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Promoter Selectivity of the *Bacillus subtilis* RNA Polymerase σ^A and σ^H Holoenzymes

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The σ^H of *Bacillus subtilis* directs transcription of a large number of early sporulation genes, whereas the principal σ factor, σ^A, is essential for the transcription of the genes for vegetative growth and early sporulation. We have purified σ^A and σ^H proteins, and characterized their properties. The genes encoding σ^A or σ^H were separately cloned into an expression vector under the control of T7 promoter. Both proteins were overproduced in *Escherichia coli* BL21(DE3) and purified from inclusion bodies after solubilization with guanidine hydrochloride. Antigenicities and N-terminal amino acid sequences of the overproduced proteins were used to identify both proteins. Unlike σ^A protein, σ^H protein showed a DNA-binding ability. To compare the promoter selectivity of the σ^A protein with that of the σ^H protein, transcription *in vitro* of 16 promoters was performed using RNA polymerase holoenzymes reconstituted from a purified core enzyme with either σ^H or σ^A. These holoenzymes correctly recognized each of the cognate promoters; σ^H-RNA polymerase recognized σ^H promoters but not σ^A promoters, and *vice versa*. A competition experiment for core RNA polymerase using σ^A and σ^H revealed that σ^A had a stronger affinity. We propose that the predicted replacement of a σ subunit in a holoenzyme from σ^A to σ^H *in vivo* at late logarithmic growth phase may require an additional factor, or the modification of a core enzyme or σ factor.

Key words: *Bacillus subtilis*, RNA polymerase, sigma factor.

The RNA polymerase of *Bacillus subtilis* is composed of at least four different subunits, α, β, β', and σ. Promoter recognition requires the association of core RNA polymerase ($\alpha_2\beta\beta'$) with one of a family of σ factors (*1, 2*). The primary σ factor in growing *B. subtilis* cell, σ^A, is homologous to σ^{70} of *Escherichia coli* (*3*). Although the σ^A protein is present throughout sporulation, its activity decreases markedly during the first 2 h of sporulation (*4*). An alternative σ factor, σ^H, appears during vegetative growth and the early sporulation phase, and is essential for sporulation (*5-18*). Other alternative σ factors, σ^E, σ^F, σ^G, and σ^K, have been identified as sporulation-specific σ factors (*1*). While σ^A and σ^H are produced prior to asymmetric cell division, the activities of other sporulation-specific σ factors appear sequentially and are restricted to one of the two cell types, the mother cell and the forespore, which are formed during the early phase of sporulation. RNA polymerase containing one of these σ factors directs transcription from different subsets of promoters at the proper time and location during development (*19-21*). Each of the successive σ factors in a developmental process would have to either bind more tightly to core RNA polymerase or be made in larger amounts than the preceding σ factor (*22*). There might be other mechanisms that inactivate the preceding σ factor or modify a core enzyme. No experiments have been done in an attempt to distinguish these possibilities.

In this report, we describe a rapid procedure for obtaining pure and active σ^A and σ^H in large quantities. We then compared their promoter recognition specificity, core binding activity, and other properties to elucidate a part of the mechanism underlying developmentally regulated exchange of σ factors.

MATERIALS AND METHODS

Overexpression and Purification of σ^H—A DNA fragment that contains the entire σ^H protein coding region (*spo0H*) of the *B. subtilis* chromosome was made by PCR with *B. subtilis* JH642 (*trpC2, pheA1*) DNA and an LA-PCR kit (Takara Shuzo). To create an appropriate ribosome-binding sequence for *E. coli* and an effective initiation methionine codon, PCR-based, oligonucleotide-directed mutagenesis was carried out with a pair of oligonucleotide primers, 5′-AGAAGGAGGGATCGGAATGAATCTACAGAA-3′ (SD and Met codon are underlined) and 5′-CTATTACAAACTGATTTCGC-3′. PCR reactions were carried out for 30 cycles with 1 ng of *B. subtilis* DNA. Each cycle was at 95°C for 1 min (denaturation), 55°C for 1.5 min (annealing), and 72°C for 2 min (extension). The PCR product was cloned into pGEM-T (Promega). A plasmid with the *spo0H* (*sigH*) gene inserted downstream of the T7 promoter was selected and called pGEMTSigH. *E. coli* BL21(DE3) (*23*) harboring pGEMTSigH was grown in LB medium (1,000 ml) containing ampicillin (100 μg/ml) at 37°C. When the

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Abbreviations: IPTG, isopropyl-β-D(−)-thiogalactopyranoside; LB, Luria broth; PMSF, phenylmethylsulfonyl fluoride.

optical density at 600 nm (OD_{600}) was 0.6, IPTG (1 mM) was added to the culture and the cells were collected by centrifugation at 10,000 × *g* for 10 min after a 3 h incubation. The pellet was resuspended in 20 ml of TGED buffer [50 mM Tris-HCl (pH 8.0), 20% (v/v) glycerol, 1 mM EDTA, 0.3 mM DTT, 2 mM PMSF] containing lysozyme (100 μg/ml) and lysed by sonication. The cell debris was removed by centrifugation at 10,000 × *g* for 10 min. The resulting pellet was extracted with 20 ml of TGED buffer containing 6 M guanidine hydrochloride and centrifuged at 10,000 × *g* for 10 min. The supernatant was diluted gradually to 1,000 ml with TGED buffer. The protein solution was then passed through a DE52 column which was equilibrated with the same buffer. The flow-through fraction was pooled and precipitated with 40% (w/v) ammonium sulfate. The protein pellet was collected by centrifugation and was dissolved in 5 ml of TGED buffer and dialyzed against TGED buffer. The amount of protein was determined with a Bio-Rad protein determination kit with BSA as the standard.

