

The core-independent promoter-specific interaction of primary sigma factor

Hsin-Yi Yeh, Tsung-Ching Chen, Kung-Ming Liou, Hsiu-Ting Hsu, Kuei-Min Chung, Li-Ling Hsu and Ban-Yang Chang*

Institute of Biochemistry, National Chung-Hsing University, Taichung 40227, Taiwan, ROC

Received September 2, 2010; Revised September 22, 2010; Accepted September 23, 2010

ABSTRACT

Previous studies have led to a model in which the promoter-specific recognition of prokaryotic transcription initiation factor, sigma (σ), is core dependent. Most σ functions were studied on the basis of this tenet. Here, we provide *in vitro* evidence demonstrating that the intact *Bacillus subtilis* primary sigma, σ^A , by itself, is able to interact specifically with promoter deoxyribonucleic acid (DNA), albeit with low sequence selectivity. The core-independent promoter-specific interaction of the σ^A is -10 specific. However, the promoter -10 specific interaction is unable to allow the σ^A to discern the optimal promoter spacing. To fulfill this goal, the σ^A requires assistance from core RNA polymerase (RNAP). The ability of σ , by itself, to interact specifically with promoter might introduce a critical new dimension of study in prokaryotic σ function.

INTRODUCTION

The bacterial RNA polymerase holoenzyme (holo RNAP) is composed of the catalytic core RNAP, $\alpha_2\beta\beta'$ and the transcription initiation factor, sigma (σ). The functions of σ have been studied since 1969 (1). It is now clear that σ confers upon holo RNAP the ability to recognize and melt promoter deoxyribonucleic acid (DNA) during transcription initiation (2–6).

The bacterial σ factors are classified into the σ^{70} and σ^{54} families (7). The σ^{70} family members in all bacteria can be subdivided into four groups. The Group 1, or primary, σ s are responsible for the transcription of house-keeping genes and are essential for viability, whereas the Groups 2–4, or alternative, σ s are required for more specialized functions such as stress responses. The primary σ s have four conserved regions (Regions

1–4) with each having 2–4 subregions, whereas the alternative σ s of Groups 3–4 lack Region 1 (7–9). Regions 2.4, 3.0 and 4.2 are thought to interact with the -10 , extended -10 (or TG motif) and -35 promoter elements, respectively, according to the results of genetic and biochemical analyses of σ in the context of holo RNAP (10–15). The idea for direct promoter interaction of σ independent of core RNAP has been put to the test (16–21); however, it was only observed for a group 3 σ , the σ^D of *Bacillus subtilis* (17). Thus, it was generally believed that the primary σ has to associate with core RNAP prior to interaction with promoter DNA.

The inability of primary σ , by itself, to bind promoter DNA was once ascribed to the auto-inhibition of σ through direct interaction between Regions 1.1 and 4.2 of the σ (22,23). However, the studies of a *Thermotoga maritima* σ revealed that this inhibition is owing to an indirect steric and/or electrostatic mechanism (24) or to the close proximity of the negatively charged Region 1.1 to the positively charged promoter recognition domains of σ , which makes σ adopt a compact structure incompatible with DNA binding (25). Multiple switches are thought to be built into σ to alleviate the auto-inhibition (26). They include the interactions between the flap domain of the β -subunit of core RNAP and Region 4 of the σ (27,28) as well as between the coiled coil domain of the β' -subunit and Region 2.2 of the σ (29). Although the switch mechanism may explain why σ is unable to bind promoter DNA in a core-independent manner, direct evidence supporting the hypothesis that σ , by itself, is unable to bind promoter DNA is still lacking. Actually, the failure to detect the core-independent promoter-specific interaction of σ could be attributed to the use of a σ devoid of promoter DNA-binding conformation or the use of an inappropriate condition for assaying the binding interaction. Since how σ recognizes promoter DNA would affect how we elucidate the functional mechanisms of σ and core RNAP during transcription initiation, we re-investigated this issue.

*To whom correspondence should be addressed. Tel: 886 4 2285 3486; Fax: 886 4 2285 3487; Email: bychang@mail.nchu.edu.tw

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Besides the recognition of -10 element, TG motif and -35 element by σ , the spacer DNA, which separates the -10 and -35 elements and is important for a promoter to function, is also proposed to be recognized by σ (30). In general, it is the length of the spacer DNA (spacing), rather than the sequence (except the TG motif), which is highly homologous (31–33). Usually, the 17 ± 1 bp spacing confers the optimal activity on promoters recognized by the primary σ (34–39). The main roles of the spacer DNA are thought to hold a more favorable orientation of the -10 element relative to the -35 element for initial binding of RNAP and for facilitating subsequent formation of a competent RNAP–DNA open complex (40–46). However, the mechanism leading to the recognition of optimal promoter spacing by RNAP remains unclear. Using a series of glutathione *S*-transferase (GST)-fused partial σ^{70} polypeptides, which exhibit specific binding to either one or both of the -10 and -35 elements of *tac* promoter, for competition assay, it was inferred that the σ^{70} , by itself is a molecular ruler, which is able to discern the optimal spacing of the *tac* promoter (23,30). In this study, we also re-examined the inference using the *B. subtilis* σ^A .

Here, we provide *in vitro* evidence demonstrating that the house-keeping *B. subtilis* σ^A is able to bind specifically to the -10 element of promoters independent of core RNAP. However, the σ^A is unable to discern the optimal promoter spacing. To fulfill this goal, it requires the assistance from core RNAP.

MATERIALS AND METHODS

Overproduction and purification of σ^A

Methods used for overproduction and purification of σ^A in inclusion bodies are similar to those reported previously (47). The cell lysate was centrifuged at 7500g to harvest the σ^A -containing inclusion bodies that were then denatured with TEDG buffer [10 mM Tris–hydrochloric acid (HCl), pH 7.9, 0.1 mM ethylenediaminetetraacetic (EDTA), 0.1 mM dithiothreitol (DTT), 0.2 mM phenylmethanesulfonyl fluoride (PMSF), and 5% glycerol] supplemented with 200 mM NaCl and 7 M guanidine–HCl (Gn–HCl), and refolded through drop-by-drop dilution of the denatured protein with the TEDG buffer containing 200 mM NaCl until the concentration of Gn–HCl was ~ 55 mM. The refolded σ^A was centrifuged at 7500g to remove unfolded proteins. The soluble σ^A in the supernatant was then precipitated with 40% (w/v) ammonium sulfate and resuspended in TEDG buffer containing 200 mM NaCl before further purification with the molecular sieving columns, Superdex HR-200 and Superdex SD-75.

To overproduce soluble σ^A , *Escherichia coli* BL21 (DE3)/pCD2 or *E. coli* BL21 (DE3)/pCD2M, which is able to express the wild-type (Wt) σ^A or the mutant (Mu) σ^A with Gln-196-Ala and Thr-199-Ala substitutions, respectively, was grown at 37°C to an OD₅₅₀ of 0.5 before being transferred to 16°C. After incubation of the culture at 16°C for 10 min, isopropyl β -D-1-thiogalactopyranoside (IPTG) (final concentration 0.4 mM) was added to induce

the expression of σ^A . Fifty minutes later, rifampicin (final concentration 100 μ g/ml) was added into the culture which was further incubated at 16°C for 12 h. The *E. coli* cells were harvested and disrupted with a microfluidizer in TEDG buffer supplemented with 100 mM NaCl and then centrifuged at 7500g. The soluble σ^A in the supernatant was absorbed with 20 g of diethylaminoethyl cellulose (DEAE)-cellulose pre-equilibrated with TEDG buffer containing 100 mM NaCl. The DEAE cellulose was packed into a column and a NaCl gradient from 0.1 to 0.45 M was applied. The major σ^A -containing fractions were pooled and the σ^A were precipitated with 40% (w/v) ammonium sulfate and resuspended in TEDG buffer containing 200 mM NaCl before further purification with the molecular sieving columns, Superdex HR-200 and Superdex SD-75.

Both the refolded and soluble σ^A purified had homogeneities higher than 95% and were stored in storage buffer (20 mM Tris–HCl, pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 200 mM KCl, 0.1 mM DTT, 0.2 mM PMSF and 50% glycerol).

