J. Biochem. 132, 29–36 (2002)

The Importance of Region 2.1 in Sustaining the Functional Structure of the *Bacillus subtilis* σ^A Factor[1]

Chao-Tsai Liao,[*] Hsuen-Chun Liao,[†] Wei-Cheng Huang,[†] Wen-Horng Wang,[†] and Ban-Yang Chang[†,2]

[*]*Chungtai Institute of Health Sciences and Technology, Taichung 40227, Taiwan, Republic of China; and* [†]*Institute of Biochemistry, National Chung-Hsing University, Taichung 40227, Taiwan, Republic of China*

Received March 12, 2002; accepted April 18, 2002

Region 2.1 of the σ factor is once proposed to be involved in core binding, and certain bulky hydrophobic amino acids in region 2.1 are thought to make contact with the conserved isoleucine residues in the promoter −10 binding region on the same protein. To examine the roles of the contact between these two regions in σ^A structure and function, σ^A factor with L145A, I149A, or Y153A was created, and the effects of each substitution on the growth of *Bacillus subtilis* and on the structural and functional properties of σ^A were analyzed. Our data revealed that the growth potential of *B. subtilis* was significantly affected by each of the substitutions of σ^A at elevated temperature. The growth defect was most pronounced with the strain containing L145A-σ^A; it possessed a low growth potential even at 37°C. In parallel, changes in the structural stability and core-binding activity of σ^A and in the promoter-binding and transcription activities of σ^A-RNA polymerase were observed for each of the substitutions, with the most drastic effects exerted by L145A. Clearly, region 2.1 of σ^A has extra functions, such as the binding of RNA polymerase to promoter DNA, other than the known core-binding ability. Moreover, the multiple effects of each of the substitutions on σ^A demonstrate that the contacts between the hydrophobic amino acids in region 2.1 and those in the promoter −10 binding region are critical to the maintenance of the functional σ^A structure and that L145 in region 2.1 plays an important role in this respect.

Key words: core-binding activity, promoter-binding activity, sigma factor, structure stability, transcription activity.

The prokaryotic RNA polymerase is composed of a σ factor and a core enzyme which contains α_2, β, and β′ subunits. The σ factor confers the specificity of promoter recognition and the transcription of specific genes. Amino acid sequence alignment of various σ factors in the σ^{70} family has revealed four regions of homology (*1, 2*). Two of them, designated as regions 2.4 and 4.2, have been shown in several σ factors to interact with the promoter DNA (*3–11*). Region 2.4, which is supposed to recognize the promoter −10 DNA, forms an amphiphilic α-helix with three isoleucines located at four-residue intervals (*12*). The three isoleucines on the hydrophobic face of the amphiphilic α-helix are critical to ensure a proper folding and to sustain the functional structure of the *Bacillus subtilis* σ^A factor (*13*).

In our previous study of the amphiphilic α-helix of the promoter −10 binding region (region 2.4) of the *B. subtilis* σ^A factor (*12, 14, 15*), we found that the double Ile-to-Ala substitutions at positions 198 and 202 on the hydrophobic face of the promoter −10 binding helix generated a temperature-sensitive (Ts) σ^A factor that formed multimeric structure predominantly at low temperature and was prone to denaturation and aggregation at elevated temperature. The monomeric form of the Ts σ^A, which possesses a looser conformation than the wild-type (Wt) monomer, was scarcely observed *in vitro* (*13*). It is believed that the aggregative tendency and looser conformation of the Ts σ^A are responsible for the significant reduction in core binding of the Ts σ^A at restrictive temperature (*13*). By analogy with the crystal structure of a partial peptide fragment of the *E. coli* σ^{70} (*16*), it is assumed that the loose conformation of the monomeric Ts σ^A and the tendency to aggregation of the multimeric Ts σ^A are attributable, at least in part, to the loss of contact between the conserved isoleucine residues (I194, I198, and I202) on the hydrophobic face of the promoter −10 binding helix and the bulky hydrophobic amino acids (L145, I149, and Y153) in the putative core-binding region, region 2.1, on the same protein (Fig. 1; *17–19*).

The necessity for all known σ factors to bind to the core enzyme has led to the assumption that the core-binding regions of σ must be highly conserved (*2, 20–23*). Several regions of σ have been reported to be involved in core binding. The earliest reported was the amino acid sequence of region 2.1 of the *Escherichia coli* σ^{70} (*17, 18*). Single-amino-acid substitutions at positions 68 and 94 in this region of the *B. subtilis* σ^E factor have also been found to affect core binding (*19*). Conserved region 2.2 has also been implicated in core binding. A single-amino-acid change (Q80N) in this region of the *E. coli* σ^{32} reduces its affinity for core RNA polymerase (*24*). Mutations that lie outside regions 2.1 and

[1] This research was supported by the National Science Council, Taiwan, Republic of China (NSC 89-2311-B-005-058).

[2] To whom correspondence should be addressed. Tel: +886-4-2287-4754, Fax: +886-4-2286-1905, E-mail: bychang@mail.nchu.edu.tw

2.2 of σ^{32} and reduce the core-binding activity of σ^{32} have also been reported (*25*). They include mutations in the promoter –35 binding region (region 4.2) and a 24-amino-acid deletion in region 3 of σ^{32} (*26*). Thus, more than one specific region of σ seems to be involved in core binding. However, it remains unclear whether all of the putative core-binding sites are really involved in contact with core RNA polymerase. It is conceivable that mutations at these positions simply alter the functional conformation of σ, which in turn lowers the core-binding activity. Moreover, detailed analyses of the extra functions of the core-binding regions or the side effects of mutation (s) in core-binding regions on σ structure and function are still lacking.

We are interested in understanding the roles of the hydrophobic amino acids (L145, I149, and Y153) in region 2.1 and the importance of their interaction with the hydrophobic amino acids in the promoter –10 binding helix. To this end, we constructed three *B. subtilis sigA* mutants in which a hydrophobic amino acid residue in region 2.1 of σ^A was replaced with alanine. We then analyzed the structural stability, core-binding activity, promoter-binding activity, and transcription activity of each of the mutant σ^A factors. Our data revealed that the substitution of alanine for each of the bulky hydrophobic amino acids, especially L145A, affects the structural stability, promoter-binding activity, and transcription activity of the *B. subtilis* σ^A factor. The multiple effects demonstrate that region 2.1 is critical to sustain the functional σ^A structure besides the putative core-binding activity.

