J. Biochem. 126, 461-469 (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[1]

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Received April 30, 1999; accepted June 18, 1999

The promoter −10 binding region of the *Bacillus subtilis* σ^A factor forms an amphiphilic α-helix with three conserved isoleucines located at four-residue intervals. To investigate the structural and functional roles of the three isoleucine residues, we constructed a series of *sigA* mutants with single and double Ile-to-Ala substitutions on the hydrophobic face of this α-helix and isolated intragenic revertants with either same-site or second-site suppressor that partially restores the structural stability and transcription activity of the mutant σ^A factors. Our data show that the three conserved isoleucine residues (Ile-194, Ile-198, and Ile-202) are involved in the hydrophobic core packing of σ^A; they affect differentially and additively the structure and function of σ^A, with the central isoleucine residue (Ile-198) playing the most important role. By analogy with the crystal structure of a σ^{70} peptide, it is apparent that interdigital interactions exist between the three conserved isoleucine residues and certain hydrophobic amino acids in region 2.1 of σ^A. They include at least the van der Waals contacts between Ile-194 and both Leu-145 and Ile-149, between Ile-198 and both Ile-149 and Tyr-153, as well as between Ile-202 and Tyr-153. The same-site suppressors, Val-194 and Val-198, restore the structural stability and transcription activity of σ^A by repacking the hydrophobic core of σ^A. The second-site suppressor (S291F) appears to be allele-specific, but it is not as effective as the same-site suppressors in restoring σ^A structure and function.

Key words: α-helix, *Bacillus subtilis*, hydrophobic amino acids, sigma factor, suppressor mutant.

The specificity of promoter utilization by sigma factors of the σ^{70}-type is dictated by two regions of the σ protein that make sequence-specific contacts with the −10 and −35 regions of promoter DNA. Region 4.2 at the C-terminus of σ contains a helix-turn-helix motif and is thought to interact specifically with the −35 region of the promoter (*1-5*). Region 2.4 forms an amphiphilic α-helix with three isoleucine residues located at four-residue intervals (*6, 7*). Amino acids on the hydrophilic face of this helix are believed to participate in sequence-specific contacts with the promoter −10 DNA as reported for *Escherichia coli* σ^{70} as well as for *Bacillus subtilis* σ^A, σ^E, σ^F, and σ^H (*5, 8-14*). Thus, amino acids on the opposite hydrophobic face of the promoter −10 binding helix must play a different role. In other words, the promoter −10 binding helix must possess a second role besides the well characterized DNA-binding property.

It is well known that the *B. subtilis* σ^A and *E. coli* σ^{70} recognize the same consensus sequence of promoter DNA (*15*) but fail to bind the promoter unless they associate with the RNA polymerase core enzyme and undergo a conformational change (*16-19*). In addition, both σ factors are able to associate with the same core enzyme (*20*), and both of them possess four conserved regions (*21, 22*) as well as a protease-sensitive site which could be a domain boundary near the end of conserved region 2 (*16, 22-24*). The two σ factors, therefore, seem to share similarities in both structure and function. Recently, the crystal structure of a σ^{70} peptide encompassing regions 1.2 to 2.4 was resolved (*25*). In line with the prevailing view, the promoter −10 binding region (region 2.4) does form an amphiphilic α-helix extended from region 2.3, with the hydrophilic face exposed to the solvent and the hydrophobic face contributing to the hydrophobic core. The latter is created by clustering of the conserved hydrophobic amino acids in both regions 1 and 2, including the three conserved isoleucine residues on the promoter −10 binding helix (*25*).

We have been interested in characterizing the second role of the promoter −10 binding helix. Endeavours have been made to analyze the properties of mutant σ^A factors by changing either the hydrophobicity (reducing the size and hydrophobicity of amino acid side chain) or the helicity (breaking the helix) of the amphiphilic α-helix. Both types of mutations rendered σ^A functionally inactive at an

[1] This research was supported by National Science Council of the Republic of China grants NSC 85-2311-B-005-027 and NSC 86-2311-B-005-037.

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elevated temperature and structurally unstable even at the permissive temperature (*26, 27*). The deleterious effects support the existence of important packing interactions between the conserved isoleucines on the hydrophobic face of the promoter −10 binding helix and certain amino acid(*s*) in the hydrophobic core region of σ^A, which are indispensable for maintaining a stable and functional σ^A structure.

To further understand the contribution of each of the conserved isoleucines to σ^A structure and function, and the location of amino acids with which the three conserved isoleucines interact, we constructed a series of mutant σ^A factors with changes made at each of the conserved isoleucines and looked for their intragenic suppressors. Structural and functional analyses of the mutant σ^A factors revealed that the three conserved isoleucine residues played differential and additive roles in σ^A structure and function. By analogy with the crystal structure of a partial σ^{70} peptide, amino acids which interact interdigitally with the conserved isoleucines were proposed.

MATERIALS AND METHODS

Construction of the sigA Mutants and Screening for Revertants—The *sigA* genes of *B. subtilis* DB1008 and DB1009 were constructed by overlapping extension polymerase chain reaction (*28, 29*). The overlapping primer sequences were as follows: B8, 5′-TCGGCCGCGGCGCGTGTAATCGCC-3′; C8, 5′-CACGCGCCGCGGCCGATCAGGCGAGAAC-3′; B9, 5′-GGCGCGTGTGGCCGCCTGTCTGATCCAC-3′; C9, 5′-GGCGGCCACACGCGCCATTGCCG-3′. The mutant *sigA* genes were integrated, respectively, into the chromosome of *B. subtilis* DB2 for the construction of *B. subtilis* DB1008 and DB1009 using the same method as that reported for the construction of *B. subtilis* DB1003 and DB1005 (*6*). To look for spontaneous revertants of the *sigA* mutants, an overnight culture of each *sigA* mutant grown in 2×SG liquid medium at 37°C (*30*) was plated on fresh 2×SG agar plates containing chloramphenicol (5 μg/ml) and incubated at high temperature (55°C). The revertants always formed larger colonies than the corresponding *sigA* mutants at high temperature and were easy to obtain.

