigma factors. In *Transcription: Mechanisms and Regulation*. pp. 1–17, Raven Press, New York.

Helmann, J. D. & Chamberlin, M. J. (1988). Structure and function of bacterial σ factors. *Annu. Rev. Biochem.* **57**, 839–872.

Helmann, J. D., Márquez, L. M. & Chamberlin, M. J. (1988a). Cloning, sequencing, and disruption of the *Bacillus subtilis* σ^{28} gene. *J. Bacteriol.* **170**, 1568–1574.

Helmann, J. D., Masiarz, F. R., & Chamberlin, M. J. (1988b). Isolation and characterization of the *Bacillus subtilis* σ^{28} factor. *J. Bacteriol.* **170**, 1560–1567.

Jaehning, J. A., Wiggs, J. L. & Chamberlin, M. J. (1979). Altered promoter specificity by a novel form of *Bacillus subtilis* RNA polymerase. *Proc. Natl Acad. Sci. USA*, **76**, 5470–5474.

Juang, Y. L. & Helmann, J. D. (1994). The δ subunit of *Bacillus subtilis* RNA polymerase: an allosteric effector of the initiation and core-recycling phases of transcription. *J. Mol. Biol.* **239**, 1–14.

Krummel, B. & Chamberlin, M. J. (1989). RNA chain initiation by *Escherichia coli* RNA polymerase. Structural transitions of the enzyme in early ternary complexes. *Biochemistry,* **28**, 7829–7842.

Kuroda, A. & Sekiguchi, J. (1993). High-level transcription of the major *Bacillus subtilis* autolysin operon depends on expression of the sigma D gene and is affected by a *sin (flaD)* mutation. *J. Bacteriol.* **175**, 795–801.

Lahm, A. & Suck, D. (1991). DNase I-induced DNA conformation. 2 Å structure of a DNase I-octamer complex. *J. Mol. Biol.* **221**, 645–667.

Lazarevic, V., Margot, P., Soldo, B. & Karamata, D. (1992). Sequencing and analysis of the *Bacillus subtilis lytRABC* divergon: a regulatory unit encompassing the structural genes of the *N*-acetymuramoyl-l-alanine amidase and its modifier. *J. Gen. Microbiol.* **138**, 1949–1961.

Leirmo, S. & Record, M. T., Jr (1990) Structural, thermodynamic and kinetic studies of the interaction of $E\sigma^{70}$ RNA polymerase with promoter DNA. In *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin, Heidelberg.

Lonetto, M., Gribskov, M. & Gross, C. A. (1992). The σ^{70} family: sequence conservation and evolutionary relationships. *J. Bacteriol.* **174**, 3843–3849.

Lonetto, M. A., Brown, K. L., Rudd, K. E. & Buttner, M. J. (1994). Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial σ factors involved in the regulation of extracytoplasmic functions. *Proc. Natl. Acad. Sci. USA*, **91**, 7573–7577.

Losick, R. & Pero, J. (1981). Cascades of sigma factors. *Cell*, **25**, 582–584.

Margot, P., Mauel, C. & Karamata, D. (1994). The gene of the *N*-acetylglucosaminidase, a *Bacillus subtilis* 168 cell wall hydrolase not involved in vegetative cell autolysis. *Mol. Microbiol.* **12**, 535–545.

Márquez, L. M., Helmann, J. D., Ferrari, E., Parker, H. M., Ordal, G. W. & Chamberlin, M. J. (1990). Studies of σ^D-dependent functions in *Bacillus subtilis*. *J. Bacteriol.* **172**, 3435–3443.

Márquez-Magaña, L. M. & Chamberlin, M. J. (1994). Characterization of the *sigD* transcription unit of *Bacillus subtilis*. *J. Bacteriol.* **176**, 2427–2434.

Meisenberger, O., Pilz, I. & Heumann, H. (1980). Small-angle X-ray study of DNA-dependent RNA polymerase subunit σ from *Escherichia coli*. *FEBS Letters*, **112**, 39–41.

Merrick, M. J. (1993). In a class of its own—the RNA polymerase sigma factor σ^{54} (σ^N). *Mol. Microbiol.* **10**, 903–909.

Mirel, D. B. & Chamberlin, M. J. (1989). The *Bacillus subtilis* flagellin gene (*hag*) is transcribed by the σ^{28} form of RNA polymerase. *J. Bacteriol.* **171**, 3095–3101.

Mirel, D. B., Lustre, V. M. & Chamberlin, M. J. (1992). An operon of *Bacillus subtilis* motility genes transcribed by the σ^D form of DNA polymerase. *J. Bacteriol.* **174**, 4197–4204.

Mirel, D. B., Lauer, P. & Chamberlin, M. J. (1994). Identification of flagellar synthesis regulatory and structural genes in a σ^D-dependent operon of *Bacillus subtilis*. *J. Bacteriol.* **176**, 4492–4500.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1990). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sasse-Dwight, S. & Gralla, J. D. (1988). Probing the *Escherichia coli glnALG* upstream activation mechanism *in vivo*. *Proc. Natl Acad. Sci. USA*, **85**, 8934–8938.

Siegele, D. A., Hu, J. D., Walter, W. A. & Gross, C. (1989). Altered promoter recognition by mutant forms of the σ^{70} subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **206**, 591–603.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enymol.* **185**, 60–89.

Waldburger, C., Gardella, T., Wong, R. & Susskind, M. M. (1990). Changes in conserved region-2 of *Escherichia coli* sigma-70 affecting promoter recognition. *J. Mol. Biol.* **215**, 267–276.

Wiggs, J. L., Gilman, M. Z. & Chamberlin, M. J. (1981). Heterogeneity of RNA polymerase in *Bacillus subtilis*: evidence for an additional σ factor in vegetative cells. *Proc. Natl Acad. Sci. USA*, **78**, 2762–2766.

Wu, C. W., Yarbrough, L. R., Hillel, Z. & Wu, F. Y.-H. (1975). Sigma cycle during *in vitro* transcription: demonstration by nanosecond fluorescence depolarization spectroscopy. *Proc. Natl Acad. Sci. USA*, **72**, 3019–3023.

Zhou, Y. N., Walter, W. A. & Gross, C. A. (1992). A mutant σ^{32} with a small deletion in conserved region 3 of σ has reduced affinity for core RNA polymerase. *J. Bacteriol.* **174**, 5005–5012.

Zuber, P., Healy, J., Carter, H. L., III, Cutting, S., Moran, C. P., Jr & Losick, R. (1989). Mutation changing the specificity of an RNA polymerase sigma factor. *J. Mol. Biol.* **206**, 605–614.

Edited by R. Schleif

(Received 13 December 1994; accepted 3 April 1995)