MATERIALS AND METHODS

Construction of sigA Mutants—A mutant *sigA* gene containing the I145A, I149A, or Y153A substitution was constructed by overlapping extension polymerase chain reaction (PCR) as described previously (*12*). Each mutation was designed to disrupt the possible contacts between L145, I149, and Y153 in region 2.1 and the three isoleucines on the hydrophobic face of the promoter –10 binding helix of the *B. subtilis* σ^A factor. The forward, reverse, and overlapping primer sequences used for the synthesis of mutant *sigA* genes were as follows: A, 5′-GTACGAATTCGACCAAGTAAAAGAG-3′; D, 5′-CGTACCTGCAGGGATCCGTATACGCTTACAATAGAAAAT-3′; B145, 5′-CTGACAACGGCCCGCAGGTTCGC-3′; C145, 5′-CCTGCGGGCCGTTGTCAGTATCGC-3′; B149, 5′-CCGTTTTGCGGCACTGACAACAA-3′; C149, 5′-GTTGTCAGTGCCGCAAAACGGTATG-3′; B153, 5′-GCGTCCGACGGCCCGTTTTGCGATAC-3′, C153, 5′-CGCAAAACGGGCCGTCGGACGCGGTATG-3′. Besides the amino acid substitutions, an *Hae*III restriction site was introduced into the overlapping primer sequences a few codons away from the mutated sites. The *Hae*III site enabled us to keep track of the mutant *sigA* genes and to monitor them after their integration into the *B. subtilis* chromosome. In the synthesis of mutant *sigA* genes, the ABx and CDx (x represents 145, 149, and 153) fragments complementary at the mutated regions were first synthesized by PCR and gel purified. The two DNA fragments were then mixed and subjected to another run of PCR to synthesize the mutant ADx fragments. These DNA fragments were further digested with *Eco*RI and *Bam*HI before being used to replace the *sigA* fragment on the pBY1T2 plasmid, which harbours a *sigA* gene with an Ala-197-Pro substitution (*27*). The pBYx plasmids thus constructed were finally used to transform *B. subtilis* DB2 (*trpC*).

Transformation of *B. subtilis* DB2 with pBYx plasmids devoid of the replication origin of *B. subtilis* but having a chloramphenicol resistance marker produced *B. subtilis* cells that contained both intact and truncated alleles of *sigA* gene in a direct repeat (*28, 29*). Since the intact *sigA* allele might be a mutant copy and the designed amino acid substitution might destabilize σ^A or affect its activity, we expected that some of the transformants might be defective in growth, especially at elevated temperature. To confirm the *sigA* mutants, co-transformation and direct sequencing of the mutant *sigA* genes were performed as mentioned previously (*12, 17*).

Overexpression and Purification of σ^A Factor—To overexpress the σ^A factor with L145A, I149A, or Y153A substitution, each of the *sigA* genes was first fished out from the corresponding strain of *B. subtilis* using PCR with the forward primer (OP1, 5′-CTGCAGAGATCTGAATTCGTTGCAAGCTTTGG-3′) and the reverse primer (MI2, 5′-CGTACCTGCAGGGATCCGTATACGCTTACAATAGAAAAT-3′). After digestion with *Eco*RI and *Pst*I, each mutant *sigA* gene was cloned into pT7-5 (*30*), a vector used for protein overexpression. Methods for overexpression and purification of σ^A were the same as those reported previously (*31*).

Analysis of the Core-Binding Activity of σ^A by Glycerol Density Gradient Centrifugation—To prepare samples for glycerol density gradient centrifugation (*19*), culture of *B. subtilis* was grown in 2× SG medium at 37°C to an optical density (A_{550}) of 0.6, then aliquoted into two portions. One aliquot was kept at 37°C; the other was transferred to 49°C Each aliquot (40 ml) was incubated at the designated temperature for 5 min, pelleted by centrifugation, and resuspended with 1.4 ml of lysis buffer (10 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM DTT, 1 mM PMSF, 0.5 mg/ml lysozyme, and 10% glycerol). The cell suspension was incubated on ice for 30 min, then treated in a French press at 20,000 psi. Brij-58 (5%, v/v) was added to the resulting cell lysate at a concentration of 0.5% (v/v), and the lysate was further centrifuged at 7,000 rpm in a Hitachi RPR20-2 rotor. Then 1.1 ml of the supernatant was layered on a 10-ml glycerol density gradient (15–30%), centrifuged at 4°C 35,000 rpm in a Hitachi RPS40T rotor for 28 h, and the sample was fractionated with a fractionator (Isco). For quantitative analysis of σ^A and β′, proteins in equal volumes of the fractionated samples were subjected to SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose paper, and probed with anti-σ^A, and anti-β′ antibodies. The protein bands which represent σ^A and β′ on the blots were scanned with a reflective densitometer. This enabled us to determine the amounts of σ^A and β′ in the fractionated samples.

The Degradation Half-Lives of σ^A—To determine the degradation rate of σ^A *in vivo*, each *B. subtilis* strain was grown in glucose minimal medium (GMM) (*32*) supplemented with 0.004% tryptophan at 37°C to an A_{550} of 0.4 (measured with a Milton Roy Company Spectronic 20D spectrophotometer), and the culture was separated into two portions. One portion was further incubated at 37°C; the other was transferred to 49°C. After 9 min, each culture was pulse-labeled with L-[^{35}S]methionine (1,200 Ci/mmol) at a concentration of 20 μCi/ml for 5 min, then chased with non-radioactive methionine at a concentration of 5 mM.

Sampling of the labeled cultures was started 2 min later. At the designated time points, 0.5 ml of each culture was removed, pelleted by centrifugation at 4°C for 10 min, and incubated at 37°C for 10 min in 40 μl of lysis buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 0.1 μg ml^{-1} lysozyme, 0.2 mM PMSF). Then 20 μl of detergent solution (1 % [w/v] SDS, 1 % [v/v] Triton X-100) was added to each sample to disrupt the cells. The sample was then heated to 90°C for 3 min, and the insoluble cell debris in the sample was removed by centrifugation. To specifically precipitate σ^A and GroEL in the supernatant simultaneouly, a 50-μl aliquot of the sample was diluted with 450 μl of incubation buffer (1% [v/v] Triton X-100, 0.1% [w/v] SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0) prior to the addition of 1 μl each of anti-σ^A and anti-GroEL antibodies. The mixture was incubated overnight at 4°C with gentle shaking (100 rpm), then 60 μl of swollen protein A–Sepharose CL-4B in incubation buffer was added to the sample mixture before shaking for another 2 h at 4°C The Sepharose resin was pelleted, washed once with incubation buffer (1 ml) and once with Tris-HCl buffered saline solution (100 mM NaCl, 10 mM Tris-HCl, pH 8.0). After vacuum-drying, proteins absorbed by the resin were solubilized with 30 μl of Laemmli sample buffer (*33*) for 10 min at 90°C A 10-μl sample of the solubilized protein solution was electrophoresed on SDS–polyacrylamide gel. The protein gels were stained with Coomassie Blue, vacuum-dried, and exposed to X-ray film. Radioactivities of the target protein bands were measured with an Ambis Radioactivity Image Reader.