Confirmation of the sigA Mutants and the Intragenic Revertants—Cotransformation and direct DNA sequencing were adopted to confirm the *sigA* mutants (*B. subtilis* DB1008 and DB1009) as well as the revertants. In the former assay, the chromosomal DNA extracted from the *sigA* mutant was transformed into *B. subtilis* DB2. Since a *cat* gene has been integrated into the chromosome of the *sigA* mutant in the vicinity of the *sigA* gene (about 1.5 kb apart) (*6*), a high cotransformation efficiency of chloramphenicol resistance and specific morphology of the *sigA* mutant would be expected if the chromosome was transformed into *B. subtilis* DB2. As expected, about 60 to 80% of the chloramphenicol-resistant transformants were found to show similar morphology of the *sigA* mutant at elevated temperature. Similar results were obtained for the intragenic revertants which contained mutations in *sigA*, as confirmed by direct sequencing of the *sigA* DNA amplified from the chromosome of revertants using a specific set of primers (*6*). The revertants, which had cotransformation efficiencies as low as a few percent or less, were considered extragenic, since DNA sequencing revealed that they were devoid of secondary mutations in the *sigA* gene.

Determination of the Degradation Rates of the Mutant σ^A Proteins—The method for the determination of σ^A degradation was the same as that reported previously (*26*).

Comparison of the Activities of Mutant σ^A Factors at Elevated Temperature—The transcription activities of mutant σ^A factors were determined by analyzing the rates of GroEL induction in all of the *B. subtilis* strains before and after heat shock. Briefly, *B. subtilis* was grown in 2× SG medium at 37°C to a cell density (A_{550}) of 0.4 (referred to as time zero) and then transferred to 49°C. Cell samples were harvested at different time points within the first 30 min after the upshift of temperature. After pelleting and resuspending the cells with 50 μl of 0.5×SET buffer (20% sucrose, 50 mM Tris-HCl, pH 7.6, 50 mM EDTA, 2 mM PMSF), lysozyme was added to digest the cell wall. The cells were then broken by adding 50 μl of sample application buffer (*16*). An equal volume of cell lysate from each of the *B. subtilis* cultures was assayed immunologically for the level of GroEL. Band density of GroEL was scanned with a Bio Image System from the Millipore. The levels of GroEL expressed at 49°C were normalized to that expressed at 37°C for each *B. subtilis* strain.

RESULTS

Construction of sigA Mutants and Screening of Revertants—The impact of changes in hydrophobicity and helicity of the promoter −10 binding helix on σ^A structure and function (*26, 27*) suggests that there are important packing interactions between the conserved isoleucine residues which position one after another on the hydrophobic face of the promoter −10 binding helix (Fig. 1, a and b) and certain amino acids in the hydrophobic core region of σ^A (*16, 23*). In order to evaluate the importance of each of the conserved isoleucine residues and to locate their interacting amino acids, our first approach was to construct a series of *sigA* mutants with Ile-to-Ala substitution(s) at the three conserved positions and screen for their intragenic revertants (see "MATERIALS AND METHODS").

The *sigA* mutants constructed were *B. subtilis* DB1003 (σ^A_{I194A}), DB1005 ($\sigma^A_{I198A,I202A}$), DB1008 (σ^A_{I202A}), and DB1009 (σ^A_{I198A}) (Fig. 1c and Ref. *6*). Probably due to the insensitivity of *B. subtilis* DB1008 to temperature elevation, its revertant was difficult to obtain. The revertants of *B. subtilis* DB1003, however, appeared at a frequency of about 10^{-7}, and those of *B. subtilis* DB1005 and DB1009 at about 10^{-6}. On the basis of the colony sizes of the revertants at elevated temperatures and the cotransformation frequencies of their chromosomal DNA (see "MATERIALS AND METHODS"), the revertants of each *sigA* mutant were classified into two (*i.e.*, *B. subtilis* DB1003) or three (*i.e.*, *B. subtilis* DB1005 and DB1009) types. Those having higher cotransformation frequencies (about 40 to 60%) were thought of as intragenic revertants. The intragenic revertant of *B. subtilis* DB1003 was named DB1003R1, and those of *B. subtilis* DB1005 or DB1009 were designated as DB1005R1 and DB1005R3, or as DB1009R4 and DB1009-R7, respectively (Table I).

Identification of Intragenic Suppressors of the Primary Mutant σ^A Factors—The intragenic suppressors for each *sigA* mutation were identified by sequencing of the *sigA*

genes of at least 12 individual intragenic revertants of the corresponding *sigA* mutant (Table II). Three different intragenic suppressors, A194V, A198V, and S291F, were found in the sequenced *sigA* genes. *B. subtilis* DB1005R3 $\sigma^{A}{}_{I198V,I202A}$ and DB1009R4 $\sigma^{A}{}_{I198V}$ harbored the identical same-site suppressor, A198V. The Ala-to-Val substitution was also found in *B. subtilis* DB1003R1 $\sigma^{A}{}_{I194V}$. The suppression of both I194A and I198A by the Ala-to-Val substitution suggests that bulky hydrophobic amino acids are essential to the functional σ^{A} structure at high temperature. This is reasonable, since valine has a comparable size and hydrophobicity (*31*) to the isoleucine residue at the corresponding position (Fig. 1c). In contrast, DB1005R1 $\sigma^{A}{}_{I198A,I202A,S291F}$ and DB1009R7 $\sigma^{A}{}_{I198A,S291F}$ contained the same second-site suppressor, S291F. The exclusive identification of S291F as the second-site suppressor for the two mutant σ^{A} factors containing the I198A substitution suggests that the suppressor is allele-specific. A possible mechanism for this apparent allele-specific suppression will be discussed later.