Mutagenesis of the Gln-196 and Thr-199 in the Region 2.4 of σ^A

The *sigA* gene with Gln-196-Ala and Thr-199-Ala substitutions was constructed using the QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene). The plasmid, pCD2, which contains the full-length *sigA* gene, was used as the DNA template for mutagenesis. The two primers used for codon replacement are as follows: *sigA*-196-199A-F: 5'-GCTACGTGGTGGATCAGAGCGGCGAT TGCACGCGCCATTGCCGATC-3' and *sigA*-196-199A-R: 5'-GATCGGCAATGGCGCGTGCAATCGCCGCT CTGATCCACCACGTAGC-3'. The plasmid containing the correct mutant *sigA* gene was named pCD2M.

Construction of the spacing variants of the *B. subtilis* *trnS* promoter DNA

The *trnS* spacing variants with a 14- to 21-bp spacing (Figure 6A) were created by the QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene). The templates and primer pairs used for the construction were shown in Supplementary Table S1. All of the resultant spacing variants were sequenced to be correct and were named as *trnS*-X, in which X indicates the length of spacer ranging from 14 to 21 bp.

Construction of the Mu G3b and *trnS*-17 promoters used for competition assay

The mutation at the -10 , -35 element or TG motif of G3b or *trnS*-17 promoter was created using the QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene). The templates and primer pairs used for the construction were shown in Supplementary Table S1. All of the promoter mutations were confirmed by DNA sequencing.

To prepare competitor DNA, the Wt and Mu G3b promoter DNA fragments (182 bp in length, spanning from +67 to -115 of the G3b promoter) were synthesized by polymerase chain reaction (PCR). The Wt G3b promoter DNA-containing plasmid, pCT-G3b and the

pCT-G3b-derived plasmids containing mutation at -10, -35 element, TG motif or either two elements of the G3b promoter were used as templates and BC1041-BamHI (5'-GAGCTCGGATCCAGAGAACGTAGACAACA A-3') and BC1046-HindIII (5'-CTGCAGAAGCTTGCC ATTTCTTCGTCCCACCTTTC-3') as primers. Similarly, the Wt and Mu *trnS*-17 promoter DNA fragments (95 bp in length, spanning from +15 to -80 of the *trnS*-17 promoter) were synthesized by PCR. The Wt *trnS* promoter DNA-containing plasmid, pOtrnS and the pOtrnS-derived plasmids containing mutation at either -10, -35 or both elements of the *trnS*-17 promoter were used as templates, and trnSF5 (5'-CATTATAAAAGTCG TCACG-3') and trnSR3 (5'-TGTGAATAATGAGACAA GAC-3') were used as primers. The promoter DNA fragments thus synthesized were further run on a 2% agarose gel, cut out from the gel, and purified using the Micro-Elute DNA Clean/Extraction Kit (GeneMark). The purified DNA was diluted with DNA-binding buffer [50 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 160 mM KCl, 0.1 mM EDTA, 0.1 mM DTT and 5% (v/v) glycerol] before being used.

Synthesis and labeling of the promoter DNA fragments

The Wt and Mu G3b promoter DNA were synthesized by PCR using the pCT-G3b or the pCT-G3b-derived plasmids containing mutation at -10, -35 or both elements of the G3b promoter as DNA templates and BC1041-BamHI and BC1046-HindIII as primers. The promoter DNA fragments of *trnS*-X were synthesized by PCR using the pOtrnS (containing *trnS*-17) and pOtrnS-derived plasmids containing *trnS*-X (X is from 14 to 21 except 17) as templates and trnSF5 and trnSR3 as primers. To label the 5'-end of promoter DNA fragments, either reverse or forward primer was labeled by [γ -³²P]-adenosine triphosphate (ATP) prior to PCR. For labeling template strand DNA, reverse primer (BC1046-HindIII or trnSR3) was ³²P-labeled; for labeling non-template strand DNA, forward primer (BC1041-BamHI or trnSF5) was ³²P-labeled. To label primer, 20 pmol of the primer DNA was mixed with 20 pmol of [γ -³²P]-ATP and 10 U of T4 polynucleotide kinase (Roche) in 20 μ l of phosphorylation buffer (50 mM Tris-HCl, pH 8.2, 10 mM MgCl₂, 0.1 mM EDTA, 5 mM DTT and 0.1 mM spermidine), then the mixture was incubated at 37°C for 2 h. The labeled promoter DNA fragments were run on a 2% agarose gel, cut out from the gel and purified using the Micro-Elute DNA Clean /Extraction Kit (GeneMark). The purified DNA was diluted with DNA-binding buffer before being used.

Electrophoretic mobility shift assay

For electrophoretic mobility shift assay (EMSA), the σ^A or σ^A -RNAP was incubated with ³²P-labeled G3b (1 nM) or *trnS* (0.5 nM) promoter DNA at 37°C for 10 min. The final volume of the binding mixture was adjusted to 10 μ l by adding the DNA-binding buffer. The binding of σ^A to promoter DNA was performed in the presence of 0.1 μ M heparin. The complex of σ^A and promoter DNA was then run on a 5% non-denaturing polyacrylamide gel in 1 \times

tris-acetate-EDTA (TAE) buffer (40 mM tris-acetate, pH 8.5 and 2 mM EDTA) at 4°C. The complex of σ^A -RNAP and promoter DNA was run at room temperature. Finally, the gel was dried and analyzed with a Fuji BAS 2500-phosphorimager. For reconstitution of holo RNAP, core RNAP and 10-fold molar excess of σ^A were incubated at 37°C for 10 min.

In EMSA-based competition assay, the preformed σ^A -G3b promoter DNA complex obtained by incubating ³²P-labeled G3b promoter DNA (1 nM) with an amount of σ^A in the presence of 0.1 μ M heparin at 37°C for 10 min was challenged with the cold Wt, Mu G3b promoter DNA or non-promoter DNA (*tgbp2* cDNA of *Bamboo mosaic virus*) for another 10 min. Then, the samples were run on a 5% non-denaturing polyacrylamide gel in a 4°C-cold chamber. Similarly, the σ^A -*trnS*-X promoter DNA complex was preformed by mixing ³²P-labeled *trnS*-X promoter (0.5 nM) with σ^A in the presence of 0.1 μ M heparin. Then, the cold Wt or Mu *trnS*-17 promoter DNA was added. The mixture was further incubated at 37°C for 10 min before being electrophoresed at 4°C. The fractional retention of the preformed binary complex was measured as a function of the molar ratio of competitor DNA to labeled promoter DNA.

DNase I footprinting assay

The σ^A or σ^A -RNAP was incubated with 0.5 nM ³²P-labeled G3b or *trnS* promoter DNA for 30 min at various temperatures (20, 25 and 37°C). The final volume of the binding mixture was 40 μ l and the final concentrations of buffer components in the binding mixture were 41.6 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 0.1 mM DTT, 0.35 mM EDTA, 60 mM KCl, 100 μ g/ml bovine serum albumin (BSA) and 17.6% glycerol. After incubation, an appropriate amount of DNase I (Roche) was added to digest the DNA. For binding at 20°C, 0.6 (for G3b promoter) or 0.25 U (for *trnS* promoter) of DNase I was added; for binding at 25°C, 0.4 U (for G3b promoter) of DNase I was added; for binding at 37°C, 0.25 (for G3b promoter) or 0.1 U (for *trnS* promoter) of DNase I was added. After a proper period of time, 40 μ l of stop buffer (50 mM Tris-HCl, pH 7.9, 200 mM NaCl, 20 mM EDTA and 1 mg/ml glycogen) was added to stop the digestion reaction. The digested DNA was extracted with phenol-chloroform. The DNA fragments in the upper aqueous layer were collected and precipitated with 0.3 M NaOAc and 2.5-fold volumes of 100% ethanol, washed twice with 70% ethanol, heat dried (60°C), dissolved in 10 μ l of loading buffer (98% deionized formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol) and electrophoresed with an 8% (w/v) polyacrylamide/8 M urea sequencing gel in 1 \times tris-borate-EDTA (TBE) buffer (90 mM tris-boric acid, pH 8.5 and 2 mM EDTA).