In Vitro Transcription Assay of the Wt and Mutant σ^A-RNA Polymerases—Core enzyme with His-tagged β′ and σ^A was prepared according to the protocols reported previously (*31, 34*). Methods for the preparation of reconstituted RNA polymerase and *in vitro* transcription were modified from a previously reported version (*31*). The RNA polymerase holoenzyme was obtained by mixing 15 μl of core enzyme (3 μg) with 15 μl of purified σ (2.0 μg) on ice for 10 min. To this solution was added, 25 μl of pCT20 or pCT24 plasmid DNA (0.3 μg) harboring the P1P2 promoter of *B. subtilis sigA* operon or the G3b promoter of ϕ29 phage (*35*), respectively. This RNA polymerase and promoter DNA mixture was incubated on ice for 10 min, then transferred to the designated temperature (37 or 49°C). Subsequently, 60 μl of reaction cocktail (40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 100 mM KCl , 0.4 mM DTT, 0.2 mM each of UTP, CTP, GTP, ATP, 3 μCi [α-^{32}P]ATP, and 5% of glycerol) prewarmed at the designated temperature was added to start the transcription reaction. The reaction was allowed to proceed for 1 h, then the sample was immediately placed on ice, and 60 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) was added. Finally, the sample was run on a 6% denaturing polyacrylamide gel, and autoradiography was performed after electrophoresis and vacuum-drying of the gel.

Preparation and Labeling of the G3b Promoter DNA—The 135-bp G3b promoter DNA was synthesized by PCR using a pCoiZA-derived plasmid containing the G3b promoter DNA as template (*36*). The two primers used for the synthesis were BC1041-*Pst*I (5′-GCTGGTCTGCAGAACGTAGACAACAACC-3′) and BC1048-*Xba*I (5′-GCGTCGTCTAGAATTTGTAGACTCTGTATC-3′). To label the G3b promoter DNA, 2.5 μg of the DNA fragment was first treated with calf intestinal alkaline phosphatase, recovered by electroelution, and resuspended in 100 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA). Then, 2 μl of polynucleotide kinase (10 U/μl), 20 μl of 10× kinase buffer (500 mM Tris-HCl, pH 8.2, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM dithiothreitol, 1 mM spermidine), 12.5 μl of γ-^{32}P-ATP (10 μCi/μl), and 65.5 μl of H_2O were added to the DNA solution. The reaction mixture was incubated at 37°C for 1 h, then extracted twice with phenol-chloroform. The labeled DNA in the upper aqueous phase was precipitated with 0.3 M sodium acetate, washed with 70% of ethanol, dried in vacuum, and finally dissolved in 100 μl of TE buffer.

Promoter-Binding Activities of Wt and Mutant σ^A-RNA Polymerases—Gel-retardation assay was used to analyze the promoter-binding activity of Wt or mutant σ^A-RNA polymerase. Core RNA polymerase was first reconstituted with σ^A factor on ice for 10 min in binding buffer (50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 125 mM KCl, and 10% glycerol). ^{32}P-labeled G3b promoter DNA was added to the reconstituted holoenzyme solution, and the mixture was incubated on ice for 10 min, then transferred to 37°C for another 20 min of incubation. The molar ratio of promoter DNA, core enzyme, and σ^A in the binding mixture was 1:50:500, with 2.24×10^{-9} M of the promoter DNA. After binding, 3 μl of loading buffer (50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 125 mM KCl, and 70% glycerol, 0.4% bromophenol blue) was added to the binding mixture, and the sample was subjected to 5% non-denaturing polyacrylamide gel electrophoresis in 1× TPE buffer (90 mM Tris-phosphate and 2 mM EDTA). The gel was prerun at 100 V for 30 min before applying the samples.

RESULTS

Construction and Growth Potential of the B. subtilis sigA Mutants—On the basis of the very high conservation of amino acids in both regions 2.1 and 2.4 among σ factors (*1, 2*) and the crystal structure of a partial peptide fragment of the *E. coli* σ^{70}, it has been assumed that L145, I149, and Y153 in region 2.1 of the *B. subtilis* σ^A factor interact interdigitally with I194, I198, and I202 on the hydrophobic face of the promoter –10 binding helix on the same protein (Fig. 1; *35*). To investigate the roles of these interdigital interactions in σ^A structure and function, *sigA* mutants with the L145A, I149A, or Y153A substitution in σ^A were constructed, and confirmed by chromosomal DNA co-transformation as well as DNA sequencing of the mutant *sigA* genes (see "MATERIALS AND METHODS"). Since alanine is a better helix former than other amino acids (*37*), we expected that the second helical structure in region 2.1 would not be disrupted by the substitutions. We also expected that some of the *sigA* mutants might grow slowly at elevated temperature, as seen for the *sigA* mutants with Ile-to-Ala substitution(s) on the hydrophobic face of the promoter –10 binding helix (*12, 35*). This is because each of the substitutions may result in the loss of contact between regions 2.1 and 2.4 of σ^A, which in turn leads to structural and functional defects of σ^A. As expected, the growth potentials of the *sigA* mutants with the L145A, I149A, or Y153A substitution were affected to different extents at elevated temperature, and the most drastic effect was observed with the strain containing L145A-σ^A (Fig. 2). This strain was defective in growth even at 37°C. These results indicated that the func-

tion of σ^A was influenced by each of the substitutions, especially by L145A on the second helix of region 2.1, and supported the notion that there were direct contacts between the hydrophobic amino acids on the hydrophobic faces of promoter –10 binding helix and in region 2.1 of σ^A as shown in Fig. 1. Moreover, they seemed to indicate that the contact between I194 and L145 is most critical for sustaining the functional conformation of σ^A.

The constitutively low growth potential of *B. subtilis* with L145A-σ^A (Fig. 2) is quite different from that observed for the Ts *sigA* mutant (*B. subtilis* DB1005) with Ile-198, 202-Ala substitutions on the hydrophobic face of the promoter –10 binding helix of σ^A, of which the growth potential is comparable to that of the Wt at 37°C (*12*). We speculated that the replacement with alanine of the bulky hydrophobic amino acid on the second helix of region 2.1 had affected the structure of σ^A differently from the reported effect of the Ile-to-Ala substitution in region 2.4 of σ^A (*13*).

Transcription Activities of the Mutant σ^A-RNA Polymerases—To answer why *B. subtilis* with L145A, I149A, or Y153A in σ^A had a lower growth potential than the Wt, especially at elevated temperature, we measured the relative transcription activities of the mutant σ^A-containing RNA polymerases on the G3b promoter of *B. subtilis* ϕ29 phage and the P2 promoter of *B. subtilis sigA* operon *in vitro*. The mutant σ^A-RNA polymerases were obtained by enzyme reconstitution. Figure 3 shows the results of *in vitro* transcription assays. Clearly, the RNA polymerase containing L145A-σ^A had lower transcription activity than those that containing the Wt or either of the other two mutant σ^A factors (Fig. 3). With G3b promoter as DNA template, the relative activity of RNA polymerase containing the Wt-, L145A-, I149A-, or Y153A-σ^A was in the ratio of 1:0.11: 0.65:0.84 at 37°C and 1:0.27:0.39:0.43 at 49°C. With P2 promoter as DNA template, the relative transcription activities of the same RNA polymerases were 1:0.1:0.31: 0.70 and 1:0.05:0.19:0.38 at 37°C and 49°C, respectively. Evidently, the function of σ^A in transcription was influenced by each of the amino acid substitutions in region 2.1 and the effect was most pronounced with L145A.