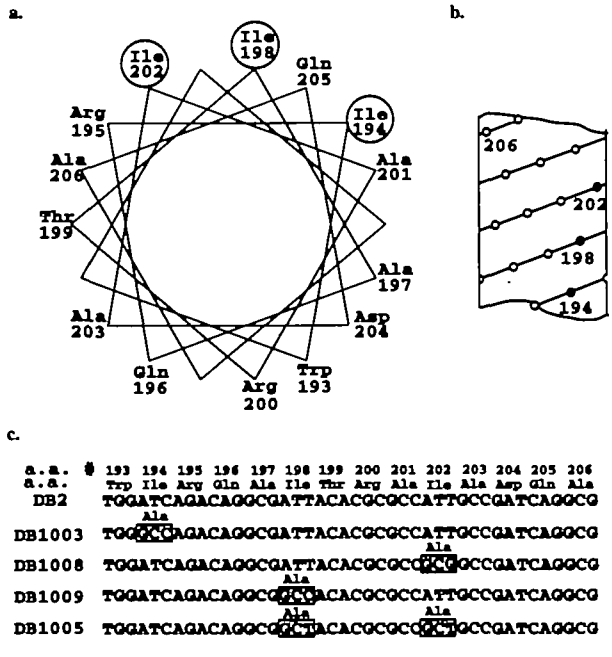

Fig. 1. **Helical wheel and helical cylinder representations of the proposed promoter −10 binding helix of *B. subtilis* σ^{A} factor.** a: Amphiphilic α-helix of the promoter −10 binding region of wild-type σ^{A}. The three isoleucine residues investigated in the study are circled. b: Cylindrical plot of the α-helix in the promoter −10 binding region of σ^{A}. The three isoleucine residues are indicated by solid circles. c: Amino acid substitutions in the α-helix of the promoter −10 binding region of the mutant σ^{A} factors. The top two lines indicate the amino acid sequence of the promoter −10 binding helix of the wild-type σ^{A}. The bottom five lines indicate the sense strand DNA sequences of the wild-type (DB2) and the four *sigA* mutants (*B. subtilis* DB1003, DB1008, DB1009, and DB1005). The sites of amino acid substitutions for the mutant *sigA* genes are boxed.

The Effects of sigA Mutations on the Growth of B. subtilis—To investigate the relative importance of each conserved isoleucine and each suppressor to σ^{A} structure and function, and their relation to the temperature sensitivity of *B. subtilis*, we compared the growth potentials of different *sigA* mutants grown in glucose minimal medium (*32*) at elevated temperature (Fig. 2 and Table III). As shown in Fig. 2a, the substitution(s) of alanine for isoleucine at different conserved position(s) affected differently the growth of *B. subtilis* when the growth temperature was elevated from 37 to 50.5°C. The influence was most pronounced with the DB1005 $\sigma^{A}{}_{I198\&202A}$ and DB1009 $\sigma^{A}{}_{I198A}$. Cell densities of *B. subtilis* containing those two σ^{A} factors declined after the upshift of temperature, despite the resumption of DB1009 growth a few hours later. DB1003 $\sigma^{A}{}_{I194A}$ slowed down the growth of *B. subtilis* only moderately, suggesting that I194A was less influential than I198 & 202A and I198A on the function of σ^{A}. The effect of DB1008 $\sigma^{A}{}_{I202A}$ on the growth of *B. subtilis* was not apparent; this was consistent with the difficulty in obtaining a suppressor.

The growth potentials of the *sigA* mutants were improved by their corresponding suppressors (Fig. 2, b, c, and d). However, the two suppressors shared by DB1005 $\sigma^{A}{}_{I198\&202A}$ and DB1009 $\sigma^{A}{}_{I198A}$, A198V and S291F, did not improve *B. subtilis* growth to the same extent (Fig. 2, c and d). Better growth of *B. subtilis* was obtained with A198V than with S291F, suggesting that the same-site suppressor was more effective than the second-site suppressor on σ^{A} function at high temperature.

Differential and Additive Effects of the Conserved Isoleucine Residues on the Structural Stability and Transcrip-

TABLE I. ***B. subtilis* strains used in the study.**

Strain	Description	Source of reference
DB2	*trpC2 sigA* (Wt)	*B. subtilis* W168
DB1003	*trpC2 sigA* (I194A) Cm^{r}	Ref. *6*
DB1003R1	Revertant of *B. subtilis* DB1003 *trpC2 sigA*(I194A) Cm^{r}	This study
DB1005	*trpC2 sigA* (I198A, I202A) Cm^{r}	Ref. *6*
DB1005R1	Revertant of *B. subtilis* DB1005 *trpC2 sigA* (I198A, I202A, S291F) Cm^{r}	This study
DB1005R3	Revertant of *B. subtilis* DB1005 *trpC2 sigA* (I198V, I202A) Cm^{r}	This study
DB1008	*trpC2 sigA* (I202A) Cm^{r}	This study
DB1009	*trpC2 sigA* (I198A) Cm^{r}	This study
DB1009R4	Revertant of *B. subtilis* DB1009 *trpC2 sigA* (I198V) Cm^{r}	This study
DB1009R7	Revertant of *B. subtilis* DB1009 *trpC2 sigA* (I198V, S291F) Cm^{r}	This study

TABLE II. **The intragenic suppressors of *B. subtilis* primary mutant sigma-A factors.**

Primary *sigA* mutant	Primary mutation	Reversion	Base substitution	Intragenic revertant
DB1003	I194A	A194V	GCC→GTC(198)	DB1003R1
DB1008	I202A	None	None	None
DB1009	I198A	1.A198V	GCC→GTC(198)	DB1009R4
		2.S291F	TCT→TTT(291)	DB1009R7
DB1005	I198A, I202A	1.A198V	GCT→GTT(198)	DB1005R3
		2.S291F	TCT→TTT(291)	DB1005R1

