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The Importance of a Proper Helical Structure in the Promoter —10 Binding Region to *Bacillus subtilis* c?^{A} Structure and Function[1]

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Two *Bacillus subtilis sigA* mutants with amino acid substitutions tending to disrupt the structure of the promoter —10 binding helix of *B. subtilis* <r^A factor were constructed. *B. subtilis* DBlOOl which contained an A197P substitution was very sensitive to temperature elevation. *B. subtilis* DB1002 had a T199G substitution and was low in growth potential at the elevated temperature. Degradation of *a*^{A} in *B. subtilis* DBlOOl ($t_{1/2}$=63.2min) and DB1002 (£„$_2$ = 186.0 min) occurred readily even at 37°C; however, <y^A in *B. subtilis* DB2 (wild-type) was fairly stable at the same temperature. The activities of both DBlOOl and DB1002 <7^A factors on *groE* promoter (<y^A-type) were lower than those of the wild-type counterpart at both permissive and restrictive temperatures. The failure of a higher <y^A concentration to suppress the Ts phenotype of DBlOOl indicated that the temperature sensitivity of *B. subtilis* DBlOOl was due to altered function, rather than insufficient concentration, of cr^A in the cells. Taken together, our results suggest that the helicity of the promoter —10 binding helix is essential to the packing interaction in the hydrophobic core region of *cf*^{A}, which helps to maintain the stable and functional *0*^{A} structure.

Key words: sigma factor, a-helix, structural stability, transcription activity, temperature sensitivity.

The prokaryotic RNA polymerase *a* factor confers specificity of promoter recognition and initiation of transcription *(1, 2).* Sequence alignment analysis of known sigma factors has revealed four conserved regions in this group of proteins *{3,4).* The 4.2 region at the C - terminus of *Bacillus subtilis* <r^A has similarity to the helix-turn-helix DNA-binding motif which is believed to interact with the — 35 region of promoter DNA (5, *6).* The 2.4 region which is supposed to recognize promoter —10 DNA *(7, 8)* is a strong amphiphilic *a*-helix with 3 isoleucine residues located at four-residue intervals (3, *4, 7, 9, 10).* Recently, the crystal structure of a o*^{70} peptide fragment, encompassing regions 1.2 to 2.4, was solved. In accordance with the prevailing view, the promoter —10 binding region (region 2.4) does form an amphiphilic #-helix extending from region 2.3 with the hydrophobic face contributing to the hydrophobic core created by clustering of the hydrophobic amino acids, including the three conserved isoleucine residues and those in conserved regions 1 and 2 *(11).* Thus, amino acids which confer altered —10 DNA recognition properties should be on the hydrophilic face of the amphiphilic *a*-helix in *Escherichia coli a*^{10} as well as *B. subtilis* <r^A, *a*^{e}, *o*$^?$, and <r^H (6, *7, 12-14).*

We have previously demonstrated that free <x^A is unable to bind promoter DNA *(15)* and that it changes conformation during the association with RNA polymerase core enzyme *(16).* The inability of free <r^A to interact directly with promoter DNA could be due to the not-readily-exposed nature of the promoter —10 binding region, which is within the most hydrophobic domain of o'^{A} and is resistant to protease attack *(17).* The hydrophobic and unexposed nature of the promoter —10 binding region suggests that its role may not be restricted to promoter recognition. This region might also contribute to the structural stability and transcription activity of <r^A. Therefore, amino acid substitutions which decrease the hydrophobicity (or the size of the hydrophobic side chain of the amino acid residue) on the hydrophobic face of the promoter —10 binding helix were designed to investigate the roles of the three conserved hydrophobic isoleucine residues in o*^{A} structure and function. Two amino-acid substitutions, I198A and I202A, were found to cause the temperature sensitivity of *B. subtilis* DB1005 *(9).* The <x^A factor of the temperature-sensitive (Ts) *sigA* mutant was defective in transcription under heat-shock conditions and was much more easily degraded than the wild-type <r^A at both permissive and nonpermissive temperatures *(18, 19).* These results suggest that there is an important packing interaction between the hydrophobic face of the promoter —10 binding helix and some other region of <r^A in the hydrophobic core region of o'^{A}. Thus, disruption of this helix would be very detrimental to o'^{A} due to the loss of the proper packing force.