Overexpression and Purification of σ^A—A DNA fragment that contains the entire σ^A protein coding region of *B. subtilis* JH642 chromosome was made by PCR. Appropriate restriction sites to generate a hexahistidine-tag in the C-terminus of σ^A were included in the PCR primers (5′-TAGAATTCGTTGCAAGCT-3′ and 5′-TGCTCGAGGAAATCTTTCAAACGTT-3′; underlines denote *Eco*RI and *Xho*I sites used for cloning). The PCR product was cloned into the pET21b *Eco*RI-*Xho*I site (Novagen). This construct was called pETSigA. *E. coli* BL21 (DE3) containing pETSigA was cultured as described above. Cells were suspended in 40 ml of a binding buffer [Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM imidazole] and then sonicated until they were lysed. The cell debris was centrifuged and resuspended in a 20 ml binding buffer containing 6 M guanidine hydrochloride. After incubation for 1 h at room temperature, the insoluble materials were removed by centrifugation at 20,000 × *g* for 20 min. The supernatant was loaded on 1 ml of Ni^{2+}-NTA agarose resin (Qiagen) equilibrated with a binding buffer containing 6 M guanidine hydrochloride. The column was washed with 10 ml of a binding buffer containing 6 M guanidine hydrochloride. σ^A was eluted with a 20-ml imidazole gradient from 5 to 500 mM in the binding buffer containing 6 M guanidine hydrochloride. To refold σ^A, the peak fractions (3 ml) were pooled and diluted step-wise with guanidine-HCl (4, 2, and 0 M) in a dialysis procedure. The amount of protein was determined as described above.

Core RNA Polymerase Preparation—B. subtilis UOT-1850 (*trpC2 lys1 aprEΔ3 nprE18 nprR2 spo0HΔHB*) cells (*24*) were grown at 37°C in 4 liters of LB medium. Cells were harvested, washed with A buffer [10 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 1 mM EDTA, 0.3 mM DTT, 0.1 M KCl, 2 mM PMSF], suspended in 50 ml of TGED buffer containing 100 μg/ml lysozyme, and lysed by an ultrasonic device. After centrifugation at 18,000 × *g* for 40 min, core RNA polymerase was obtained by Polymin P precipitation and DE52 chromatography. The fractions containing RNA polymerase, as judged by the presence of $\beta\beta'$ in SDS-PAGE, were loaded onto a Bio-Rex 70 column (BioRad) equilibrated with TGED buffer (*25*). Core RNA polymerase was recovered by elution with a linear gradient of NaCl from 0 to 1.0 M. The peak fractions, as judged by SDS-PAGE, were pooled and rechromatographed on a second Bio-Rex 70 column in the same manner to eliminate trace amounts of σ factors (Fig. 1C). σ^A or σ^H protein was not detected by Western blot analysis with an anti-σ antibody.

Template DNAs—The templates used in this study are as follows: *abrB* P2 (*26*); *kinC* (*27, 28*); *xyl* (*29*); *orf253/146* (*30*); *spo0H* (*6, 31*); *spo0A* Ps/Pv (*10, 32*); *spoVG* (*33*); *orf283* (*30*); *sigA* P4 (*12*); *citG* P2 (*14*); *kinA* (*10, 34*); *spo0F* P2/*orfS* (*35*); *gnt* (*36*); *secA* (*37*). The structure of the truncated template DNAs and the transcripts directed by the promoter located on each template are summarized in Fig. 2.

For template DNA fragments, PCR reactions were carried out for 30 cycles with 1 ng of *B. subtilis* JH642 DNA. Each cycle was 95°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min. PCR products were electrophoresed on a non-denaturing polyacrylamide gel and purified. The primer sequences were as follows:

abrB: 5′-GTTTCCAAGACATTACTGAC-3′/5′CGATAGGAATAACTACACGT-3′

kinC: 5′-AAGGCGGAGTGATATCATCT-3′/5′-AACAGGCCAATTAATCGGGT-3′

xyl: 5′-GTATGATTTAGTACATAGCG-3′/5′-TGAGCCATGTTATTTCCTCC-3′

orf253/orf146: 5′-GGAACAATGGCATCTGCTTG-3′/5′-GCGGATCAATATCTACCAGC-3′

spo0H: 5′-GCTTCTGAGAGAGGTAGAAA-3′/5′-CGGTACTTCGTAATCAAGTA-3′

spo0A Pv/Ps: 5′-AGCAAGCTGACTGCCGGAGT-3′/5′-CCAGCTCTCGATTATCATCA-3′

spoVG: 5′-GCTTTATGACCTAATTGTGT-3′/5′-CGTAATCTTACGTCAGTAAC-3′

orf283: 5′-AAGAAGAACTGAATGCAGGC-3′/5′-CAGCAATCTCCGGCTCCTGT-3′

sigA P4: 5′-GAGAACGATTTATTACACTG-3′/5′-CAGAGTCCAAAGTAGTTTCG-3′

citG P2: 5′-AAGGATTCCCGCGTATAT-3′/5′-TTGGGCGCCCCAAAATTTAT-3′

kinA: 5′-GATAAGATCAATAAAATTAT-3′/5′-CCAAGACTGCATGAATATCG-3′

spo0F P2/*orfS*: 5′-GCTTGAGCAAGCAGCCGCGC-3′/5′-AGCGCCTGCAGGCCGTTCGC-3′

gnt: 5′-AGCTTTCCTTAAAAGAATCA-3′/5′-GAATTCGAGCTCGGTACCCG-3′

secA: 5′-GTCCGAGGTGCATAACGAGG-3′/5′-AAGCTTCAACAAGAAGATCA-3′

In Vitro Transcription—To reconstitute a holoenzyme, the core enzyme (1 pmol) was mixed with a fourfold excess of σ protein in the transcription buffer [18 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM $MgCl_2$, 20 μM EDTA, 8 mM 2-mercaptoethanol, 10% (v/v) glycerol] and incubated for 15 min on ice. DNA template (0.1 pmol) was mixed with a holoenzyme and incubated for 3 min at 37°C in 42.5 μl of a transcription buffer. After the pre-incubation, 7.5 μl of a nucleotide/heparin mixture {1.2 mM each ATP, GTP, CTP, 0.4 mM UTP, 120 kBq (3.24 μCi) [α-^{32}P]UTP [Institute of Isotopes, 15 TBq (405 Ci)/mmol], 1.33 mg/ml heparin} was added to allow single-round RNA synthesis. RNA synthesis was carried out for 5 min to produce run-off transcripts and was terminated by adding 50 μl of a stop solution (40 mM EDTA, 300 μg/ml yeast tRNA). Transcripts were precipitated with ethanol, dissolved in

the sample buffer [80% (v/v) of formamide, 0.1% (w/v) SDS, 8% (v/v) glycerol, 8 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] and analyzed by electrophoresis on polyacrylamide gels containing 8 M urea. Gels were analyzed with a Bioimage analyzer BAS2000 (Fuji Film).