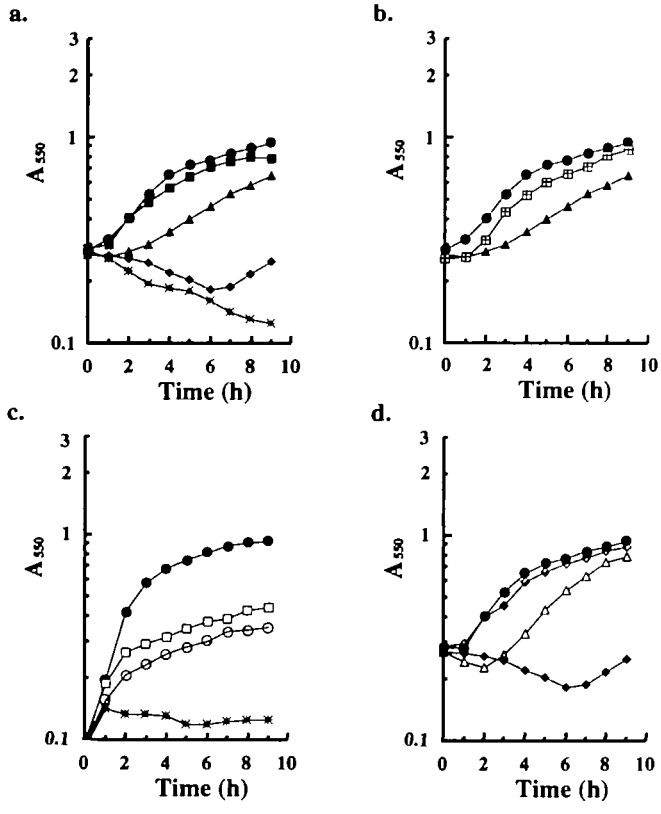

Fig. 2. **Effects of temperature elevation on growth of *B. subtilis*.** a: Effects of temperature elevation on growth of *B. subtilis* DB2 and the *sigA* mutants *B. subtilis* DB1003, DB1008, and DB1009. b: Effects of temperature elevation on growth of *B. subtilis* DB2, DB1003, and DB1003R1. c: Effects of elevated temperature on growth of *B. subtilis* DB2, DB1005, DB1005R1, and DB1005R3. d: Effects of temperature elevation on growth of *B. subtilis* DB2, DB1009, DB1009R4, and DB1009R7. *B. subtilis* strains were grown in glucose minimal medium at 37°C to an optical density (A_{550}) of about 0.2 before being transferred to 50.5°C (a, b, and d) or grown in glucose minimal medium at 49°C after inoculation with an initial optical density (A_{550}) of about 0.08 (c). The symbols ●, ▲, ■, ◆, *, ⊞, ○, □, ◇, and △ represent the growth of *B. subtilis* DB2, DB1003, DB1008, DB1009, DB1005, DB1003R1, DB1005R1, DB1005R3, DB1009R4, and DB1009R7, respectively.

tion Activity of σ^A Factors—To further investigate the contribution of each of the conserved isoleucines to σ^A structure, the stabilities of the mutant σ^A factors were compared at 37 and 49°C. Our data showed that the stability, in terms of degradation rates, of the mutant σ^A factors at 37°C in descending order was DB1008 $\sigma^A{}_{I202A}$ > DB1003 $\sigma^A{}_{I194A}$ > DB1009 $\sigma^A{}_{I198A}$ > DB1005 $\sigma^A{}_{I198\&202A}$ (Fig. 3a) with the calculated half-lives of 251, 154, 109, and 59 min, respectively (Table III). The difference in degradation half-life among the three σ^A factors with single Ile-to-Ala substitution indicates that Ile-194, Ile-198, and Ile-202 are not of equal importance to σ^A structure. The shorter half-live of $\sigma^A{}_{I198A}$ relative to $\sigma^A{}_{I202A}$ and $\sigma^A{}_{I194A}$ indicates that Ile-198 is more important than the other two isoleucines to the structural stability of σ^A. Moreover, the lower structural stability of $\sigma^A{}_{I198\&202A}$ compared with $\sigma^A{}_{I202A}$ and $\sigma^A{}_{I198A}$ suggests that Ile-198 and Ile-202 act in concert to sustain the structural stability of σ^A. All of the σ^A factors, whether wild-type or mutant, became easily degraded at 49°C (Table III). However, the degradation is not responsible for the temperature sensitivity of *B. subtilis*, since overexpression of the temperature-sensitive σ^A factor could not restore the growth of *B. subtilis* DB1005 at elevated temperature. Insufficient activity of the mutant σ^A factor at elevated temperature was the major cause for such a growth defect (*26*).

TABLE III. **The extent of temperature sensitivity of *B. subtilis* and the half-lives of SigA degradation in *B. subtilis*.**

Strain	Temperature sensitivity[a]	Degradation half-lives (min)[b]	
		37°C	49°C
DB2	1	>600	34
DB1003	6	154	25
DB1003R1	3	>600	33
DB1008	4	251	30
DB1009	8	109	23
DB1009R4	2	>600	33
DB1009R7	7	128	23
DB1005	10	59	23
DB1005R1	9	74	23
DB1005R3	5	158	24

[a]Relative sensitivity to tempeature elevaiton; 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 50.5°C. [b]The half-lives of SigA degradation were determined according to the regression lines shown in Fig. 2.

Since σ^A is solely responsible for the transcriptional control of GroEL (*26, 33, 34*), the relative importance of each of the conserved isoleucines to σ^A activity was investigated by comparing the rates of GroEL induction in the *sigA* mutants at 49°C (Fig. 4a). Our data showed that none of the GroEL inductions in the *sigA* mutants reached the level of *B. subtilis* DB2. Besides, the rate of GroEL induction in *B. subtilis* DB1003, DB1008, or DB1009 each bearing a single Ile-to-Ala substitution in σ^A was much higher than that observed in *B. subtilis* DB1005 possessing double Ile-to-Ala substitutions. These results suggest that the conserved isoleucines act in a cooperative manner to maintain σ^A function at high temperature. The rate of GroEL induction in the *sigA* mutants in decending order was *B. subtilis* DB1008 ($\sigma^A{}_{I202A}$) > DB1003 ($\sigma^A{}_{I194A}$) > DB1009 (σ^AI198A), indicating that Ile-194, Ile-198, and Ile-202 assume unequal responsibility for σ^A function, and the most important part is played by Ile-198.