L145A, I149A, and Y153A Cause Structural Instability of σ^A Even at 37°C—The decreased transcription activity of a mutant σ factor may be attributed to structural instability (*38–40*), aggregation of folding intermediate (*13*), or low core-binding activity of the mutant σ factor (*17, 24–25*). It could also be a result of low promoter-binding activity, change in promoter specificity (*5, 9–11*), blocking of transcription initiation (*41*), or defective open complex formation of the mutant σ-containing RNA polymerase (*42, 43*). Since L145, I149, and Y153 in region 2.1 were assumed to interact with I194, I198, and I202, we suspected that the substitutions, L145A, I149A, and Y153A may lead to structural instability of σ^A as seen for the Ile-to-Ala substitutions in region 2.4 due to the loss of hydrophobic contacts with the three conserved isoleucine residues (*35*). As suspected, the structural stability of σ^A was significantly affected by L145A, I149A, or Y153A. The degradation half-lives of the Wt and the three mutant σ^A factors at 37°C

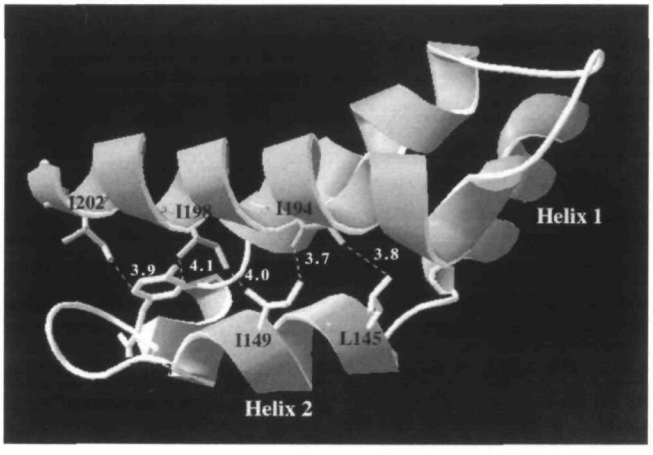

Fig. 1. **The proposed interdigital interactions between the three isoleucine residues on the hydrophobic face of the promoter –10 binding helix (region 2.4) and the hydrophobic amino acids in region 2.1 of the *B. subtilis* σ^A factor.** The diagram was drawn with Swiss-PdbViewer v3.7b2 based on analogy with the *E. coli* σ^{70} factor, of which the amino acid residues in these two regions are very highly conserved among the primary σ factors (*2*). The amino acid residues involved in the interactions are completely identical in the *E. coli* σ^{70} and *B. subtilis* σ^A factors. The α-helix of region 2.4, which starts from I194, extends from the helix of region 2.3. The three conserved isoleucine residues (I194, I198, and I202) on the hydrophobic face of the promoter –10 binding helix are shown in tan. L145 and I149 on the hydrophobic face of the second helix and Y153 in the loop of region 2.1 are shown in green. The proposed interdigital interactions are indicated by dashed lines with the distance (Å) between the contact atoms indicated.

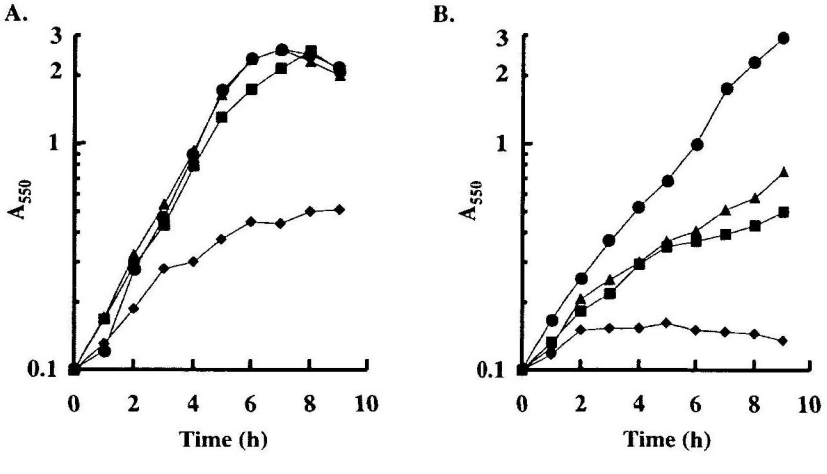

Fig. 2. **Growth potentials of the *B. subtilis sigA* mutants.** Each strain of *B. subtilis* was grown in 2× SG liquid medium at (A) 37 and (B) 49°C The symbols ●, ▲, ■, and ◆ represent the strain of *B. subtilis* containing Wt-, Y153A-, Y149A-, and L145A-σ^A, respectively.