Gel Shift Assay—Various amounts of σ^A or σ^H were incubated with a 5′-end-labeled promoter DNA fragment (0.1 pmol) in a 25 μl binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 50 μg/ml BSA, 50 μg/ml poly(dI-dC):poly(dI-dC), 1 mM DTT] for 5 min at 37°C. Reaction products were analyzed by electrophoresis through 4% polyacrylamide slab gel in a 33 mM Tris-phosphate buffer (pH 7.8). Following electrophoresis, the gels were analyzed with a Bioimage analyzer BAS2000.

DNase I Footprint Assay—DNase I footprinting was done essentially by the procedure of Aiba (*38*). A 5′-end-labeled, *spoVG* promoter DNA fragment (0.1 pmol) and proteins (as indicated in the legend to Fig. 8) were incubated in 100 μl of the footprinting buffer [20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM DTT, 0.1 mM EDTA, 50 μg/ml BSA] for 5 min at 37°C. DNase I (Takara Shuzo) was added at a concentration of 1 U/ml, and incubation was continued for 15 s at 37°C. Then 25 μl of DNase I stop solution [1.5 M sodium acetate (pH 5.3), 20 mM EDTA, 100 μg/ml yeast tRNA] was added. The mixture was treated with phenol, and the material was precipitated with ethanol. The products were analyzed on 8% PAGE containing 8 M urea. The gels were analyzed with a Bioimage analyzer, BAS2000.

RESULTS

Overproduction and Purification of σ^H—B. subtilis spo0H (sigH) gene was cloned into pGEM-T under the control of the strong promoter of bacteriophage T7. The resultant recombinant plasmid pGEMTSigH was transferred to *E. coli* BL21(DE3) containing a chromosomal copy of the T7 RNA polymerase gene which is under the control of the *lac* operator. Synthesis of σ^H protein was induced with IPTG. After IPTG was added, the growth of the cells stopped as the cells started to accumulate σ^H protein (27 kDa). Densitometric scanning revealed that the overproduced 27-kDa protein constituted approximately 30% of the total cellular protein (Fig. 1A, lane 2). The overproduced σ^H protein was found to be in the form of inclusion bodies that sedimented with the cell debris in the broken cells. Guanidine hydrochloride was chosen as the denaturing agent because it has been used successfully to extract and renature *E. coli* σ^{70} (*39*) and *B. subtilis* σ^A (*40*) from inclusion bodies. The renatured protein was recovered by passing the lysate through DE52. Almost all contaminating proteins were adsorbed on DE52 except σ^H. Using this procedure, we were able to purify about 20 mg of σ^H from a 500-ml culture. The identity of the overproduced σ^H was confirmed by amino-terminal sequencing of the first 15 amino acid residues, which completely matched those predicted from the DNA sequence. Further, the identification of σ^H was confirmed by its reactivity to anti-σ^H antibody (Fig. 1A, lanes 5-8) (*24*).

Overproduction and One-Step Purification of Histidine-Tagged σ^A—The *B. subtilis* σ^A gene was cloned into pET21b and placed under the control of the T7 promoter. We engineered a variant σ^A subunit carrying hexahistidine at its C-terminus. The resultant recombinant plasmid pETSigA was transferred to *E. coli* BL21 (DE3). Synthesis of the His-tagged σ^A was induced with IPTG. After IPTG induction, growth inhibition of the cell was observed, as reported (*40*). Densitometer scanning revealed that the overproduced σ^A protein (55 kDa) made up approximately 30% of the total cellular protein (data not shown). As in the case of σ^H, the overproduced σ^A protein was also found to be in the form of inclusion bodies. After solubilization with guanidine, the σ^A was loaded onto a Ni^{2+}-NTA column equilibrated with a Ni^{2+}-NTA-buffer containing 6 M guanidine-HCl. Proteins that were bound to the column were eluted with 5-600 mM imidazole gradients under denaturing conditions. The major 55-kDa protein eluted with the imidazole gradient was identified as σ^A by immunoblot analysis with anti-σ^A antibody (Fig. 1B) (*40*). Refolding of the denatured σ^A protein was carried out by stepwise dilution of guanidine-HCl (4, 2, and 0 M) in a dialysis procedure. We were able to purify about 40 mg of σ^A from a 500-ml culture. The first 15 amino acid residues of overproduced σ^A are the same as those predicted from DNA sequence analysis.

Promoter Selectivity of the Reconstituted σ^A- or σ^H-RNA Polymerase—To examine promoter recognition of the reconstituted σ^A- or σ^H-RNA polymerase, we carried out *in*

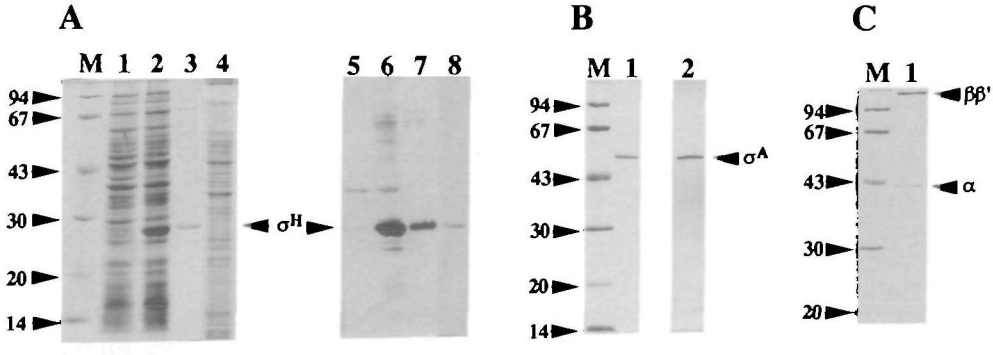

Fig. 1. **SDS-PAGE of purified σ^A and σ^H proteins.** A: Purification of σ^H (lanes 1 to 4) and Western blot analysis (lanes 5 to 8). Total proteins (10 μg) from IPTG (1.0 mM)-treated *E. coli* BL21 (DE3) cells containing pGEM-T (lanes 1 and 5), total proteins (10 μg) from IPTG (1.0 mM)-treated *E. coli* BL21 (DE3) cells containing pGEMT-SigH (lanes 2 and 6), and purified σ^H (0.5 μg) (lanes 3 and 7), and total proteins (10 μg) from *B. subtilis* JH642 that was grown at 37°C in 2×SG medium (*24*) to T_1 (1 h after the end of exponential growth) (lanes 4 and 8) were analyzed by 12.5% gel. B: Purified σ^A-C-his (0.5 μg) (lane 1) and its Western blot analysis (lane 2). C: Core RNA polymerase (0.3 μg) (lane 1). Lane M, molecular weight standards ($M_r \times 10^{-3}$). The positions of purified proteins are indicated.