Restoration of Structural Stability and Transcription Activity of the Mutant σ^A Factors by the Same-Site and Second-Site Suppressors—To investigate the effects of the same-site and second-site suppressors on the structural stabilities of the mutant σ^A factors, the half-lives of the mutant and their corresponding suppressor mutant σ^A factors were compared. Our data showed that in all cases the suppressor mutant σ^A factors were more stable than their corresponding mutant σ^A factors at 37°C in terms of degradation rate (Fig. 3, b, c, and d). The degradation half-lives of DB1003 $\sigma^A{}_{I194A}$ and DB1003R1 $\sigma^A{}_{I194V}$ were 154 and >600 min, respectively (Fig. 3b and Table III). Furthermore, the degradation half-lives of DB1005 $\sigma^A{}_{I198\&202A}$, DB1005R1 $\sigma^A{}_{I198\&202A,S291F}$, and DB1005R3 $\sigma^A{}_{I198V,I202A}$ were 59, 74, and 158 min, respectively (Fig. 3c and Table III), and those of DB1009 $\sigma^A{}_{I198A}$, DB1009R7 $\sigma^A{}_{I198A,S291F}$, and DB1009R4 $\sigma^A{}_{I198V}$ were 109, 128, and >600 min, respectively (Fig. 3d and Table III). Clearly, restoration of the structural stabilities of DB1005 and

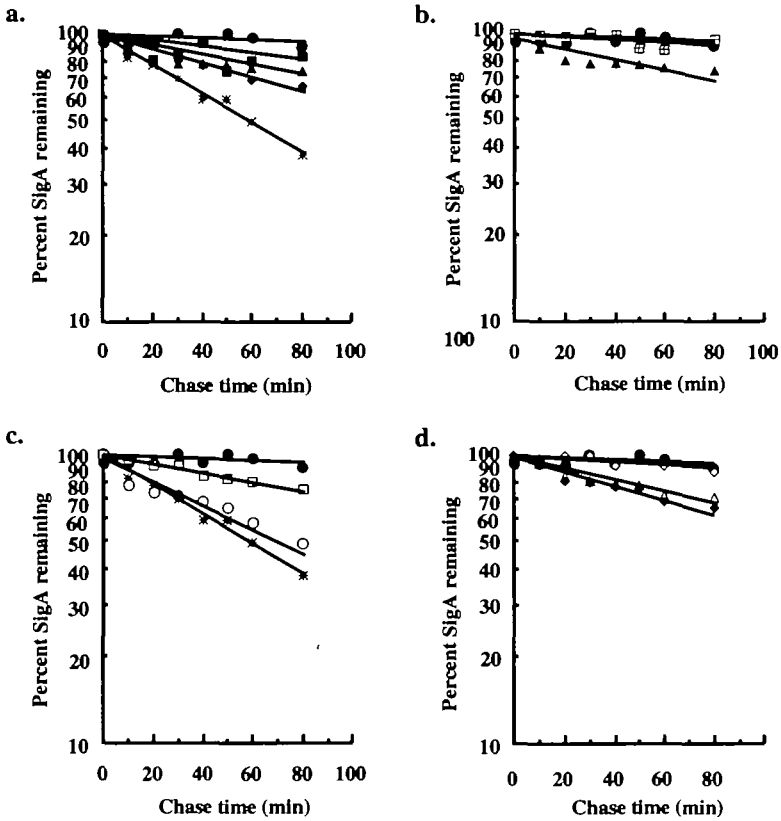

Fig. 3. **Degradation of σ^A proteins at 37°C.** a: The regression lines of the average of triplicate determinations of *B. subtilis* DB2, DB1003, DB1008, DB1009, and DB1005 σ^A factors remaining in the cells after being pulse-labeled with ^{35}S-methionine (*26*). b: The regression lines of the average of triplicate determinations of *B. subtilis* DB2, DB1003, and DB1003R1 σ^A factors remaining in the cells. c: The regression lines of the average of triplicate determinations of *B. subtilis* DB2, DB1005, DB1005R1, and DB1005R3 σ^A factors remaining in the cells. d: The regression lines of the average of triplicate determinations of *B. subtilis* DB2, DB1009, DB1009R4, and DB1009R7 σ^A factors remaining in the cells. The standard deviation was about 15% of the average. Each point represents the ratio of cpm of σ^A at each time point/cpm of σ^A at time zero. The result of each degradation line was repeated at least twice and it was reproducible. The symbols ●, ▲, ■, ◆, *, ⊞, ○, □, ◇, and △ represent degradation of σ^A in *B. subtilis* DB2, DB1003, DB1008, DB1009, DB1005, DB1003R1, DB1005R1, DB1005R3, DB1009R4, and DB1009R7, respectively.