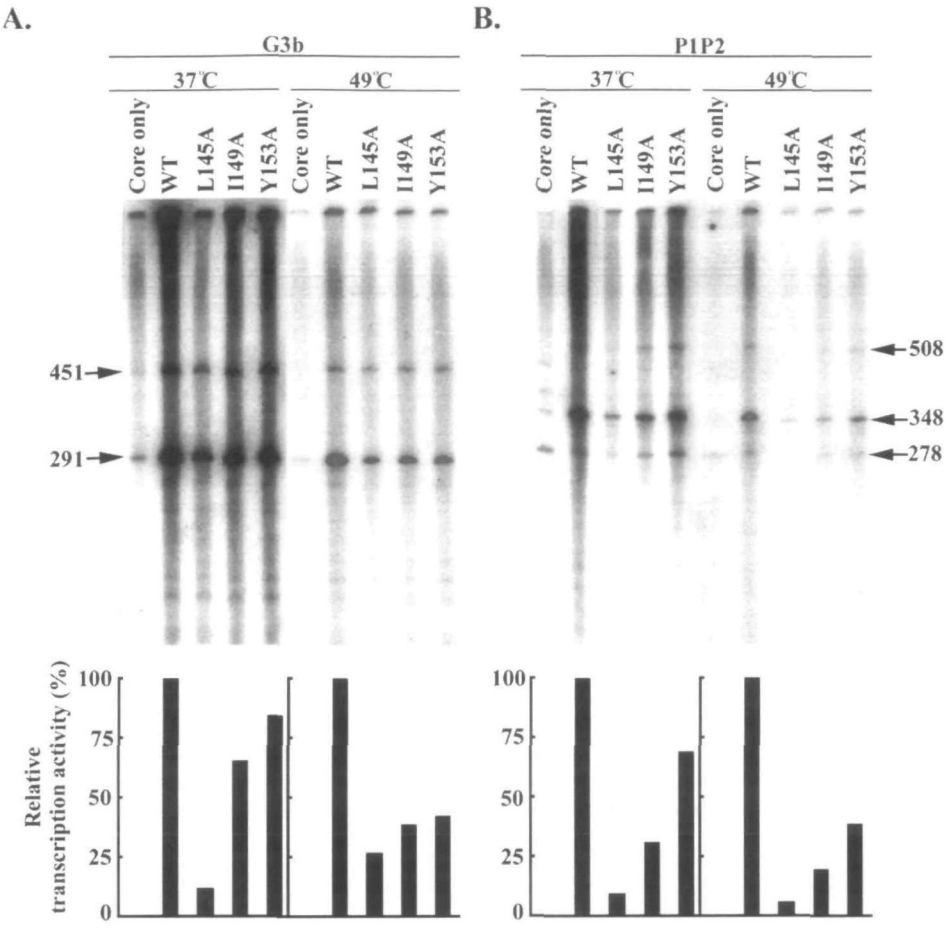

Fig. 3. ***In vitro* transcription activities of the Wt and mutant σ^A-RNA polymerases on different promoters.** A: Transcription activities of the Wt and mutant σ^A-RNA polymerases on the G3b promoter of *B. subtilis* ϕ29 phage at 37 and 49°C. B: Transcription activities of the Wt and mutant σ^A-RNA polymerases on the P1P2 promoter of *B. subtilis sigA* operon at 37 and 49°C. For each promoter template, the pattern of RNA transcripts (top) and the relative transcription activities (bottom) of the Wt and mutant σ^A-RNA polymerases are shown. Transcription from either promoter would produce two transcripts due to the presence of T1T2 terminators of the *E. coli rrnB* operon (*45*), which are located downstream of the G3b and P1P2 promoters on the pCT24 and pCT20 plasmids, respectively. The two transcripts from the G3b promoter are 291 and 451 nt in length, and those from the P1P2 promoter are 348 and 508 nt in length. The transcript of 278 nt in length was the RNA product transcribed from the putative P8 promoter (no physiological significance has been identified yet) of the *B. subtilis sigA* operon (*35*). To calculate the relative transcription activity, the total band density of the two major transcripts synthesized by a specific mutant σ^A-RNA polymerase was divided by that obtained for the Wt counterpart at the designated temperature.

were >600, 129, 417, and 292 min, respectively (Table I), indicating that L145 is more critical than I149 and Y153 to the structural stability of σ^A. Like the mutant σ^A factors with Ile-to-Ala substitution(s) in the promoter –10 binding region (*35*), all of the tested σ^A factors, whether Wt or mutant, became unstable at elevated temperature (49°C) with degradation half-lives ranging from 26 to 38 min. Since the structural stabilities of the Wt and mutant σ^A factors were similar at high temperature, it is likely that the reduction in growth potential of *B. subtilis* containing L145A-σ^A, I149A-σ^A, or Y153A-σ^A is not the result of structural instability of mutant σ^A factors. Other factors must be responsible for the decreased growth potential of the *sigA* mutants at high temperature.

Effect of L145A, I149A, or Y153A in σ^A on Core-Binding Activity of RNA Polymerase Holoenzyme—The structural instability of L145A-, I149A-, or Y153A-σ^A (Table I), and the location of these substitutions in region 2.1, which is thought to interact with core RNA polymerase, raised the possibility that each of the substitutions might affect the core-binding activity of σ^A. To check this possibility, cell lysate of each *sigA* mutant prepared at 37°C or 49°C was subjected to glycerol density gradient centrifugation. During the centrifugation, σ^A factors were separated into free and core-associated states. The free σ^A was sedimented in the top fractions (mostly fractions 1–9), whereas the core-associated σ^A was in the bottom ones (fractions 10–19). Distribution of σ^A in the core fractions (as indicated by the presence of β' subunit) for each *B. subtilis* strain was analyzed by Western blotting and the results are shown in Fig. 4. Both the Wt and mutant σ^A factors were able to associate with core RNA polymerase at both 37 and 49°C, although the core-binding activity of the tested σ^A factors varied. The percentages of σ^A bound to core RNA polymerase at 37°C were about 37.6, 25.6, 25.9, and 38.5% for the Wt-, L145A-, I149A-, and Y153A-σ^A, respectively. The corresponding figures at 49°C were about 27, 17.9, 21.1, and 30.8%. Since

TABLE I. **Temperature sensitivity of *B. subtilis* strains and the half-lives of σ^A degradation in them.**

sigA strain	Temperature sensitivity[a]	Degradation half-life (min)[b]	
		37°C	49°C
Wt	4	>600	34
L145A	1	129	38
I149A	3	417	27
Y153A	2	292	26

[a]Relative sensitivity to temperature elevation; the higher the value, the higher the sensitivity to temperature elevation. The values were determined by comparing the growth potentials of all tested *B. subtilis* strains at 49°C. [b]The half-lives of σ^A degradation were determined as described in "MATERIALS AND METHODS." The half-lives of GroEL in different strains were also measured and used as an internal control. They are about 150 min at 37°C and about 45 min at 49°C.