vitro transcription from several promoters. The structures of the truncated DNA templates and the transcripts expected for each template are summarized in Fig. 2. The major transcripts from the templates were of the sizes expected if transcription had been initiated from the *in vivo* start sites of the respective promoters (Fig. 3). The σ^H-RNA polymerase correctly recognized the promoters (*orf146, spo0A* Ps, *spoVG, orf283, sigA* P4, *citG* P2, *kinA*, and *spo0F* P2), which are expected to be transcribed by σ^H-RNA polymerase *in vivo*, but did not recognize the σ^A promoters. Similarly, the σ^A-RNA polymerase recognized σ^A promoters (*abrB* P2, *kinC, gnt, xyl, orf253, spo0H, secA*, and *spo0A* Pv), but did not recognize the σ^H promoters. Hexahistidine appears to interfere with neither the interaction between the σ^A subunit and core RNA polymerase nor the recognition of the σ^A promoter sequence by the σ^A. These *in vitro* results indicate that a strict difference in promoter selectivity exists between the two molecular species of the RNA polymerase, as observed *in vivo*.

The transcription of *spo0F* is supposed to be regulated by dual promoters (P1 and P2) (*41*). During vegetative growth, transcripts from both promoters are detected, although the upstream promoter (P1) appears to be weak. It has been hypothesized that, at the onset of sporulation, Spo0A-P binds to site 1 of the *spo0F* P1 promoter which is recognized by σ^A-RNA polymerase, and Spo0A-P then prevents the transcription from P1 promoter and stimulates the transcription from the P2 promoter which is recognized by the σ^H-RNA polymerase (*42*). We could detect an σ^H-dependent transcript from P2 promoter, but the *spo0F* P1 promoter could only be partially recognized by σ^A-RNA polymerase *in vitro* (Fig. 3, lane 15), because the expected σ^A-dependent transcript from P1 promoter must be 193 bases long. However, an σ^A-dependent transcript with *spo0F* DNA was 126 bases long (Fig. 3, lane 15) which is the expected transcript from the major promoter of *orfS*. The *orfS* gene is located upstream of *spo0F* and is transcribed on the strand opposite the *spo0F* gene. Two start sites, minor and major ones, for the transcription of *orfS* have been reported (*35*). In the upstream region of the *orfS* transcription initiation site, the sequence 5′-TCTG-AG-17 bp-TATACA-3′ exists, which is similar to that of the σ^A promoter.

In addition, some other not yet identified RNAs transcribed by σ^A-RNA polymerase were also detected in the other reactions (Fig. 3, lanes 17, 19, and 23). Judging from the sizes, we assume the locations of the promoters of these RNAs to be as shown in Fig. 2; *i.e.*, 5′-CTTCTA-17 bp-AATTTT-3′ to be on the strand opposite *kinA*, 5′-ATC-GAA-17 bp-TTATAA-3′ to be on the same strand as *sigA* P4, and 5′-GGATAC-17 bp-TATTTT-3′ to be on the strand opposite *orf283*.

Next, the capacity of each promoter to accept RNA polymerase was estimated by measuring the quantities of RNAs detected in Fig. 3 and was used to determine the order of promoter strength. All of the promoters were saturated with the holoenzymes within 3 min of the preincubation (data not shown). The results are summarized in Fig. 4. However, there seems no correlation between the degree of −10 and −35 consensus and the amounts of the transcripts.

Competition of σ^A and σ^H Binding to Core RNA Polymerase—The transcription of the five σ factor genes, *sigH, sigE, sigK, sigF*, and *sigG*, depends on the action of the preceding σ factor in the developmental sigma cascade (*19, 20*). According to this cascade theory, the subsequent σ may possess higher affinity to the core RNA polymerase than to the preceding one (*22*). To test this, the affinity of the two species of σ subunits, σ^A and σ^H, to the core enzyme was examined. First, the σ saturation curves for maximum transcription by a fixed amount (1 pmol per assay) of a core enzyme were compared between the two σ subunits using σ^A-specific (*kinC*) and σ^H-specific (*spoVG*) promoters. The amount of σ subunit required for the maximum transcription of each promoter at a fixed amount (1 pmol) of a core enzyme was roughly 4 pmol, and the saturation curves for the two σ subunits were essentially the same (Fig. 5). Next, *in vitro* transcription was carried out using a fixed amount of core enzyme and various amounts of σ^A and σ^H in the presence of *kinC* and *spoVG* templates. The core enzyme