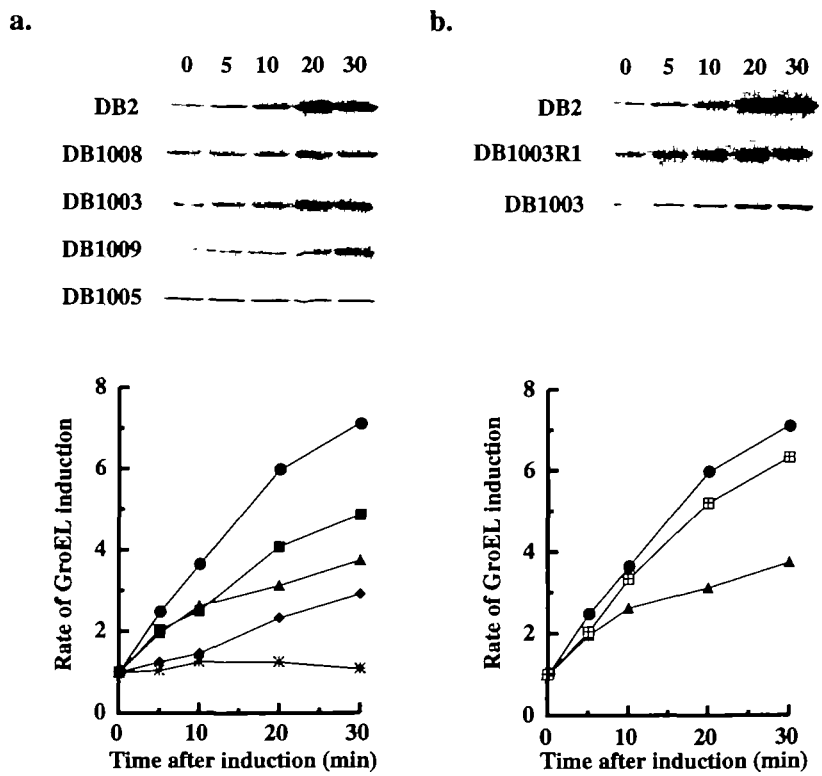

Fig. 4. **Comparison of σ^A activity at 49°C in terms of the rate of GroEL induction.** Immunological determination of GroEL induction was performed as reported previously (*7*). a: Rates of GroEL induction in *B. subtilis* DB2 and the *sigA* mutants. b: Rates of GroEL induction in *B. subtilis* DB2, DB1003, and DB1003R1. Numbers above the upper panel of each figure indicate the time points (in min) at which cell cultures of *B. subtilis* were sampled after the upshift of temperature from 37 to 49°C. The lower panel of each figure indicates rates of GroEL induction within the first 30 min after heat shock. The rates of GroEL induction were calculated by dividing the band densities of GroEL at 49°C by that at 37°C for each *B. subtilis* strain after scanning with a Bio Image system from Millipore. The symbols ●, ▲, ■, ◆, *, and ⊞ represent rates of GroEL induction in *B. subtilis* DB2, DB1003, DB1008, DB1009, DB1005, and DB1003R1, respectively.

DB1009 σ^A factors were more significant with the same-site suppressor (A198V) than with the second-site suppressor (S291F).

The activities of the mutant σ^A factors were also improved by both the same-site and second-site suppressors (Figs. 4b and 5, a and b). As shown in Fig. 5a, DB1005R1

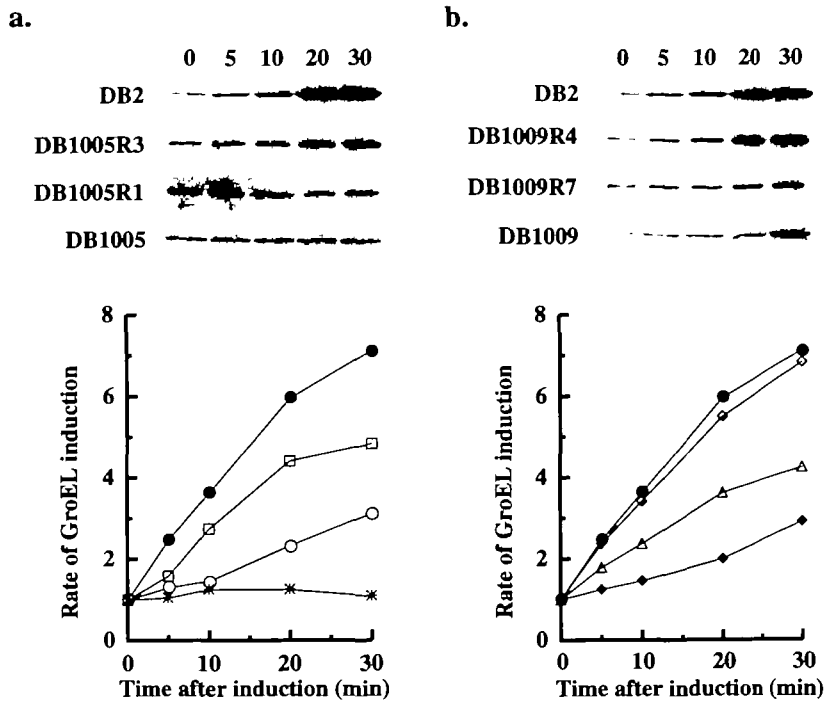

Fig. 5. **Comparison of σ^A activity at 49°C in terms of the rate of GroEL induction.** Immunological determination of GroEL induction was performed as reported previously (7). a: Rates of GroEL induction in *B. subtilis* DB2, DB1005, DB1005R1, and DB1005R3. b: Rates of GroEL induction in *B. subtilis* DB2, DB1009, DB1009R4, and DB1009R7. Numbers above the upper panel of each figure indicate the time points (in min) at which cell cultures of *B. subtilis* were sampled after the upshift of temperature from 37 to 49°C. The lower panel of each figure indicates rates of GroEL induction within the first 30 min after heat shock. The rates of GroEL induction were calculated by dividing the band densities of GroEL at 49°C by that at 37°C for each *B. subtilis* strain after scanning with a Bio Image system from Millipore. The symbols ●, ◆, *, ○, □, ◇, and △ represent rates of GroEL induction in *B. subtilis* DB2, DB1009, DB1005, DB1005R1, DB1005R3, DB1009R4, and DB1009R7, respectively.