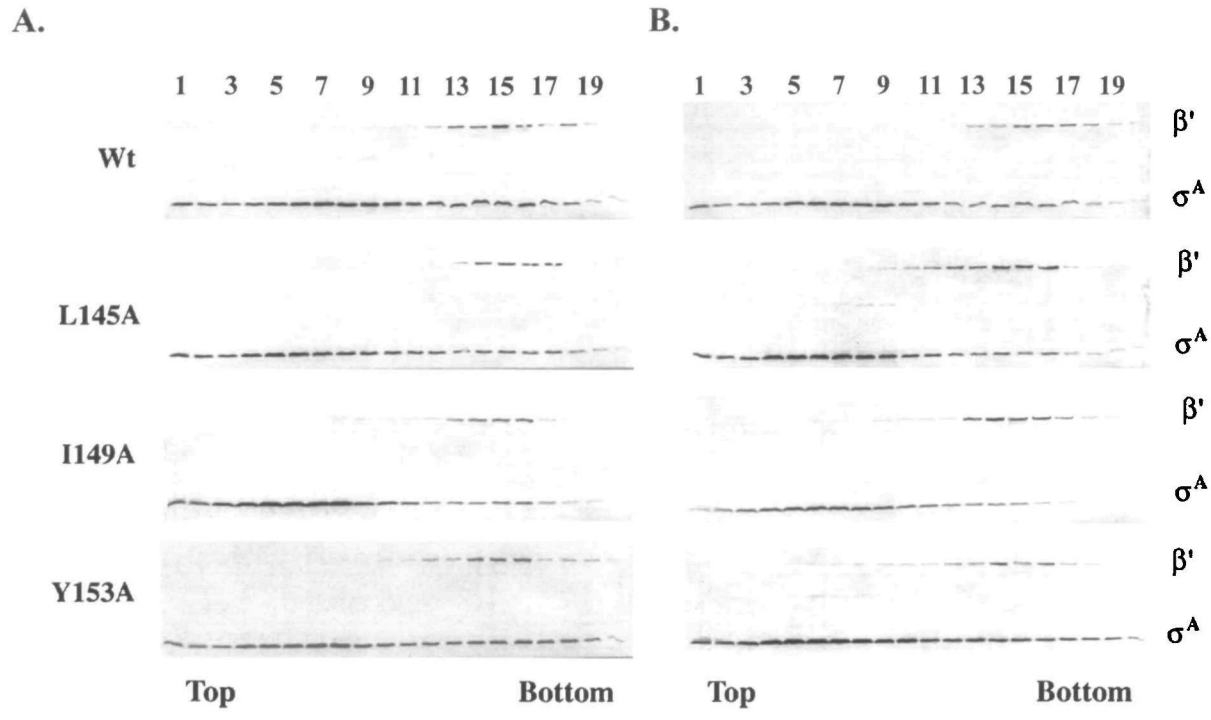

Fig. 4. **Core-binding activities of the Wt and mutant σ^A factors as determined by glycerol density gradient centrifugation and Western blot analyses.** A and B show the distributions of σ^A and β′ in the gradient samples of cell lysates of different *B. subtilis* strains grown at 37 and 49°C, respectively. The contents of σ^A in the tested strains of *B. subtilis* were very similar as analyzed by Western blotting. Methods for preparation of the gradient samples and detection of the distributions of σ^A and β′ are as described in "MATERIALS AND METHODS." Numbers shown above the figures are the fraction numbers from the top to the bottom of the glycerol density gradient. The core enzyme-containing fractions are indicated by the presence of β′ subunit. The percentage of σ^A associated with core enzyme was obtained by dividing the total band density of core-associated σ^A by the total band density of σ^A in the gradient for each strain of *B. subtilis*.

about 17.9% of the total cellular σ^A remained associated with core RNA polymerase in the strain of *B. subtilis* containing L145A-σ^A after the upshift of temperature to 49°C, it can be argued that the mechanism responsible for the low growth potential of *B. subtilis* containing L145A-σ^A at elevated temperature was very different from that responsible for the Ts phenotype of the *sigA* mutant, *B. subtilis* DB1005, of which the incapability of core binding of the Ts σ^A is attributed to its tendency to denaturation aggregation (*13*) and the loose conformation of the monomeric form of the Ts σ^A.

Replacement of L145, I149, or Y153 of σ^A with Alanine Decreased the Promoter-Binding Activity of Mutant σ^A-RNA Polymerase—Since the relatively high core-binding activity of L145A-σ^A (about 66% [17.9/27] of that of the Wt at 49°C) could not explain the low activity of the L145A-σ^A-RNA polymerase on transcribing the G3b or P2 promoter (about 27 or 5% of the Wt σ^A-RNA polymerase activity, respectively, at 49°C; Fig. 3), it seemed that the Leu-to-Ala substitution at position 145 of σ^A must have affected the process of transcription, subsequent to core binding of σ^A. The first possibility was that the L145A-σ^A-RNA polymerase was defective in promoter binding. This idea was examined by measuring the promoter-binding activity of RNA polymerase containing the Wt-, L145A-, I149A-, or Y153A-σ^A. As shown in Fig. 5, only L145A-σ^A had a significant effect on binding of RNA polymerase to the G3b promoter at 37°C; the amount of promoter DNA bound by the L145A-σ^A-RNA polymerase was about 34.5% of that of the Wt-σ^A-RNA polymerase. No significant difference in the activity of promoter binding was observed for RNA polymerase harboring the Wt-, I149A-, or Y153A-σ^A at the same temperature. However, a clear difference in the promoter-binding activity of RNA polymerase was observed as the temperature was elevated to 49°C; the relative amounts of promoter DNA bound by the Wt-, L145A-, I149A-, and Y153A-σ^A containing RNA polymerases were 100, 19.7, 62.36, and 78.45%, respectively. Thus, the three hydrophobic amino acids in region 2.1, especially L145, are essential to the promoter-binding function of σ^A-RNA polymerase. Moreover, the close correlation between the reduction in promoter binding of RNA polymerase containing L145A-, I149A-, or Y153A-σ^A and the reduced growth potentials of the corresponding *B. subtilis* strains at 49°C suggests that it is the promoter-binding activity of RNA polymerase which determines the capability of the mutant *B. subtilis* to grow at elevated temperature.

DISCUSSION

We have shown that L145A, I149A, and Y153A in region 2.1 of the *B. subtilis* σ^A factor change the structural stability and core-binding activity of σ^A, as well as the promoter-binding activity of σ^A-RNA polymerase. The pleiotropic effects of these mutations on σ^A are consistent with the facts that region 2.1 is within the most hydrophobic core region (*13, 31–35*), is probably involved in core binding (*17, 19*), and is in close contact with the promoter –10 binding

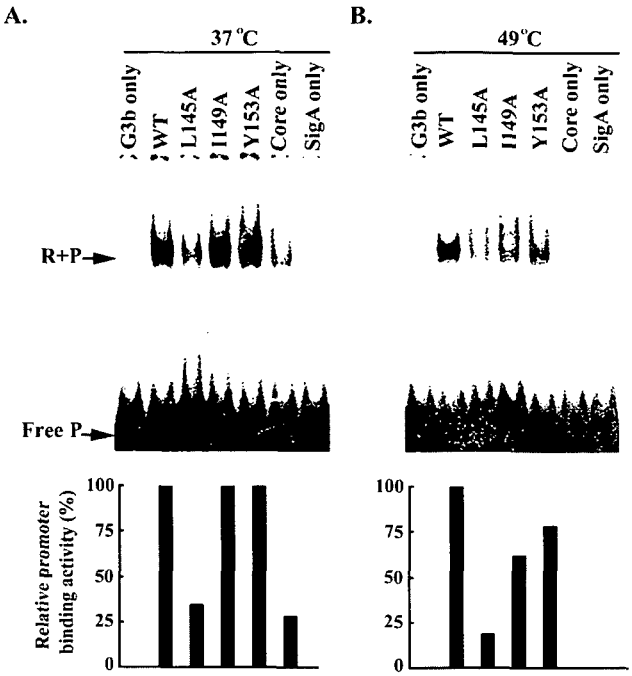

Fig. 5. **Promoter-binding activities of the Wt and mutant σ^A-RNA polymerases as determined by gel retardation assays.** A and B show the promoter-binding activities of the Wt and mutant σ^A-RNA polymerases on the G3b promoter of *B. subtilis* ϕ29 phage at 37 and 49°C, respectively. The upper part of each figure depicts the amount of promoter DNA retarded by RNA polymerase containing the Wt-, L145-, I149-, or Y153-σ^A factor. Free P indicates the unbound G3b promoter DNA. P+R indicates the RNA polymerase and G3b promoter DNA complex. The lower part of each figure indicates the relative promoter-binding activity of RNA polymerase containing the Wt-, L145-, I149-, or Y153-σ^A factor. To calculate the relative promoter-binding activity, the band density for a specific mutant σ^A-RNA polymerase and promoter DNA complex (P+R) was divided by that obtained for the Wt counterpart at the designated temperature.

helix (*16, 35*). They also demonstrate that the interdigital interactions between the three bulky hydrophobic amino acid residues (L145, I149, and Y153) in region 2.1 and the three isoleucine residues (I194, I198, and I202) in the promoter –10 binding helix (Fig. 1) are critical for the maintenance of the functional σ^A structure.