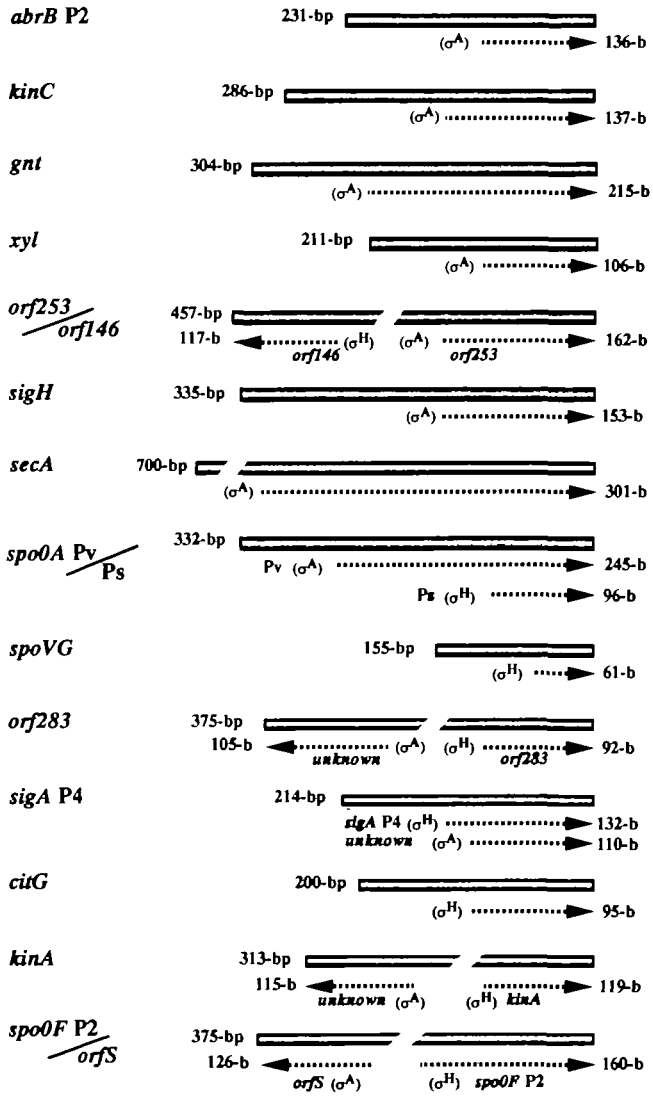

Fig. 2. **Structure of DNA templates and expected RNA products.** Truncated DNA templates carrying promoters were prepared by PCR. Bars indicate the DNA fragments. Hatched arrows indicate accurate transcripts directed by the respective DNA templates. The nucleotide lengths of DNAs and RNAs are shown. The promoter type (σ^A or σ^H) is indicated.

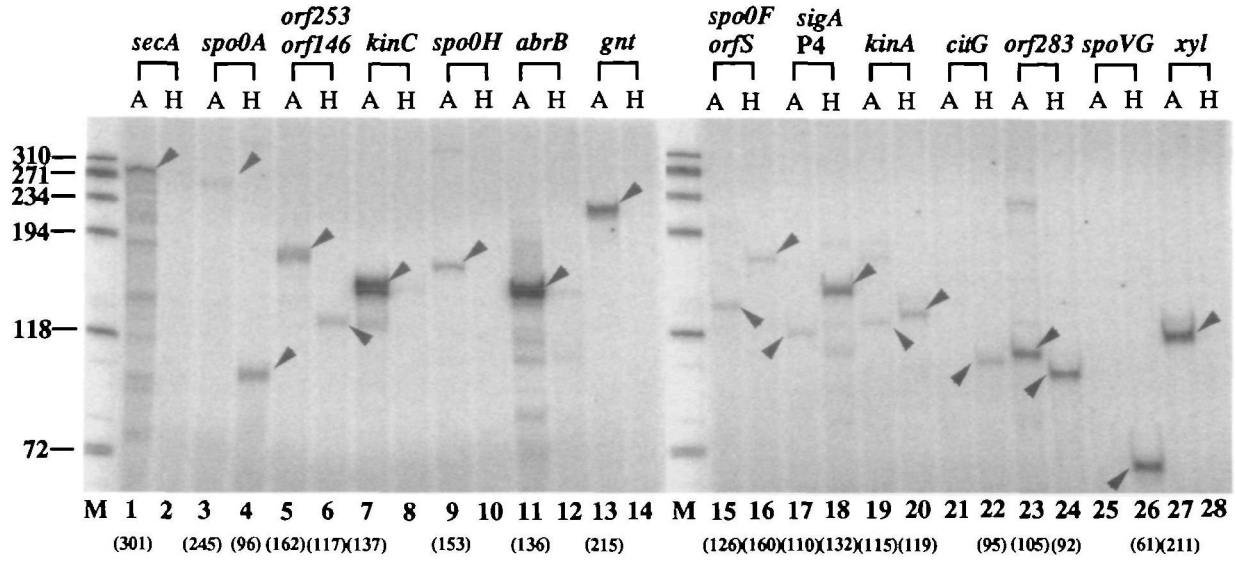

Fig. 3. ***In vitro* transcription experiments with the reconstituted Eσ^A and Eσ^H holoenzymes.** Single-round *in vitro* transcription assay was carried out with DNA templates containing the promoters. To form the open complexes, the reaction mixtures containing the reconstituted RNA polymerase and the DNA templates were pre-incubated for 3 min at 37°C before addition of the substrate solutions. Odd-numbered lanes are reactions with Eσ^A, and even-numbered lanes, with Eσ^H. The positions of specific transcripts are indicated by arrowheads. Lane M, size markers are indicated in bases. The numbers in the parentheses are numbers of bases of the expected transcripts.

[A]

σ^A Promoter		-35	-10	+1	Transcription Level(%)
abrB P2	gAcTATAAgAAcTAATTcTTAcAATcAATAgTAAAcAAAATgA	TTgAcg	ATTATTggAAAccTTgT	TATgcT	ATgAAgg	100
kinC	AAgATATTTggTAATgAATgATTTgggATAcTTTAcATATTTTA	cTcAAT	TATTTgTcgAAgAATgg	TAcAAT	AAgTAg	63
gnt	AATATggTAAAAATTTAAATAAAAATTAgAAATgAAAgTgT	TTgcAT	AAAAgAAATATTcAcgT	TATcAT	AcTTgTATA	37
xyl	AccTTTATTATATcTAATgTgTTcATgAAAAAcTAAAAAAAATA	TTgAAA	ATAcTgATgAggTTATT	TAAgAT	TAAAAT	30
orf253	TTcAATTATTTcccTTcTgATTcggAAcAAAATgATgAAAAAA	ATgAAA	cgcTTTAATcTTTTTTTgA	TAgAAT	AgAAg	10
secA	TTAATTgAAccgAcTgAATAATgAAgAgAAgccTTccgTgATg	Tccgcg	gAAggTTTTTgTTTTTc	TTATTT	gcAAATT	8
spo0H	TTgAAAAcgTTTgAAAAgTggAggcgggggAgAcTTAgATTAAg	TTgAcg	cTTTTTTgcccAATAcTg	TATAAT	ATTTcT	7
spo0A Pv	AAgcTgAcTgccggAgTTTccggcAgTTTTTTTTATTTTgATcccTc	TTcAcT	TcTcAgAATAcATAcgg	TAAAAT	ATAc	5

[B]