RESULTS

Purification of the *B. subtilis* primary σ , σ^A , with a core-independent promoter DNA-binding activity

The *B. subtilis* σ^A is a primary σ factor. To obtain the σ^A active in promoter DNA binding, we modified the

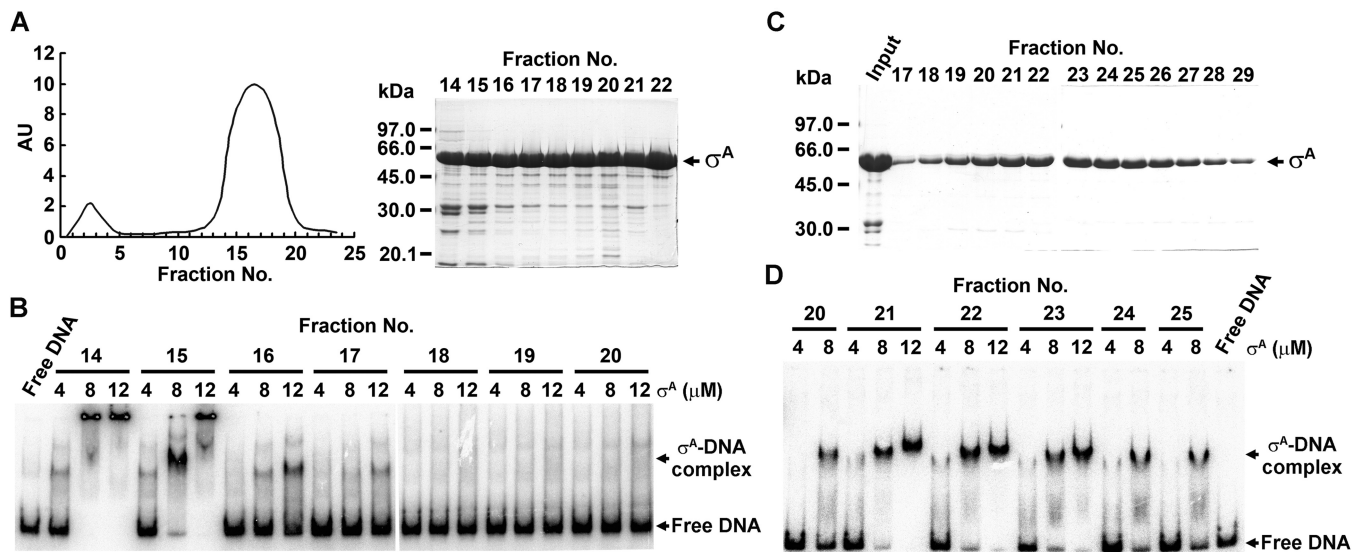


Figure 1. Purification of the *B. subtilis* σ^A with a core-independent promoter DNA-binding activity. (A and C) Purification of the *in vitro* refolded *B. subtilis* σ^A by molecular sieving columns, Superdex HR-200 (A) and SD-75 (C). AU as shown in the left panel of (A) indicates the absorption unit of the fractionated samples. The fraction no. shown on top of the SDS-polyacrylamide gels indicates the fraction of the σ^A -containing sample collected from the two columns. (B and D) The G3b promoter DNA-binding activity of σ^A fractionated by Superdex HR-200 (B) and SD-75 (D) columns. The numbers above the horizontal lines are the fractions of σ^A -containing sample collected from the two columns. The numbers shown below the horizontal lines are the concentrations (μM) of σ^A used.

methods used to purify the overexpressed σ^A from *E. coli* (16,47,48). Basically, the σ^A -containing inclusion bodies were denatured with 7 M Gn-HCl and diluted slowly with refolding buffer drop by drop, rather than stepwise. Then, the refolded σ^A was subjected to purification with two consecutive molecular sieving columns, Superdex HR-200 and SD-75, rather than with an anion exchange (DEAE cellulose) and a molecular sieving (Sephadex-G75) column. With these modifications, the σ^A with promoter DNA-binding activity was achieved. Shown in Figure 1A and B are the results of σ^A purification with Superdex HR-200 and the promoter DNA-binding activities of the fractionated σ^A as examined by EMSA. Significant binding of the tested ϕ 29 phage G3b promoter DNA was observed for the σ^A eluted earlier from the Superdex HR-200 column (fraction nos 14–16), suggesting that the σ^A active in promoter DNA binding has a relatively looser conformation. Because the σ^A sample was still low in homogeneity at this step, we further purified it with an SD-75 molecular sieving column. As shown in Figure 1C and D, the σ^A eluted from the SD-75 column (fraction nos 21–23) had a much improved homogeneity (~95%) and was also active in promoter DNA binding with a relatively high apparent dissociation constant (K_d) of about 5 μM , which is similar to that reported for the complex of *B. subtilis* σ^D and its cognate promoter (17). Because the active σ^A sample is free from the β -subunit of *E. coli* core RNAP and lack of transcription activity (Supplementary Figure S1), we assumed that the σ^A , by itself, is able to bind the G3b promoter DNA.

To rule out the possibility that the promoter DNA-binding activity of the *in vitro* refolded σ^A is just an artifact due to protein refolding, we further purified the σ^A overproduced in *E. coli* in a soluble form at low

temperature (16°C) and analyzed its promoter DNA-binding activity. Similar to the refolded σ^A , the soluble σ^A was able to bind the G3b promoter DNA independent of core RNAP (Supplementary Figure S2A and B), supporting the hypothesis that the *B. subtilis* σ^A is an intrinsic DNA-binding protein.

σ^A binds preferentially to the promoter –10 element *in vitro*

To investigate whether σ^A has preference for a specific promoter element, six Mu G3b promoter DNA with single or pairwise substitutions at –10 element, –35 element and TG motif (Figure 2A) were adopted for competition assay by EMSA. In this analysis, the binary complex preformed by σ^A and labeled Wt G3b promoter DNA was challenged with various concentrations of the unlabeled Wt, Mu G3b promoter DNA and non-promoter DNA (the full-length *tghp2* cDNA of *Bamboo mosaic virus*). As shown in Figure 2B, the preformed binary complex was relatively more sensitive to challenge with the Wt or the Mu G3b promoter DNA having base substitutions at either –35, TG or –35/TG element than to challenge with those having substitutions at –10 element (including –10, –10/TG and –10/–35) or with the non-promoter DNA. The requirement of the promoter –10 element for efficient competition was also visualized when the correlation between the fractional retention of the preformed σ^A -promoter DNA complex and the log [Competitor]/[Labeled G3b promoter DNA] was examined (Figure 2C). Clearly, σ^A binds preferentially to the –10 element of G3b promoter *in vitro*. Moreover, the high efficiency of the non-promoter DNA, albeit lower than that of the Wt