It has been reported that, of the three isoleucine residues on the hydrophobic face of the promoter –10 binding helix, I198 is most essential to sustain the normal σ^A structure and function (*35*). Thus, it was predicted that L149, which is opposite to I198, should be also most important to the structure and function of σ^A. However, it is shown here that L145 at the most N-terminal position of the second helix of region 2.1 is much more critical to σ^A structure and function than I149 and Y153, although the latter two amino acids are supposed to make more direct contact with the isoleucine residues on the promoter –10 binding helix than does L145 (Fig. 1). The reason for this discrepancy is unclear. It could be that there are other important contacts between L145 and amino acids in the neighborhood that are not deducible from the limited crystal data of the partial peptide of *E. coli* σ^{70} (*16*).

More than one specific region of the σ factor has been reported to be involved in core binding (*17–19, 22, 24–26, 44*). Among them, region 2.1 first drew our attention (*17–19*). Two mutations in this region of the *B. subtilis* σ^E factor have been found to affect the core-binding activity of σ^E. One of them, designated as *sigE*Δ61-3, contains a three-amino-acid deletion at the end of the first helix and loses core-binding activity. The other mutant σ^E factor, which contains an Arg-to-Cys substitution at position 68 of σ^E, the most N-terminal position of the second helix of region 2.1 and one that is solvent-exposed, has a reduced core-binding activity (*19*). In the present study, L145A and I149A located on the hydrophobic face of the second helix of region 2.1 slightly reduce the core-binding activity of σ^A (Fig. 4), while Y153A located in the loop region downstream the second helix of region 2.1 has a slight positive effect on core binding. The slight change in core binding of L145A-, I149A-, and Y153-σ^A may be explained by the presence of multiple core-binding sites on σ^A. In other words, the efficient binding of σ^A to core enzyme may be an additive event, and thus change of one of the binding sites may not have a drastic effect on the core-binding activity of σ^A. However, this seems to be not the case, as L145, I149, and Y153 are located within an unexposed core region and are not directly involved in core binding (Fig. 1). We think it more probable that the overall core-binding conformation of region 2.1 of the *B. subtilis* σ^A factor is altered to various extents by L145A, I149A, and Y153A.

The significantly low promoter-binding activity of RNA polymerase containing L145A-σ^A (about 34.5 and 19.7% of that of the Wt at 37 and 49°C, respectively) was unexpected (Fig. 5). It seems that this is not a result of the reduced core-binding activity of L145A-σ^A, since this mutant σ^A factor still possesses about 66% of the core-binding activity of Wt σ^A at 37 and 49°C (Fig. 4), and since the promoter-binding assay was performed under a condition in which the core enzyme was highly saturated with σ^A (the molar concentration of σ^A used for holoenzyme reconstitution was in 10-fold excess of that of core enzyme; see "MATERIALS AND METHODS"). Therefore, we favor the notion that the reduction in the efficacy of recognition or binding of the promoter –10 binding region of the L145A-, I149-, or Y153-σ^A-RNA polymerase to the promoter DNA must be attributed to improper adjustment of the promoter –10 binding region of the mutant σ^A to the cognate promoter, especially at elevated temperature. Taken together, our data suggest that the contacts between the hydrophobic amino acid residues in regions 2.1 and 2.4 of the *B. subtilis* σ^A factor are essential to sustain the functional core-binding and promoter-binding conformations of σ^A. In other words, region 2.1 may play an important role in retaining the active conformation of σ^A.

REFERENCES

1. Helmann, J.D. and Chamberlin, M.J. (1988) Structure and function of bacterial sigma factor. *Annu. Rev. Biochem.* **57**, 839–872
2. Lonetto, M., Griskov, M., and Gross, C.A. (1992) The σ^{70} family: sequence conservation and evolutionary relationships. *J. Bacteriol.* **174**, 3843–3849
3. Daniels, D., Zuber, P., and 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 cognate promoter. *Proc. Natl. Acad. Sci. USA* **87**, 8075–8079
4. Gardella, T., Moyle, H., and Susskind, M.M. (1989) A mutant