σ^H Promoter		-35	-10	+1	
spoVG	TAAcTATATccTATTTTTTcAAAAAATATTTTAAAAAcgAgc	AggA	TTTcAgAAAAAATcgTg	gAAT	TgATAcAcTA	100
orf283	AcAATAccATcATATTTTcATcAAAATTgATggATgTAcAgc	AggA	AAAAccATgAgAAAATA	gAAT	TATAAAAATg	43
sigA P4	ATTTATTAcAcTgcTgcAAAAAATccTgTcgAAcgATgAA	gggA	TTTTgcAcTAAAgcATc	gAAT	AATgTAcgAcgg	42
spo0A Ps	AAAgATATgccAcTAATATTggTgATTATgATTTTTTTAg	Aggg	TATATAgcggTTTTgTc	gAAT	gTAAAcATgTAg	30
citG	TTgAATTAAAATTAAAATcTgccAAgATcgAAAAATAAAA	AggA	TTTTTTgTgTcATTggc	gAAT	TATgATcTATTg	23
kinA	ccTTTcTTTTATTcAAAAATTgAcgTTcAccATAAgAATAgA	AggA	gAATAcTcATTTTcTAgc	gAAT	cATAcTAgg	13
orf146	TcTTATAAATTcTgcAATTTcTATgTTTTTTTgAcTTAAAAA	AggA	ATTTcTTAAAgAAAAAAA	gAAg	AAgTcAcAg	8
spo0F P2	gAAAATgcTcAgAAAATgTcgTAAAgTAgAcTATTATAATTAA	AggA	AATAggAAAATcAAAcA	gAAT	AcATAcAAT	4

Fig. 4. **Alignment of promoters tested in this study and comparison of the promoter strength.** Transcription level is expressed in arbitrary units after correction for the U content of each transcript in Fig. 3. A: Promoters transcribed by Eσ^A. B: Promoters transcribed by Eσ^H. The transcription start site, +1, and −35 and −10 position relative to the +1 site are marked. The promoter sequences (−35 and −10) proposed for each gene are underlined. A and T are shown in capital letters.

was saturated with σ factor under the conditions shown in Fig. 5. Even though the concentration of σ^A is much lower than that of σ^H, *kinC* was transcribed preferentially by Eσ^A (Fig. 6). On the other hand, *spoVG* transcribed by Eσ^H was easily blocked by increasing the amount of σ^A. Contrary to the hypothesis described above, these results suggest that the affinity of σ^H to the core enzyme is weaker than that of σ^A.

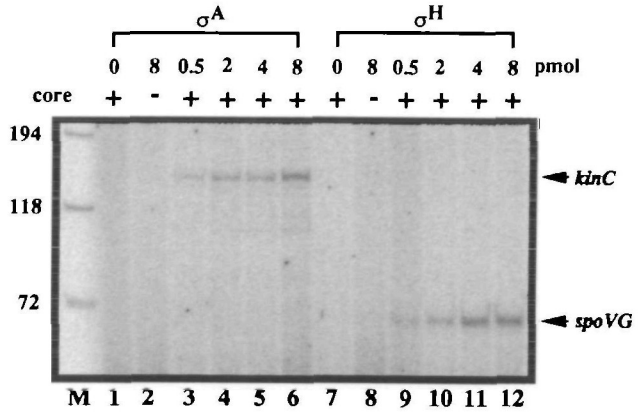

Fig. 5. **Saturation curve of σ subunit for maximum transcription.** Core enzyme (1 pmol) and various amounts of σ^A (lanes 1 to 6) and σ^H (lanes 7 to 12) were mixed and used for single-round transcription with *kinC* (lanes 1 to 6) and *spoVG* (lanes 6 to 12) template DNAs. Proteins were added at the indicated amounts.

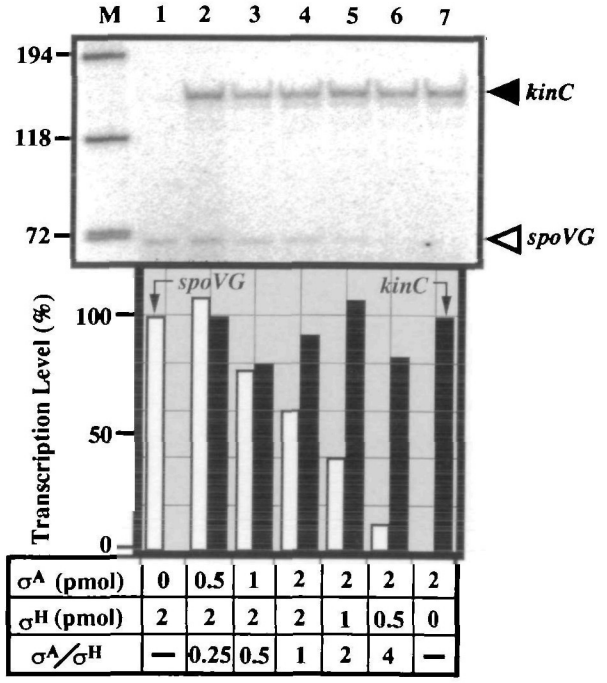

Fig. 6. **Competition of σ^A and σ^H for binding to core RNA polymerase.** The *in vitro* single-round transcription was carried out using a fixed amount of core enzyme and varying amounts of σ subunits. Core enzyme (0.5 pmol), and varying amounts of σ^A and σ^H were mixed in transcription buffer and incubated for 10 min at 0°C to form holoenzymes in a single tube. The template mixture containing *kinC* and *spoVG* template DNAs was added to the RNA polymerase mixture and incubated for 3 min at 37°C. After addition of a substrate mixture containing heparin, RNA synthesis was carried out for 5 min at 37°C. RNA was analyzed by gel electrophoresis. Intensities of bands were quantitated with a BAS-2000 Bio-Imaging Analyzer, normalized to the maximum level, and are expressed in arbitrary units. Lane M, size markers are indicated in bases.