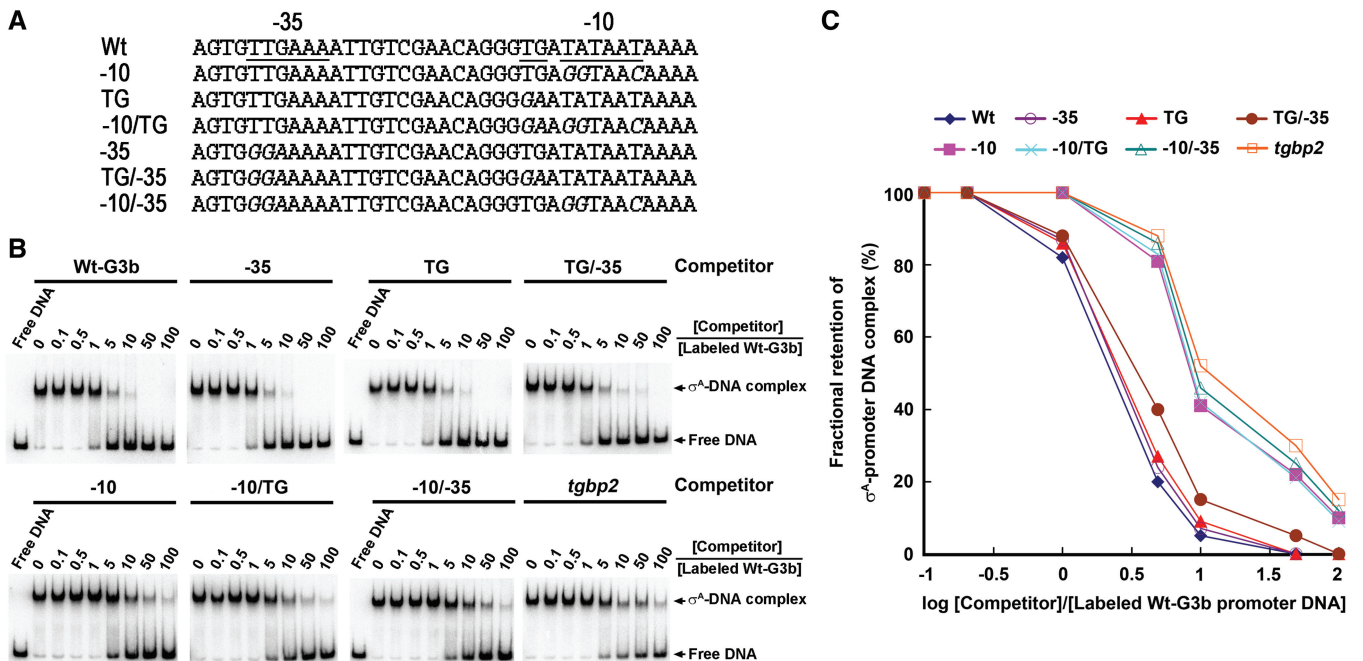


Figure 2. σ^A binds preferentially to the promoter -10 element. (A) Mutations of the G3b promoter DNA. The sequences of the -10 element, TG motif and -35 element of the Wt G3b promoter are underlined. -10 , TG, -10 /TG, -35 , TG/ -35 and -10 / -35 indicate the six promoter mutations used for competition assay. Base substitutions in each Mu promoter are italicized. (B and C) Fractional retention of the σ^A -promoter DNA complex as a function of increasing molar ratio of competitor DNA to ^{32}P -labeled G3b promoter DNA. In the EMSA-based competition assay (B), $8 \mu\text{M}$ σ^A and 1 nM ^{32}P -labeled G3b promoter DNA were used to generate the σ^A -G3b promoter DNA complex. The competitor DNA used to challenge the σ^A -G3b promoter DNA complex was shown above the horizontal lines. The molar ratios of competitor DNA to the labeled G3b promoter DNA were 0:1, 0.1:1, 0.5:1, 1:1, 5:1, 10:1, 50:1 and 100:1 as indicated under the horizontal lines. In analysis of the correlation between the fractional retention of the preformed binary complex and the $\log [\text{Competitor}]/[\text{Labeled G3b promoter DNA}]$ (C), the band density of each preformed binary complex in the absence of competitor DNA was referred to as 100%. The experiment was repeated twice and the same tendency was obtained.

G3b promoter DNA, to challenge the preformed σ^A -Wt G3b promoter DNA complex suggested that the σ^A also binds the G3b promoter DNA with low sequence selectivity.

The low sequence selectivity of σ^A *in vitro*

The low sequence selectivity of σ^A was confirmed by DNase I footprinting which probes the protein-DNA interaction in a time- and population-averaged manner. This assay was performed under various temperatures (20, 25 and 37°C) and σ^A concentrations (5, 10 and $20 \mu\text{M}$), where both partial and complete titrations of the labeled G3b promoter DNA (spanning from -115 to $+67$ of the promoter) occurred. The specific interaction of σ^A -RNAP with G3b promoter DNA was also examined for reference. The specific footprint of σ^A -RNAP on the template strand DNA spanned from about -70 to $+1$ at 20°C , and extended downstream into $+17$ at 25 and 37°C (left panel of Figure 3B). Downstream extension of the specific σ^A -RNAP footprint on the non-template strand DNA was also observed as binding temperature was increased from 20°C (-42 to $+10$) to 37°C (-42 to $+20$) (right panel of Figure 3B). Similar footprint extension was reported for *E. coli* RNAP on various promoters (49,50). Different from the above observations, the footprints of σ^A on both strands of the G3b promoter DNA were only detected at lower temperatures (20 and 25°C) with σ^A

concentration higher than $10 \mu\text{M}$ (Figure 3B). The presence of complete or nearly complete protection of the -10 region as well as partial protection near the -35 region supported the idea that σ^A binds preferentially at least to the -10 element of G3b promoter (Figure 2C). Moreover, the DNA protected by σ^A was much wider than that protected by σ^A -RNAP in both the upstream and downstream regions of the core promoter. These results indicated that, despite the preferential binding for -10 element, the σ^A also binds DNA with low sequence selectivity. The low sequence selectivity of σ^A could be substantiated by the facts that the σ^A has a similar affinity for the G3b promoter DNA and the *tgbp3* non-promoter DNA which is relatively A-T rich (Supplementary Figure S3B) and that the full-length *tgbp2* cDNA is able to compete with the Wt G3b promoter DNA for σ^A , albeit less efficiently (Figure 2B and C). Similar observations were reported for the *B. subtilis* σ^A fragment lacking Region 1 (51) and the *E. coli* GST $\sigma(360)$ (52).

The promoter-specific interaction of σ^A is -10 specific *in vitro*

To confirm that σ^A binds preferentially to the promoter -10 element or that the σ^A -promoter interaction is -10 specific *in vitro*, the footprinting patterns of σ^A on the Wt and the three G3b promoter DNA with base substitutions

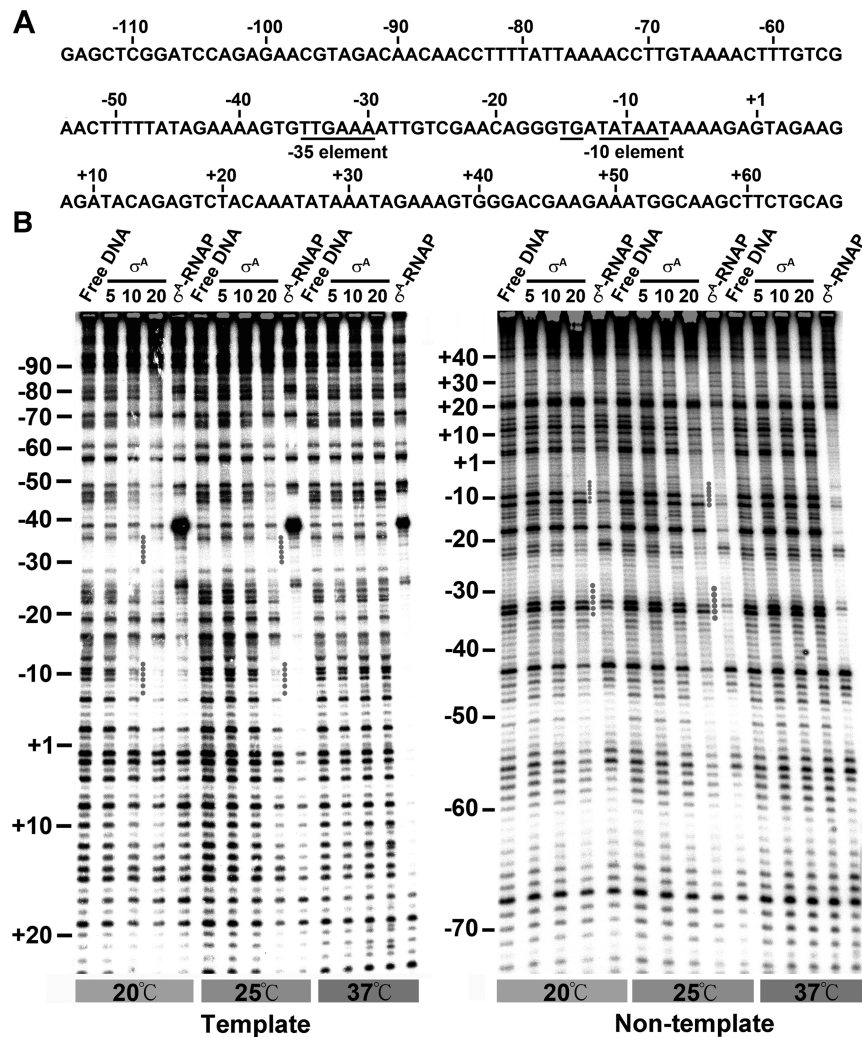


Figure 3. The low sequence selectivity of σ^A . (A) The nucleotide sequence of non-template strand DNA of the G3b promoter from -115 to $+67$. The bases of the -35 element, TG motif and -10 element are underlined. (B) The footprinting patterns of σ^A and σ^A -RNAP on the template and non-template strand DNA at the designed temperature (20 , 25 or 37°C). The concentrations (μM) of σ^A used for the footprinting assay are shown under the horizontal lines and the concentration of σ^A -RNAP used is 100 nM. The numbers shown on the left of each panel are positions relative to the transcription start site of the G3b promoter. The dotted lines indicate the -10 and -35 regions of the G3b promoter DNA protected by σ^A .