Escherichia coli σ^{70} subunit of RNA polymerase with altered promoter specificity. *J. Mol. Biol.* **206**, 579–590
5. Keener, J. and Nomura, M. (1993) Dominant lethal phenotype of a mutation in the –35 recognition region of *Escherichia coli* σ^{70}. *Proc. Natl. Acad. Sci. USA* **82**, 8124–8128
6. Kenney, T.J., York, K., Youngman, P., and Moran, C.P. (1989) Genetic evidences that RNA polymerase associated with σ^A uses a sporulation specific promoter in *Bacillus subtilis*. *Proc. Natl. Sci. Acad. USA* **86**, 9109–9113
7. Kenney, T.J. and Moran, C.P. (1991) Genetic evidence for interaction of σ^A with two promoters in *Bacillus subtilis*. *J. Bacteriol.* **173**, 3282–3290
8. Marr, M.T. and Robert, J.W. (1997) Promoter recognition as measured by binding of polymerase to nontemplate strand oligonucleotide. *Science* **276**, 1258–1260
9. Siegele, D.A., Hu, J.C., Walter, W.A., and Gross, C.A. (1989) Altered promoter recognition by mutant forms of the σ^{70} subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **206**, 591–603
10. Waldburger, C., Gardella, T., Wong, R., and Susskind, M.M. (1990) Changes in conserved region 2 of *Escherichia coli* σ^{70} affecting promoter recognition. *J. Mol. Biol.* **215**, 267–276
11. Zuber, P., Healy, J., Carter, H.L., Cutting, S., Moran, C.P., and Losick, R. (1989) Mutation changing the specificity of an RNA polymerase sigma factor. *J. Mol. Biol.* **206**, 605–614
12. Chang, B.Y. and Doi, R.H. (1993) Effects of amino acid substitutions in the promoter –10 binding region of the σ^A factor on growth of *Bacillus subtilis*. *J. Bacteriol.* **175**, 2470–2474
13. Wen, Y.D., Liao, C.T., Liou, K.M., Wang, W.H., Huang, W.C., and Chang, B.Y. (2000) Structural and functional properties of a *Bacillus subtilis* temperature-sensitive σ^A factor. *Proteins* **40**, 613–622
14. Chang, B.Y., Chen, K.Y., Wen, Y.D., and Liao, C.T. (1994) The response of a *Bacillus subtilis sigA* mutant to heat stress. *J. Bacteriol.* **176**, 3102–3110
15. Liao, C.T., Wen, Y.D., Wang, W.H., and Chang, B.Y. (1999) Identification and characterization of a stress-responsive promoter in the macromolecular synthesis operon of *Bacillus subtilis*. *Mol. Microbiol.* **33**, 377–388
16. Malhotra, A., Severinova, E., and Darst, S.A. (1996) Crystal structure of a σ^{70} fragment from *Escherichia coli* RNA polymerase. *Cell* **87**, 127–136
17. Lesley, S.A. and Burgess, R.R. (1989) Characterization of the *Escherichia coli* transcription factor σ^{70}: localization of a region involved in the interaction with core RNA polymerase. *Biochemistry* **28**, 7728–7734
18. Lesley, S.A., Brow, M.A.D., and Burgess, R.R. (1991) Use of *in vitro* protein synthesis from polymerase chain reaction-generated templates to study interaction of *Escherichia coli* transcription factors with core RNA polymrase and for epitope mapping of monoclonal antibodies. *J. Biol. Chem.* **266**, 2632–2638
19. Shuler, M.F., Tatti, K.M., Wade, K.H., and Moran, C.P. (1995) A single amino acid subsitution in σ^E affects its ability to bind core RNA polymerase. *J. Bacteriol.* **177**, 3687–3694
20. Gribskov, M. and Burgess, R.R. (1986) Sigma factors from *E. coli*, *B. subtilis*, phage SP01, and phage T4 are homologous proteins. *Nucleic Acids Res.* **14**, 6745–6763
21. Neidhardt, F.C., VanBogelen, R.A., and Lau, E.T. (1983) Molecular cloning and expression of a gene that controls the high temperature regulon of *Escherichia coli*. *J. Bacteriol.* **153**, 597–603
22. Sharp, M.M., Chan, C.L., Lu, C.Z., Marr, M.T., Nechaev, S., Merritt, E.W., Severinov, K., Roberts, J.W., and Gross, C.A. (1999) The interface of with core RNA polymerase is extensive, conserved, and functionally specialized. *Genes Dev.* **13**, 3015–3026
23. Stragier, P., Parsot, C., and Bouvier, J. (1985) Two functional domains conserved in major and alternate bacterial sigma factors. *FEBS Lett.* **187**, 11–15
24. Joo, D.M., Ng, M., and Calendar, R. (1997) A σ^{32} mutant with a single amino acid change in the highly conserved region 2.2 exhibits reduced core RNA polymerase affinity. *Proc. Natl. Acad. Sci. USA* **94**, 4907–4912
25. Joo, D.M., Nolte, A., Calendar, R., Zhou, Y.N., and Jin, D.J. (1998) Multiple regions on the *Escherichia coli* heat shock transcription factor σ^{32} determine core RNA polymerase binding specificity. *J. Bacteriol.* **180**, 1095–1102
26. Zhou, Y.M., Walter, W.A., and 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
27. Liao, C.T., Wen, Y.D., Wang, W.H., Tsai, S.S., Doi, R.H., and Chang, B.Y. (1997) The importance of a proper helical structure in the promoter –10 binding region to *Bacillus subtilis* structure and function. *J. Biochem.* **122**, 911–917
28. Saunders, V.A. and Saunders, J.R. (1987) *In vivo* and *in vitro* mutagenesis in *Microbial Genetics Applied to Biotechnology* (Saunders, V.A. and Saunders, J.R., eds.) pp. 163–218, MacMillan, New York
29. Shortle, D., Dimaio, D., and Nathan, N. (1981) Direct mutagenesis. *Annu. Rev. Genet.* **15**, 265–294
30. Tabor, S. (1990) Expression using the T7 RNA polymerase/promoter system in *Current Protocols in Moleculkar Biology* (Ausubel, F.A., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds.) pp. 16.2.1–16.2.11, Greene Publishing & Wiley Interscience, New York
31. Chang, B.Y. and Doi, R.H. (1990) Overproduction, purification, and characterization of the *Bacillus subtilis* RNA polymerase σ^A factor. *J. Bacteriol.* **172**, 3257–3263
32. Spizizen, J. (1958) Transformation of biochemical strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. USA* **44**, 1072–1078
33. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* **227**, 680–685
34. Huang, W.C. (2000) Identification and characterization of the functional domains of *Bacillus subtilis* σ^A factor. Master thesis. National Chung-Hsing University, Taiwan, ROC
35. Liao, C.T., Wang, W.H., and Chang, B.Y. (1999) Differential and additive effects of the three conserved isoleucine residues in the promoter –10 binding region on *Bacillus subtilis* σ^A structure and function. *J. Biochem.* **126**, 461–469
36. Chang, B.Y, Liao, C.T., Wen, Y.D., and Wang, W.H. (1997) The temperature sensitivity of *Bacillus subtilis* is due to insufficient activity, rather than insufficient concentration, of the mutant σ^A factor. *Microbiology* **143**, 1299–1308
37. Chou, P.Y. and Fasman, G.D. (1974) Emperical predictions of protein conformation. *Biochemistry* **13**, 222–245
38. Gopal, V. and Chatterji, D. (1997) Mutations in the 1.1 subdomain of *Escherichia coli* sigma factor and disruption of its overall structure. *Eur. J. Biochem.* **244**, 613–618
39. Grossman, A.D., Burgess, R.R., Walter, W.A., and Gross, C.A. (1983) Mutations in the *lon* gene of *E. coli* K12 phenotypically suppress a mutation in sigma subunit of RNA polymerase. *Cell* **32**, 151–159
40. Lowe, P.A., Aebi, U., Gross, C.A., and Burgess, R.R. (1981) *In vitro* thermal inactivation of a temperature-sensitive subunit mutant (*rpoD800*) of *Escherichia coli* RNA polymerase proceeds by aggregation. *J. Biol. Chem.* **256**, 2010–2015
41. Jones, C.H. and Moran, C.P. (1992) Mutant σ factor blocks transition between promoter binding and initiation of transcription. *Proc. Natl. Acad. Sci. USA* **89**, 1958–1962
42. Juang, Y. L. and Helmann, J.D. (1994) A promoter melting region in the primary factor of *Bacillus subtilis*. Identification of functionally important aromatic amino acids. *J. Mol. Biol.* **235**, 1470–1488
43. Rong. J.C. and Helmann, J.D. (1994) Genetic and physiological studies of *Bacillus subtilis* σ^A mutant defective in promoter melting. *J. Bacteriol.* **176**, 5218–5224
44. Datwyler, S.A. and Meares, C.F. (2000) Protein-protein interactions mapped by artificial proteases: where σ factors bind to RNA polymerase. *TIBS* **25**, 408–414
45. Jurgen, B., Dull, T.J., Sleeter, D.D., and Noller, H.F. (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* **148**, 107–127