DNA Binding Activity of σ^H—Deletion analysis of *E. coli* σ^{70} indicated that the amino-terminal region 1.1 (*3*) inhibited DNA binding by the carboxy-terminal domains (*43*). *B. subtilis* σ^D lacking conserved region 1.1 is able to bind DNA (*44*). Since σ^H lacks conserved region 1.1 (*3*), it is predicted to have intrinsic DNA-binding ability. To confirm this prediction, the ability of σ^H to form stable complexes with promoter DNA was investigated. Increasing the concentrations of σ^H led to the formation of a discrete band containing a DNA fragment of σ^H-dependent *spoVG* or *sigA* P4 promoter in an electrophoretic mobility shift assay (Fig. 7, A and B, lanes 1-6). When the *kinC* promoter DNA, which is transcribed by σ^A-RNA polymerase but not by σ^H-RNA polymerase, was used in the assay, the shifted band was also observed in the presence of σ^H (Fig. 7C, lanes 1-6). However, σ^A-promoter DNA complex was not observed (Fig. 7, A, B, and C, lanes 7-12). These results

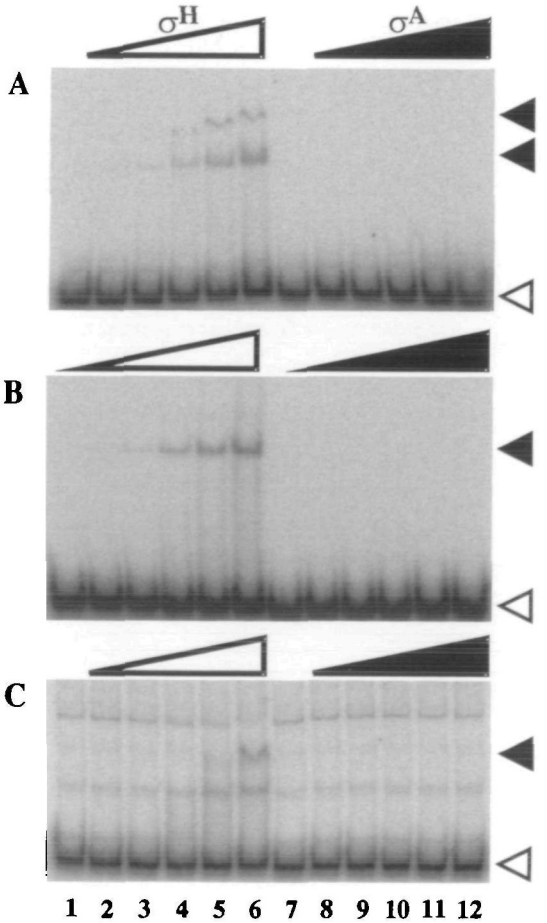

Fig. 7. **Binding of σ to DNA.** Binding of σ^A or σ^H to the template DNA was assessed in a gel mobility-shift assay. Increasing amounts of σ proteins (lanes 1 to 6 contained 0, 0.5, 1, 5, 10, 15 pmol, respectively) were allowed to bind for 10 min at 37°C to 0.1 pmol of ^{32}P-labeled DNA templates (A, *spoVG*; B, *sigA* P4; C, *kinC*). σ proteins used are indicated. Free DNA (open arrowhead) and σ-DNA complexes (closed arrowhead) are indicated on the right side. The faint bands on panel C are PCR by-products.

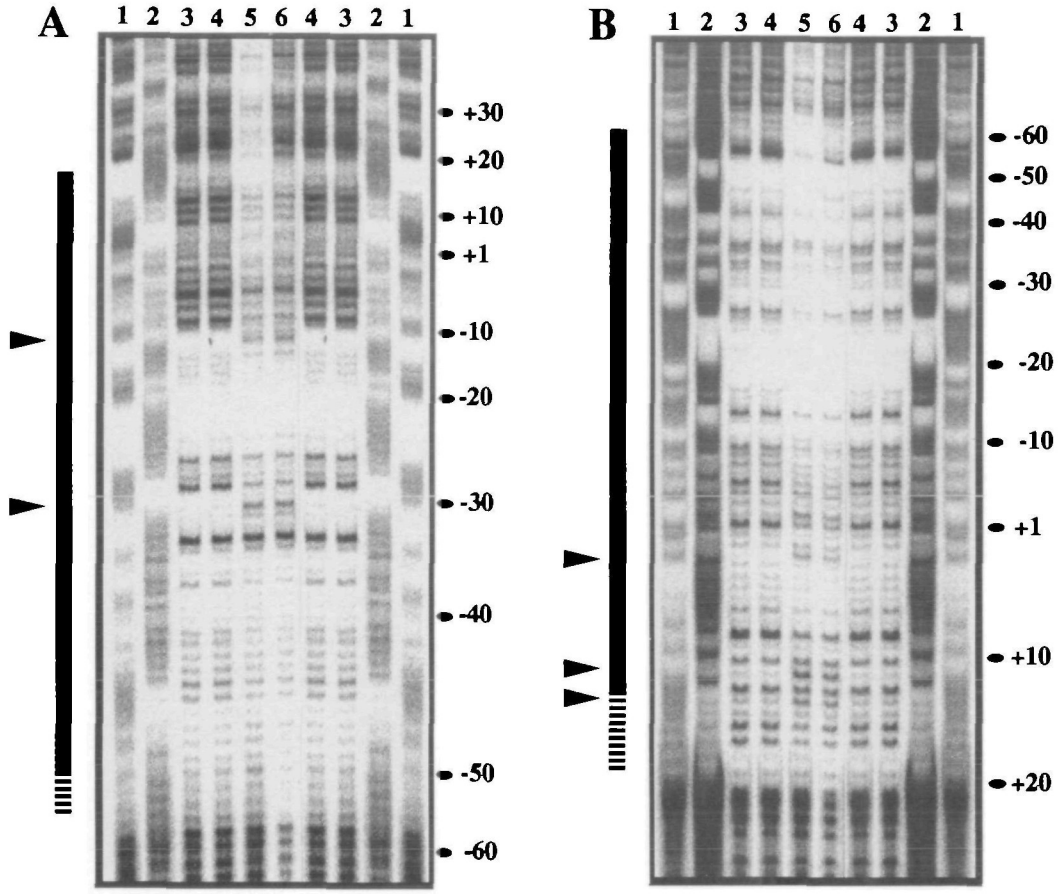

Fig. 8. **DNase I footprinting experiments on the *spoVG* promoter of σ^H.** A: Top strand. Lane 1, C and T chemical sequencing ladder; lane 2, A and G chemical sequencing ladder; lane 3, no protein control; lane 4, 3 pmol core enzyme; lane 5, 15 pmol σ^H; lane 6, Eσ^H holoenzyme reconstituted with 3 pmol of core enzyme and 6 pmol of σ^H protein. Binding reactions were carried out in footprinting buffer containing 0.1 pmol ^{32}P-labeled DNA. Nucleotide positions relative to the transcription initiation site (+1) are indicated on the right. The protected region is indicated on the left by a bar. B: Bottom strand. The lane assignments, nucleotide positions, and symbols are the same as in A.

```
5'-agcttt atgacctaat tgtgtaacta tatcctattt
3'-tcgaaa tactggatta acacattgat ataggataaa

tttcaaaaaa tattttaaaa acgagcagga tttcagaaaa
aaagtttttt ataaaatttt tgctcgtcct aaagtctttt
                    →RNA
aatcgtggaa ttgatacact aatgctttta tatagggaaa
ttagcacctt aactatgtga ttacgaaaat atatcccttt

aggtggtgaa ctactgtgga agttactgac gtaagat-3'
tccaccactt gatgacacct tcaatgactg cattcta-5'
```