at either -10 , -35 or $-10/-35$ elements were compared. The footprints of σ^A at both -10 and -35 elements were observed on both strands of each promoter DNA in the presence of a higher concentration ($20\ \mu\text{M}$) of σ^A , no matter whether there is a promoter mutation or not (Figure 4). However, the footprints of σ^A at both -10 and -35 elements (as indicated by dotted line) were only observed for the Wt and the Mu promoter DNA with base substitutions at -35 element as the σ^A concentration was decreased to $10\ \mu\text{M}$. No significant footprint of σ^A at -10 element was detected for the G3b promoter DNA with -10 or $-10/-35$ substitutions under the same condition. This result supports the idea that the *in vitro* σ^A -promoter interaction is -10 specific.

Furthermore, we compared the promoter DNA-binding activities of the Wt- and the Mu- σ^A of which Gln-196 and Thr-199, which are assumed to be critical for the sequence-selective interaction of σ^A -RNAP with -12T

of promoter DNA (51,53), are replaced with Ala. To achieve accurate comparison, we purified the Wt- and Mu- σ^A overproduced in *E. coli* in a soluble form and analyzed their promoter DNA-binding activities. A relatively weaker affinity of the Mu- σ^A , comparing the Wt, for promoter DNA was observed in EMSA (Figure 5A). Moreover, the footprints of the Mu- σ^A at -10 and -35 elements on both strands of the G3b promoter DNA were undetectable, whereas those of the Wt- σ^A were easily detected under the same condition (Figure 5B). More importantly, the -10 preference or -10 specificity became insignificant for the Mu- σ^A as revealed from competition assay using the Wt G3b promoter DNA or the G3b promoter DNA containing base substitutions at -35 element as a competitor (Figure 5C). The requirement of the conserved amino acid residues in Region 2.4 of σ^A (Figure 5) and conserved bases in promoter -10 element for efficient σ^A -promoter DNA interaction (Figure 4)

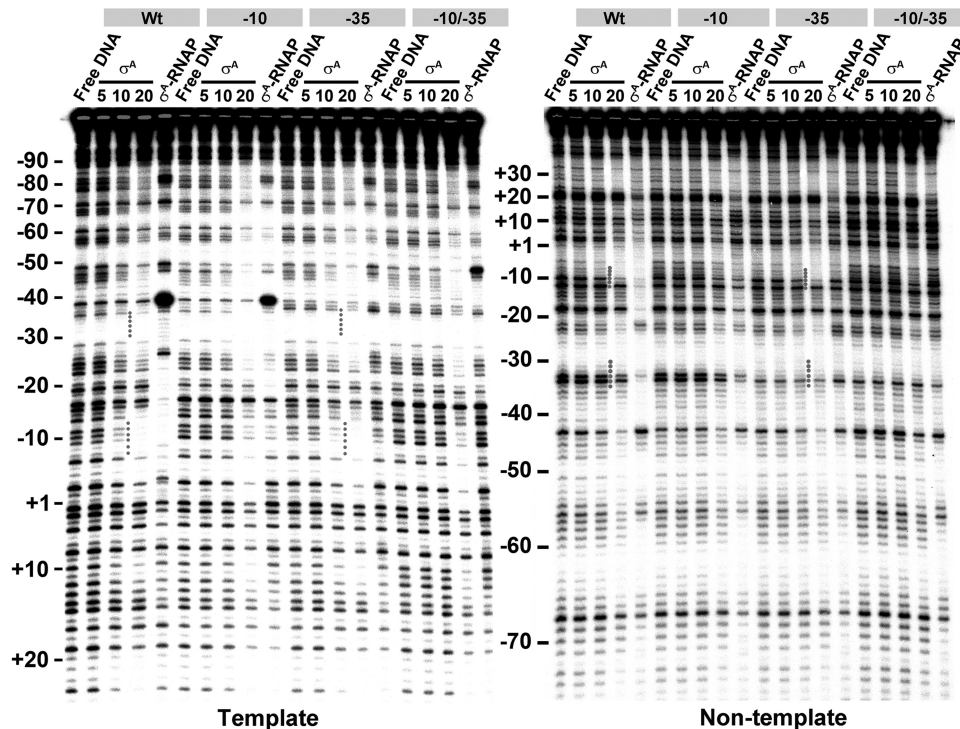


Figure 4. The effect of base substitutions on the footprinting patterns of σ^A on the G3b promoter DNA. The effect of base substitutions on the footprinting patterns of σ^A and σ^A -RNAP on the template (left panel) and non-template strand DNA (right panel) at 20°C. Wt, -10, -35 and -10/-35 indicate the Wt G3b promoter and the Mu G3b promoter containing base substitution at -10, -35 or both elements, respectively. Other details for experimental conditions and labeling of the figures are the same as those described in the legend to Figure 3.

indicated that the promoter interaction of σ^A *in vitro* is indeed -10 specific.

The -10 specific promoter interaction of σ^A is unable to allow the σ^A to discern the optimal promoter spacing *in vitro*

To see whether σ^A , by itself, is able to discern the optimal promoter spacing as reported previously (30), the spacing variants of another *B. subtilis* promoter, *trnS*, of which the -10, -35 elements, TG motif, and spacing (17 bp) are highly conserved, were constructed. The resultant spacing variants were named as *trnS*-X, in which X is the length of spacer DNA ranging from 14 to 21 bp (Figure 6A). Moreover, to diminish the potential non-specific binding of σ^A to the spacing variants in both upstream and downstream regions of the promoter, a shorter DNA fragment of each spacing variant, corresponding to the region from +15 to -80 of the *trnS*-17 promoter, was adopted to perform EMSA.

The results of EMSA are shown in Figure 6B. σ^A bound to the spacing variants with about equal efficiency; however, three distinct binding strengths of σ^A -RNAP were observed among the spacing variants. Optimal binding was seen for *trnS*-17. Moderate binding was detected for *trnS*-16 and *trnS*-18. Lowest binding was observed for the rest of *trnS* spacing variants with a shorter (14- or 15-bp) or a longer (19-, 20- or 21-bp) spacer. Since the footprint of σ^A on the *trnS* promoter was detectable at 20 but not 37°C, we thus also carried

out the binding analysis at 20°C throughout the EMSA. Similar EMSA results were obtained for both σ^A and σ^A -RNAP (Supplementary Figure S4). Therefore, σ^A , by itself, is unable to discern the optimal promoter spacing (17 ± 1 bp). To accomplish this function, the assistance from core RNAP is required.

To see whether the -10 specific interaction of σ^A also occurs with the *trnS* spacing variants, three Mu *trnS*-17 promoters with base substitutions at -10, -35 or -10/-35 elements were constructed (top panel of Figure 6C) and then used for EMSA-based competition analyses. As shown in the bottom panel of Figure 6C, the preformed binary complex of σ^A and each spacing variant was relatively more sensitive to challenge with the Wt or the Mu *trnS*-17 promoter containing base substitutions at -35 element than with those bearing base substitutions at -10 element (including -10 and -10/-35 mutations). Clearly, similar to the -10 specific G3b promoter interaction of σ^A , the σ^A also has a preference for binding to the -10 element of the *trnS* spacing variants. These results also indicated that the promoter -10 specific interaction of σ^A is unable to allow the σ^A to discern the optimal promoter spacing *in vitro*.