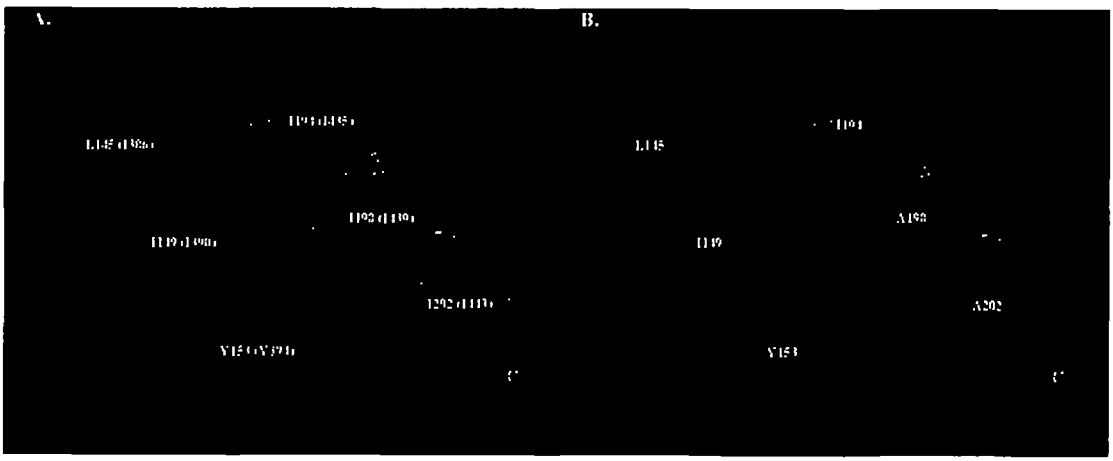

Fig. 6. **The potential van der Waals contacts between the three conserved isoleucines in region 2.4 and the opposite amino acids in region 2.1 of *B. subtilis* σ^A factor.** The structure of σ^A and the distances of putative contacts were analyzed by using the program Swiss-PdbViewer v2.2, by analogy with the crystal structure of a σ^{70} peptide (*25*). a: The van der Waals contacts in the wild-type σ^A factor. The corresponding amino acids in σ^{70} are shown in parentheses. b: The van der Waals contacts in the DB1005 σ^A factor.

$\sigma^A{}_{I198\&202A,S291F}$ and DB1005R3 $\sigma^A{}_{I198V,I202A}$ are more active than DB1005 $\sigma^A{}_{I198\&202A}$ in terms of the rate of GroEL induction. Similar results were observed for DB1009 $\sigma^A{}_{I198A}$ and its suppressors, DB1009R4 $\sigma^A{}_{I198V}$ and DB1009-R7 $\sigma^A{}_{I198A,S291F}$ (Fig. 5b). In both categories, the same-site suppressor (A198V) always conferred a much improved σ^A activity, while only little improvement was conferred by the second-site suppressor (S291F). These results indicate that Val-198 on the hydrophobic face of the promoter −10 binding helix is far more critical than Phe-291 in the turn structure of region 3.2 (*22, 35*) to restore the functional σ^A structure at elevated temperature. Apparently, these two suppressors improve σ^A activity through different mechanisms.

Interdigital Interactions between the Three Conserved Isoleucine Residues and the Hydrophobic Amino Acid Residues in Region 2.1 of σ^A—We attempted to identify the amino acid residues interacting interdigitally with the three conserved isoleucines by looking for their intragenic second-site suppressors. However, only one second-site suppressor, S291F, was obtained. The suppressor amino acid was not predicted to be in the hydrophobic core (*21, 22*) but in a turn structure of σ^A (*35*). Therefore, it was unlikely to be involved in the interdigital interactions with the conserved isoleucines in the hydrophobic core region of σ^A. We suspected that the amino acid residues opposite to the conserved isoleucines were already bulky and hydrophobic and thus escaped detection by the genetic ap-

TABLE IV. **Potential differences in van der Waals contacts between atoms in regions 2.1 and 2.4 among different *B. subtilis* sigma-A factors.**

B. subtilis strain	Contact regions 2.4 \ 2.1	Distances between contact atoms (A)				
		$L_{145}C\delta_2$	$I_{149}C\delta$	$I_{149}C\gamma_2$	$Y_{153}C\varepsilon_1$	$Y_{153}C\delta_2$
DB2	$I_{194}C\delta$	3.7(V)				
	$I_{194}C\gamma_2$		3.7(V)			
	$I_{198}C\delta$			4.0(V)		
	$I_{198}C\gamma_2$				4.1(V)	
	$I_{202}C\delta$					3.9(V)
DB1003	$A_{194}C\beta$	5.9				
	$A_{194}C\beta$		3.9(V)			
	$I_{198}C\delta$			4.0(V)		
	$I_{198}C\gamma_2$				4.1(V)	
	$I_{202}C\delta$					3.9(V)
DB1003R1	$V_{194}C\gamma_2$	5.1				
	$V_{194}C\gamma_1$		3.4(V)			
	$I_{198}C\delta$			4.0(V)		
	$I_{198}C\gamma_2$				4.1(V)	
	$I_{202}C\delta$					3.9(V)
DB1008	$I_{194}C\delta$	3.7(V)				
	$I_{194}C\gamma_2$		3.7(V)			
	$I_{198}C\delta$			4.0(V)		
	$I_{198}C\gamma_2$				4.1(V)	
	$A_{202}C\beta$					6.2
DB1009	$I_{194}C\delta$	3.7(V)				
	$I_{194}C\gamma_2$		3.7(V)			
	$A_{198}C\beta$			5.2		
	$A_{198}C\beta$				4.6	
	$I_{202}C\delta$					3.9(V)
DB1009R4	$I_{194}C\delta$	3.7(V)				
	$I_{194}C\gamma_2$		3.7(V)			
	$V_{198}C\gamma_2$			4.9		
	$V_{198}C\gamma_1$				3.9(V)	
	$I_{202}C\delta$					3.9(V)
DB1005	$I_{194}C\delta$	3.7(V)				
	$I_{194}C\gamma_2$		3.7(V)			
	$A_{198}C\beta$			5.2		
	$A_{198}C\beta$				4.6	
	$A_{202}C\beta$					6.2
DB1005R3	$I_{194}C\delta$	3.7(V)				
	$I_{194}C\gamma_2$		3.7(V)			
	$V_{198}C\gamma_2$			4.9		
	$V_{198}C\gamma_1$				3.9(V)	
	$A_{202}C\beta$					6.2

Putative van der Waals contacts with distances below 4.2 A are indicated with (V); one-letter amino-acid code is used; β, δ, γ, and ε are the positions of carbon atoms in the side chain of an amino acid residue. Not all of the van der Waals contacts between the shown atoms are included.