Fig. 9. **Summary of DNase I footprint data.** The sequence of the *spoVG* promoter is shown. The boundaries of the region protected from DNase I by Eσ^H are indicated by bars and the hypersensitive sites induced by σ^H and Eσ^H are indicated by vertical arrows. The −35 and −10 regions are underlined.

indicate that σ^H can bind to DNA, but that this binding might be non-specific. To investigate the topology of the σ^H-DNA complex, we performed a DNase I footprinting experiment using DNA containing a *spoVG* promoter. σ^H alone protected the *spoVG* promoter region both on the top and bottom strands, and the holoenzyme containing σ^H protected a more restricted region (Fig. 8, A and B, lanes 5 and 6). RNA polymerase core alone did not protect the *spoVG* promoter region (Fig. 8, A and B, lane 4). These results indicate that σ^H alone protects the *spoVG* promoter region non specifically, and that holoenzyme may recognize the *spoVG* promoter more correctly. DNase I hypersensitive sites at +14 (bottom strand), +12 (bottom strand), +3 (bottom strand), −11 (top strand), and −30 (top strand) relative to the transcription start site are induced not only by σ^H, but also by σ^H-RNA polymerase. Since this assay was done in solution, the results could be interpreted as the sum of two or more separate interactions on different DNA molecules.

DISCUSSION

Promoter Selectivity between σ^A- and σ^H-RNA Polymerases—In this study, we systematically analyzed promoter recognition by σ^A- and σ^H-RNA polymerases of *B. subtilis*. The strict promoter selectivity of each RNA polymerase is probably attributable to the conserved sequence of each cognate promoter. The sequences, TTXWHR and TAYY-YT (X=G or C; Y=A or T; W=A, G, or C; H=A or C; R=A, G, or T) separated by 17 bases were conserved in the −35 and −10 regions of σ^A-dependent promoters. The σ^H promoters analyzed, −35 and −10 regions AGGA and

GAAT separated by 17 bases, were highly conserved (Fig. 4). However, no correlation between the apparent promoter strength and the degree of consensus was apparent from our results, as described above. For all of the promoters analyzed in this paper, σ^A- or σ^H-RNA polymerase did not require any nucleotides in the pre-incubation mixture of RNA polymerase and template DNA to produce transcripts. In other words, open complex formation, which is heparin-resistant, occurred in the absence of any nucleotides, leading to the transcription of each promoter. The apparent incongruity between degree of conservation of promoters and promoter strength obtained in this study may suggest that either the strength of a promoter is determined by other elements *in vivo* or that heparin affects the initial interaction between promoter DNA and holoenzyme, resulting in weak production of transcripts.

Reconstitution of the σ Cascade In Vitro—The affinity of σ^H to core RNA polymerase is lower than that of σ^A (Fig. 7). This observation is apparently in conflict with one of the principles of the sigma cascade; each of the successive σ factors in a developmental cascade would have to bind more tightly to core RNA polymerase than the preceding σ factor. However, Hicks and Grossman (*22*) reported that relatively small changes in the amount of σ^A can cause large changes in the expression of genes controlled by σ^H. These results are most easily explained by a simple competition model, where changes in the amount of or slight inactivation of σ^A in the cell would alter the ability of σ^H to bind to free core RNA polymerase. In fact, the loss of σ^A activity and the disappearance of extractable σ^A-RNA polymerase from sporulating *B. subtilis* has been reported, although anti-σ^A antibody precipitates similar amounts of σ^A from crude extracts of vegetative and sporulating cells (*45*). These results may suggest that, once the early sporulation genes are expressed, σ^A becomes largely inactive and loses its ability to compete with the σ^H factor for the core.

Recently, several complex regulatory systems controlling selective transcription of growth-phase-specific gene in *E. coli* have been reported. The intracellular environment, such as concentrations of potassium glutamate or trehalose, or DNA configuration may be important for replacement of σ on RNA polymerase (*46*). However, little is known of the intracellular conditions controlling transcription in *B. subtilis*. The data presented in this report suggest that growth phase-coupled replacement of core RNA polymerase-associated σ subunit from σ^A to σ^H may require an additional factor or modification of the core enzyme in addition to inactivation of σ^A factor.

DNA-Binding by σ—As described in the text, σ^H protein alone bound to the DNA (Figs. 8 and 9). This is expected, since σ^H lacks N-terminal region 1.1 which has been reported to inhibit DNA binding (*43*). A threonine-to-isoleucine substitution at position 100 of σ^H suppressed the effect of G·C to A·T substitution at position −13 in the *spoVG* promoter (*33*). Substitution of threonine to alanine at position 100 of σ^H, which removes the amino acid side chain present at this position, resulted in a σ factor that was unable to discriminate *spoVG* promoter derivatives that differed by only a single base pair substitution at position −13 (*47*). Thus, these *in vivo* results suggest that the amino acid at position 100 of σ^H directly contacts the base pairs of the promoter. However, our results suggest that this binding might be non-specific (Figs. 8 and 9). Therefore, the promoter might be recognized by σ^H-RNA polymerase holoenzyme, but not by σ^H alone. The interaction between σ^H and DNA may be regulated at the level of the protein structure and/or conformational folding. Therefore, upon interaction with the core enzyme to generate holoenzyme, σ^H may undergo a conformational change that exposes the DNA-binding domain and permits sequence-specific promoter recognition. Thus, *in vivo* promoter recognition is almost certainly mediated by the holoenzyme. The physiological role of the binding ability of σ^H to DNA, however, remains unclear.

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