σ^A contacts the *trnS* spacing variants in a flexible manner, in addition to the -10 specific interaction

To confirm that σ^A binds the *trnS* spacing variants through preferential interaction with their promoter -10 elements, DNase I footprinting assay was performed.

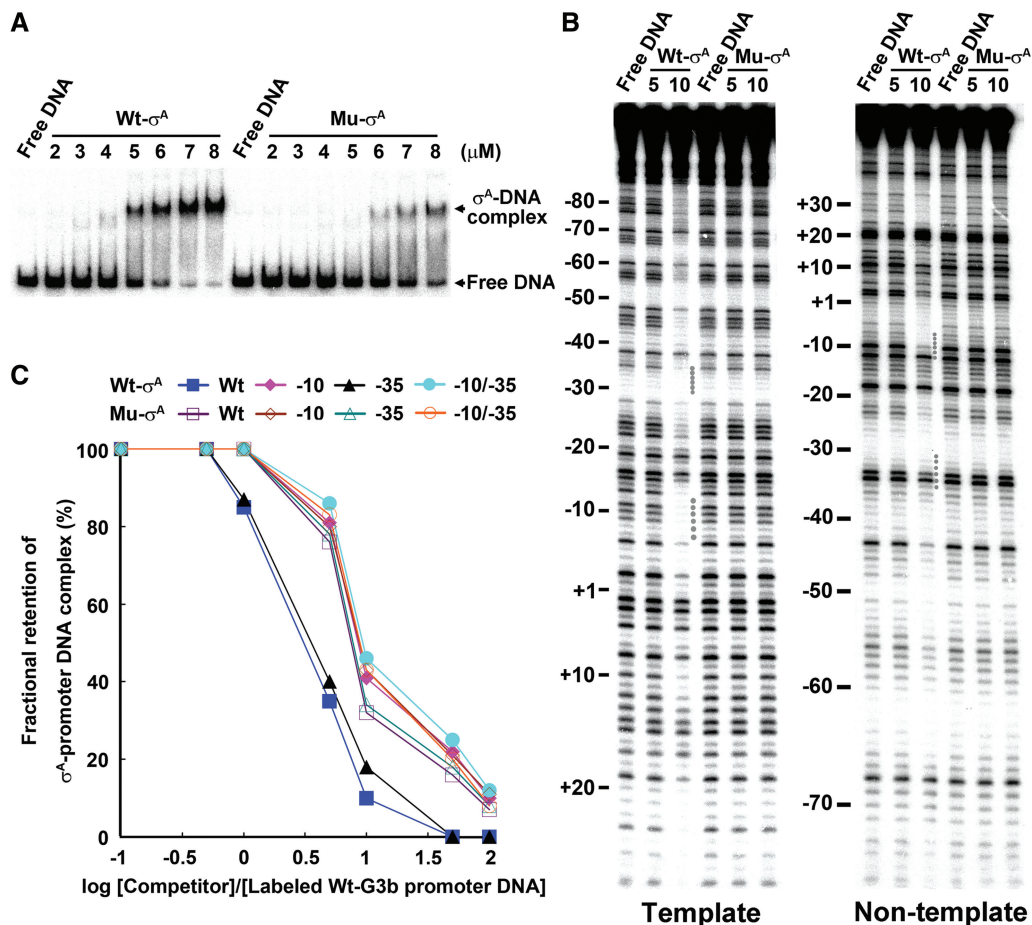


Figure 5. The effect of Gln-196-Ala and Thr-199-Ala substitutions in Region 2.4 of σ^A on the promoter DNA-binding activity of σ^A . The σ^A with Gln-196-Ala and Thr-199-Ala substitutions was designated as Mu- σ^A . (A) The effect of Gln-196-Ala and Thr-199-Ala substitutions in Region 2.4 of σ^A on the promoter DNA-binding activity of σ^A as examined by EMSA. (B) The effect of Gln-196-Ala and Thr-199-Ala substitutions in Region 2.4 of σ^A on the promoter DNA-binding activity of σ^A as examined by DNase I footprinting. In both (A) and (B), the numbers shown under Wt- σ^A or Mu- σ^A are the concentrations (μM) of σ^A . Other details for labeling of the figures are the same as those described in the legend to Figure 3. (C) The effect of Gln-196-Ala and Thr-199-Ala substitutions in Region 2.4 of σ^A on the preferential promoter -10 binding of σ^A . In the analysis of fractional retention of the Wt- or Mu- σ^A -promoter DNA complex as a function of increasing molar ratio of competitor to ^{32}P -labeled G3b promoter DNA, $8\mu\text{M}$ Wt- σ^A or $15\mu\text{M}$ Mu- σ^A was mixed with 1nM ^{32}P -labeled G3b promoter DNA to generate the σ^A -promoter DNA complex. For competition, the molar ratios of the competitor DNA to the labeled G3b promoter DNA were 0:1, 0.1:1, 0.5:1, 1:1, 5:1, 10:1, 50:1 and 100:1. The band density of each preformed binary complex in the absence of competitor DNA was referred to as 100%. The experiment was repeated twice and the same tendency was obtained.

To start with, we screened the optimal σ^A concentration for specific interaction between σ^A and *trnS*-17 promoter at 20°C . Specific footprints of σ^A on both DNA strands of the *trnS*-17 promoter, spanning from about -58 to -6 on the template and from about -59 to $+3$ on the non-template strand DNA (as encompassed by the upward and downward arrowheads), were detected when $20\mu\text{M}$ of σ^A was used for binding analysis (Supplementary Figure S5). Thus, the same condition was adopted for DNase I footprinting analyses of σ^A on other *trnS* spacing variants. As shown in Figure 7, similar protection patterns, except those in the spacers, were observed for σ^A on the *trnS* spacing variants. Moreover, clear footprint was observed at -10 element on the template strand (left panel), but at both -10 and -35 elements on the non-template strand DNA (right panel). Furthermore, there was a gradual upstream extension of

the σ^A footprint on both DNA strands (indicated by the arrowhead) as the spacing was increased from 14 to 21 bp. Since σ^A only binds specifically to the promoter -10 element of the *trnS* spacing variants (Figure 6C) and since there is a gradual upstream extension of the σ^A footprint on the *trnS* promoter DNA in response to the increase of spacer length, we favored that, in addition to the -10 specific promoter interaction, σ^A also contacts the -35 element and its upstream sequence of each *trnS* spacing variant in a flexible manner.

σ^A -RNAP, but not σ^A alone, can recognize optimal promoter spacing

To examine the effect of core RNAP on binding of σ^A to the *trnS* spacing variants, the footprints of σ^A -RNAP on the spacing variants were analyzed at both 20°C and 37°C . Shown in Figure 8A are the footprints of σ^A -RNAP on the