proaches. To check this point, we investigated the van der Waals contacts between the amino acids in the hydrophobic core of σ^A by analogy with the crystal structure of a σ^{70} peptide (*25*). Interdigital interactions between the amino acid residues in regions 2.1 and 2.4 of σ^A were detected. As shown in Fig. 6 and Table IV, Ile-194 interacts at least with Leu-145 and Ile-149, Ile-198 with Ile-149 and Tyr-153, and Ile-202 with Tyr-153 in the hydrophobic core region of the wild-type σ^A. It is also clear that the same-site suppressors (Val-194 and Val-198) shorten the distances between the contact atoms and generate new van der Waals contacts in the hydrophobic core region of σ^A when the mutant and its corresponding revertant σ^A factors were compared (Table IV). The correlation between the number of van der Waals contacts in the hydrophobic core region of σ^A (Table IV) and the degree of structural stability (Fig. 3 and Table III) as well as the transcription activity of σ^A factors (Figs. 4 and 5) indicates that sufficient interdigital packing interactions in the hydrophobic core of σ^A were essential to the functional σ^A structure.

DISCUSSION

We have investigated the relative importance of the three conserved isoleucine residues on the hydrophobic face of the promoter −10 binding helix to σ^A structure and function and have proposed the amino acid residues with which the three conserved isoleucines interact. The results pinpoint the existence of a structural role, besides the promoter DNA-binding function, of the promoter −10 binding helix of a σ factor.

The finding of valine with a bulky hydrophobic side chain as the same-site suppressor at both positions 194 and 198 of σ^A (Table II) assesses the steric and chemical requirements of bulky hydrophobic amino acids for the core packing of σ^A. These results also explain why the amino acid residues at positions 194, 198, and 202 have to be so highly conserved in known σ factors. The ability of valine at positions 194 (DB1003R1 σ^A) and 198 (DB1005R3 and DB1009R4 σ^A) (Table II) to suppress the defects of DB1003, DB1005, and DB1009 σ^A factors indicates that filling the cavities of the protein cores of the mutant σ^A factors offsets the destabilizing effect of the large-to-small amino acid substitution (Ile-to-Ala) (Fig. 6 and Table IV). Similar restabilization effects on proteins have been also reported for the well-characterized T4 lysozyme, barnase, and Gene V protein (*36-39*). The additive effects of Ile-194, Ile-198, and Ile-202 on σ^A structure and function (Figs. 3, 4, and 5) indicate that the three conserved isoleucine residues contribute to the hydrophobic core packing of σ^A through interdigital interactions with the amino acids in certain hydrophobic region of σ^A. This indication is substantiated by the results obtained from the examination of the hydrophobic core of σ^A by analogy with the crystal structure of a σ^{70} peptide encompassing regions 1.1 to 2.4 (Fig. 6 and Ref. *25*) and by the hydrophobic nature of amino acids in region 2.1 (*16*), which are conserved among group 1 σ factors and are presumed to play similar structural and functional roles (*22*).

The finding of valine at position 198, rather than 202, as the same-site suppressor of DB1005 $\sigma^A{}_{I198\&202A}$ (Table II) indicates that the amino acid residue at position 198 makes the major contribution to the hydrophobic core packing of σ^A. A similar conclusion can be drawn from the fact that DB1008 $\sigma^A{}_{I202A}$, with an isoleucine residue at position 198 of σ^A, is most resistant to high temperature among the mutant σ^A factors (Fig. 2a), and that DB1008 $\sigma^A{}_{I202A}$ is superior to DB1003 $\sigma^A{}_{I194A}$ and DB1009 $\sigma^A{}_{I198A}$ in both structural stability and transcription activity (Figs. 3a and 4a). On the basis of the contacts between regions of 2.1 and 2.4 of σ^A (Fig. 6 and Table IV), we assume that the differential effects of the conserved isoleucine residues on σ^A structure and function (Figs. 3, 4, and 5) are determined at least by the relative positions of the conserved isoleucine residues on the hydrophobic face of the promoter −10 binding helix, and by the numbers of contacts between the conserved isoleucine residues and the opposite amino acids in region 2.1.

The only intragenic second-site suppressor (S291F) we obtained is located in the turn structure of region 3.2 of σ^A (*35*). One explanation for this finding is that there is a direct interaction between the original mutation and the second-site suppressor. If this were true, we would expect to see similar extents of improvement in the structural stability and transcription activity of DB1009 σ^A by S291F and A198V. Nevertheless, the efficiency of suppression is much lower with S291F than with A198V (Figs. 3d and 5b), suggesting that Ile-198 and Ser-291 in the wild-type σ^A (or Ala-198 and Phe-291 in the revertant σ^A) are not likely to interact with each other. This proposition agrees with the fact that the conserved isoleucines are completely buried within the hydrophobic core and interact with the hydrophobic amino acids in region 2.1 (Fig. 6 and Ref. *25*), while the amino acid residue at position 291 in region 3.2 of σ is not conserved and is sometimes a charged residue (*22*).

Another interpretation of suppression by S291F is that the second-site suppressor results in an indirect compensation of the defects caused by the original *sigA* mutations. Possibly, the *sigA* mutations, I194A, I198A, and I202A, influence the overall functional structure of region 3, which in turn affects abortive transcription (*40*), core-binding (*41*), promoter-binding or other structural features of σ^A. The facts that *B. subtilis* DB1009R7, like *B. subtilis crsA47-a sigA* mutant, which is resistant to catabolite repression of sporulation, sporulates better than the wild-type *B. subtilis* DB2 in a sporulation medium (SBM) containing 1% glucose (B.-Y. Chang, unpublished results) and that the second-site suppressor (Phe-291) of DB1009 $\sigma^A{}_{I198A}$ is located right next to Phe-290, which is responsible for the catabolite-resistant sporulation of *B. subtilis crsA47* (*42, 43*), might be good indications of such a possibility. The whole set of *sigA* mutants mentioned above will be of great value for testing the validity of the conjecture and for investigating the mechanisms causing the negative dominance of DB1005 *sigA* gene (B.-Y. Chang, unpublished results) in the future.

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