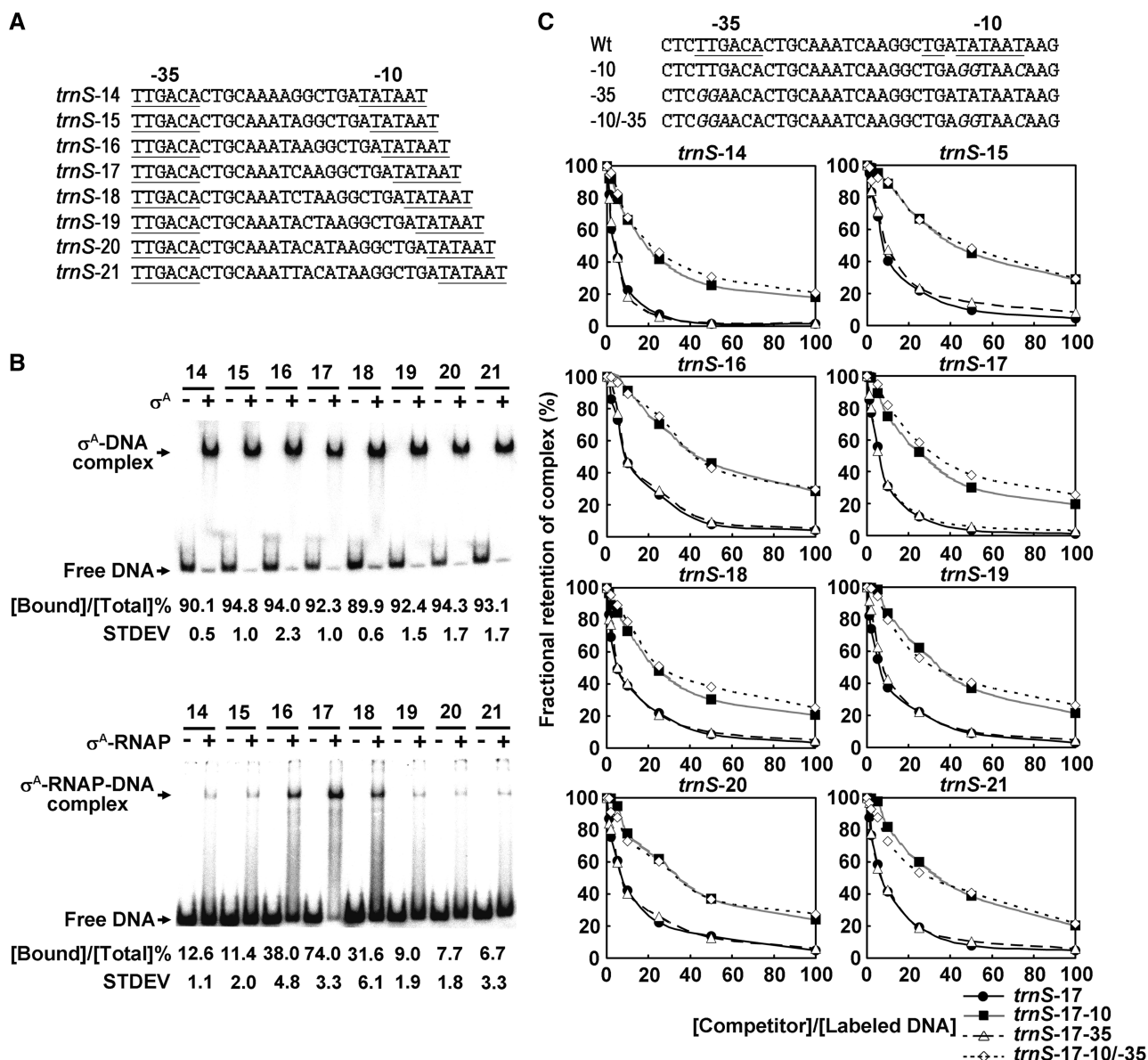


Figure 6. The promoter -10 specific interaction of σ^A is unable to allow the σ^A to discern the optimal promoter spacing *in vitro*. (A) The spacing variants of the *B. subtilis trnS* promoter used in this study. The highly conserved -10 and -35 elements of the *trnS* promoter are underlined. (B) The binding of σ^A (top panel) and σ^A -RNAP (bottom panel) to the *trnS* spacing variants. In EMSA, $10 \mu\text{M}$ σ^A or 50 nM σ^A -RNAP was used. The numbers above the horizontal lines are the spacer lengths of the *trnS* spacing variants. The plus and minus signs denote the addition and omission of σ^A or σ^A -RNAP, respectively. The values shown under each panel are the percentages of *trnS* spacing variant bound by σ^A or σ^A -RNAP ([Bound DNA]/[Total DNA] %). Each value was the average of triplicate measurements with a standard deviation (STDEV) shown. (C) Upper panel: the Wt and Mu *trnS*-17 promoters with base substitutions at either -10 , -35 or both elements (indicated by -10 , -35 and $-10/-35$) used for competition assay. The sequences of conserved -10 , -35 elements and TG motif of the *trnS*-17 promoter are underlined, and base substitutions in each Mu promoter are italicized. Lower panel: the fractional retention of the preformed σ^A -*trnS* promoter DNA complex as a function of the molar ratio of competitor DNA to ^{32}P -labeled *trnS* spacing variant ([Competitor]/[Labeled DNA]). The *trnS* spacing variant is indicated by *trnS*-X (X is the length of spacer DNA ranging from 14 to 21 bp) above each chart. In this assay, $6 \mu\text{M}$ σ^A and 0.5 nM *trnS*-X promoter DNA were used to generate the σ^A -*trnS*-X promoter DNA complex. The molar ratios of competitor DNA to the labeled *trnS* promoter DNA were 0:1, 1:1, 2:1, 5:1, 10:1, 25:1, 50:1 and 100:1. The band density of each preformed binary complex in the absence of competitor DNA was referred to as 100%.

trnS spacing variants at 20°C . Significant footprints of σ^A -RNAP at the -10 and -35 elements of both DNA strands were detected only for the *trnS* variants with an optimal spacing (16 and 17 bp). Weak or no footprint of σ^A -RNAP at the -10 element was observed for the variants with a shorter (14- or 15-bp) or a longer (18-, 19-, 20- or 21-bp) spacer; the footprint on this type of promoter was

restricted to the region spanning from the upstream of the -10 element to the position corresponding to -60 of *trnS*-17 on the template strand DNA and from the -35 element to the position corresponding to -59 of *trnS*-17 on the non-template strand DNA. Clearly, the σ^A -RNAP mainly binds the -35 element and its upstream sequence at 20°C if the promoter spacing is non-optimal.

Shown in Figure 8B are the footprinting patterns of σ^A -RNAP on both DNA strands of the *trnS* spacing variants at 37°C. Again, significant footprints of σ^A -RNAP at both -10 and -35 elements, consistent with the existence

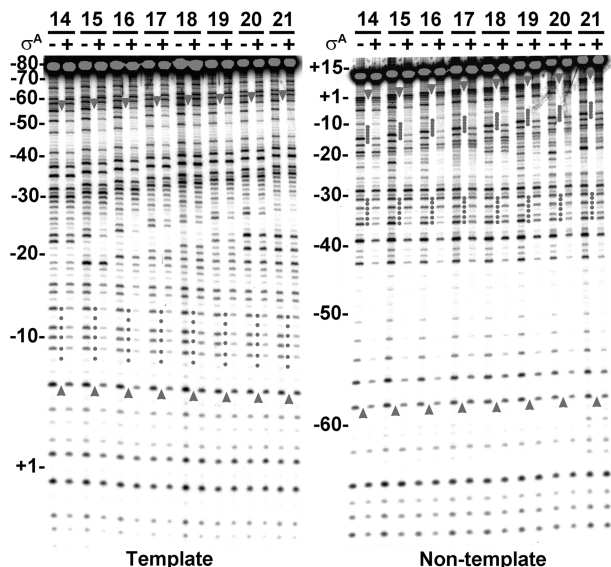


Figure 7. σ^A contacts the *trnS* spacing variants in a flexible manner in addition to the -10 specific interaction. The footprinting patterns of σ^A on the template (left panel) and non-template strand DNA (right panel) of the *trnS* spacing variants at 20°C. In this assay, 20 μ M σ^A was used for footprinting assay. The numbers above the horizontal lines are the spacer lengths of the *trnS* spacing variants. The plus and minus signs denote the addition and omission of σ^A , respectively. The numbers shown on the left of each panel are positions relative to the transcription start site of the *trnS*-17 promoter. The DNA protected by σ^A on both the template and non-template strand DNA of each spacing variant are encompassed by the upward and downward arrowheads. The promoter -10 and -35 elements protected by σ^A on each *trnS* spacing variant are indicated by dotted lines.

of specific interaction between σ^A -RNAP and the two promoter elements (Supplementary Figure S6), were observed only for the *trnS* variants with an optimal spacing, especially that with a 17-bp spacer. No or very weak footprint of σ^A -RNAP was detected on both DNA strands if the spacer is shorter (14- or 15-bp) or longer (19-, 20- or 21-bp). Taken together, these results demonstrated that core RNAP not only enables σ^A to discern the optimal promoter spacing but also to simultaneously and specifically recognize both promoter elements as previously reported (27,29).

DISCUSSION

We have documented the *in vitro* core-independent promoter-specific interaction of the *B. subtilis* primary σ factor, σ^A . This interaction is -10 specific; however, it is unable to allow the σ^A to discern the optimal promoter spacing. To fulfill this goal, the σ^A requires assistance from core RNAP.

The detection of core-independent -10 specific promoter interaction of σ^A *in vitro* is contrary to previous findings with the *E. coli* σ^{70} (22,23) and *T. maritima* σ^A (24,25), for which it has been reported that Region 1.1 of σ acts as an auto-inhibitor for σ -promoter interaction, and that the promoter interaction in the absence of core RNAP would require deletion of Region 1.1 of σ , just as seen for the σ^D of *B. subtilis* (17). Reasons responsible for these contradictory results are complicated. We thought that our ability to detect the core-independent -10 specific promoter interaction of σ^A is ascribed to the isolation of σ^A with a proper promoter DNA-binding conformation through the use of a molecular sieving column, Superdex HR-200 (Figure 1) and the use of a relatively low temperature and low ionic strength for DNase I footprinting

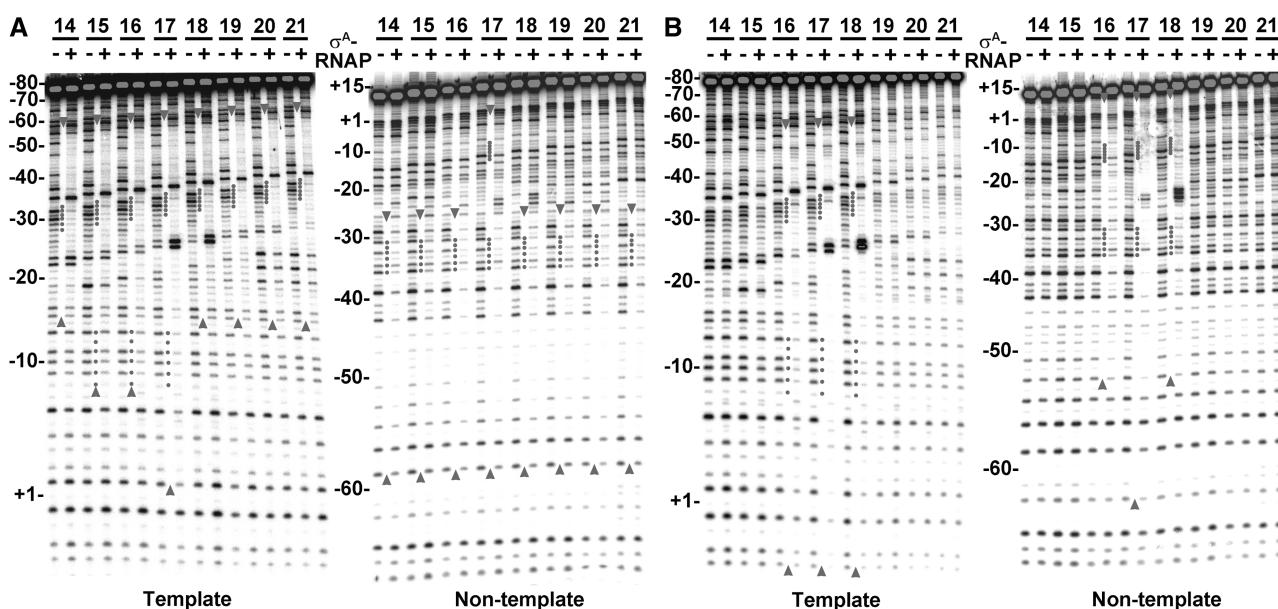


Figure 8. The footprinting patterns of *trnS* spacing variants in response to σ^A -RNAP binding. (A and B) The footprinting patterns of σ^A -RNAP on the *trnS* spacing variants at 20 (A) or 37°C (B). The concentration of σ^A -RNAP used for footprinting assay was 15 nM. Details for labeling the figures are the same as those described in the legend to Figure 7.

(Figure 3). The early elution of the σ^A active in promoter-DNA binding from the Superdex HR-200 column suggests that the σ^A has a relatively looser conformation. This idea can be supported by our preliminary observation that a σ^A preparation more active in promoter DNA binding possesses a higher intensity of tryptophan fluorescence (the two tryptophan residues of the *B. subtilis* σ^A are located just at the N-terminal end of the promoter -10-binding helix of σ^A). Possibly, the close proximity of the negatively charged Region 1.1 to the positively charged promoter recognition domains of σ^A (25) is at least to a certain extent alleviated during the slow refolding of the denatured σ^A . Moreover, the need of a low temperature and low ionic strength for the detection of an efficient interaction between σ^A and promoter DNA by DNase I footprinting suggests that the σ^A -promoter DNA interaction is an exothermic reaction, in which electrostatic and/or hydrogen bonding occur.

The preferential binding of σ^A , by itself, to the promoter -10 (TATAAT) but not -35 element *in vitro* (Figures 2B, C and 6C) is consistent with the findings that the consensus sequences of GTA(C/T)AATGGGA and TGTAGAAT, both of which contain the TATAAT-like sequence, are required for single stranded deoxyribonucleic acid (ssDNA) aptamer binding to the *Thermus aquaticus* free σ^A (54,55). It is also consistent with our observation that the -10 specific promoter interaction of σ^A is unable to allow the σ^A to discern the optimal promoter spacing (Figure 6). The lack of binding specificity of the promoter -35 element by σ^A can be attributed to the presence of Region 1.1 of σ^A , which makes σ Region 4.2 inaccessible and thus prevents the interaction between σ Region 4.2 and the promoter -35 element (22–24). The ability of σ^A to contact the promoter -35 element and its upstream sequence of each *trnS* spacing variant in a flexible manner (Figure 7) indicates that after the promoter -10 specific interaction of σ^A , an extensive σ^A -promoter contact in the -35 and its upstream region occurs. It also suggests that the structure between the two DNA-binding domains of σ^A is flexible enough for σ^A to contact the two promoter regions separated by various spacings. The structural flexibility of *E. coli* σ^{70} in recognition of the -10- and -35-like sequences has also been reported in the λ Q-engaged transcription elongation complex (56). The structural flexibility between the two DNA-binding domains of σ^A can be attributed to the existence of a relatively unstructured linker, Region 3.2, in σ^A according to the modeled structure of thermophilic eubacteria σ^A (57,58). In fact, our analysis of the structural property of the *B. subtilis* σ^A by PONDR[®] also revealed the existence of a disordered flexible Region 3 in σ^A (Supplementary Figure S7). Thus, the ability of σ^A in the context of RNAP to discern the optimal promoter spacing must be due to the change of the linker domain from an extended unfolded conformation to a hairpin loop structure fixed in the binary complex of RNAP and promoter DNA (58). This change makes the distance (~50 Å) between the two DNA-binding domains of σ only more compatible with the ~17-bp (57.8 Å) spacing (27,59).

The low sequence selectivity of σ^A (Figure 3 and Supplementary Figure S3) together with the *in vitro* -10 specific promoter interaction of σ^A (Figures 2B, C and 6C) suggests σ can either directly recognize the promoter -10 element or indirectly through non-specific interaction with the A-T rich or promoter -10-like sequences ubiquitous on the chromosome DNA, especially in the upstream region of a promoter, and through sliding to the promoter site in a way similar to that proposed for the sliding of *E. coli lac* repressor to its operator (60,61). Investigation of the existence of *in vivo* σ^A -promoter interaction, which is critical to σ function, is currently undergoing in the lab.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank the National Science Council of Taiwan, Republic of China, for supporting the research.

FUNDING

National Science Council of Taiwan, Republic of China (NSC 92-2311-B-005-021, NSC 94-2311-B-005-006, and NSC 94-2311-2752-B-005-001-PAE). Funding for open access charge: National Science Council of Taiwan, Republic of China.

Conflict of interest statement. None declared.

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