In this study, we aimed to investigate the importance of maintaining a proper helical structure in the promoter —10

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binding region to ff^A structure and function. Amino acid residues which tend to break helical structure *(20, 21)* were introduced into positions on the edge and proximal to the N-terminus of the promoter —10 recognition helix (Fig. 1). This strategy enabled us to obtain two *B. subtilis sigA* mutants which were either Ts or low in growth potential at high temperature. The a^k proteins in these two *sigA* mutants had altered structural stability and transcription activity, suggesting that the helicity, like the hydrophobicity, of the promoter —10 binding helix was essential to the packing interaction in the hydrophobic core region of ff^A, which is required for the maintenance of a stable and functional σ^A structure.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—B. subtilis strain used for the construction of *sigA* mutants was *B. subtilis* DB2 (B. *subtilis* 168 *trpC).*

The Construction of Mutant sigA Strains of B. subtilis—The mutant *sigA* genes were constructed by overlapping extension polymerase chain reaction *(22, 23).* The method used for the construction of the integration plasmids (pBYl and pBY2) which contained Ml and M2 *sigA* genes, respectively, was the same as that reported previously *(9).* The mutant *sigA* genes on the plasmids lacked a 137-bp DNA sequence for the ribosome binding site and the N-terminal 35 amino acid codons of *sigA.* The lengths of the flanking DNA sequences 5' and 3' to the mutated codon (proline or glycine) were about 480 and 680 bps, respectively. Primer sequences for the synthesis of the Ml and M2 *sigA* genes (Fig. lc) were as follows: A, 5'-GCCTGATGAAAGCCGTTC-3'; D, 5'-TGAAGTTGCTTCTTGGTC-3'; Bl, 5'-AATCGGCTGGCGGATCCACCACGTAGCA-3'; Cl, 5'-GATCCGCCAGCCGATTACACGCGCCAT-3'; B2, 5-GCGACCAATCGCCTGGCGGATCCACCACGTAGCA-3'; C2, 5'-GATCCGCCAGGCGATTGGTCGCGCCATTGCCGAT-3'. Besides the designed amino acid substitutions, a *BamHl* restriction site was incorporated into the primer sequence at amino acids 193 to 195. The *BamHl* site allowed us to keep track of the mutant *sigA* genes during their construction and to monitor them after integration into the *B. subtilis* chromosome. After integration of the mutant *sigA* gene into the chromosome of *B. subtilis,* transformants were first selected on 2xSG plates *(24)* containing chloramphenicol (5 /<g/ml) at 30°C, and then patched on fresh 2xSG plates at 52 or 55°C. Transformants which were sensitive to temperature elevation or grew slowly at the tested temperature were selected.

Confirmation of the Mutant sigA Strains—To confirm the *sigA* mutants, cotransformation and direct DNA sequencing of the mutant *sigA* genes were performed. In the former experiment, chromosomal DNAs extracted from the *sigA* mutants were transformed into DB2. Since a *cat* gene has been integrated into the chromosome of the mutant in the vicinity of *sigA* gene (about 1.5 kb apart), a high cotransformation efficiency of chloramphenicol resistance and defective growth at elevated temperature would be expected if the chromosomes of the *sigA* mutants were transformed into DB2. Just as expected, about 44% of the chloramphenicol-resistant transformants were found to be temperature-sensitive as well when DB2 was transformed with the chromosomal DNA from *B. subtilis* DB1001. It was much higher (about 80%) when the chromosomal DNA of DB1002 was transformed into DB2. The intact *sigA* DNA on the chromosome of each mutant strain was synthesized by polymerase chain reaction using a set of specific primers and then sequenced *(9). B. subtilis* DB1001 and *B. subtilis* DB1002 contained the same *sigA* mutations as those of the Ml and M2 *sigA* genes, respectively (Fig. lc).

Construction of Plasmids Containing sigA Genes Expressible in B. subtilis—The plasmid, pCX2F, which was able to express the wild-type *sigA* gene, was constructed previously (*19).* The *sigA* gene on this plasmid was controlled by the transcriptional signal (P1P2 promoter) of *sigA* operon and translational signal of *sigA* gene *(25).* The pCX2Fl plasmid which contained the *sigA* gene of *B. subtilis* DB1001 was obtained by gene conversion *(26, 27).* In this approach, the pCX2F plasmid was introduced into *B. subtilis* DB1001 and then kanamycin (km)-resistant transformants with a Ts phenotype comparable to DB1001 were screened. These transformants contained plasmids on which the wild-type *sigA* allele had been replaced with the mutant *sigA* allele. To obtain *B. subtilis* DB1001 with a homogeneous population of pCX2Fl (containingDB1001 *sigA* gene), plasmids in the primary Ts transformants were extracted and reintroduced into *B. subtilis* DB1001. The secondary Ts transformants thus obtained contained DB1001 *sigA* gene on the plasmids as examined by DNA sequencing.

Degradation Rates of a^k and GroEL Proteins—The method for determination of the degradation rates of σ^A and GroEL proteins *in vivo* was the same as that reported previously *(19).*

Determination of the Transcription Activities of Wild-Type and Mutant σ^A Factors at 37° C In Vivo by a lacZ Transcriptional Gene Fusion—To measure the activities of wild-type and mutant σ^A factors at 37°C, the integration vector which contained a *lacZ* gene under the control of *groE* promoter *(19)* was incorporated into the *aprE* locus of *B. subtilis.* The *B. subtilis* strain was then grown at 37°C in the 2 X SG medium containing erythromycin (1 ^g/ml) and harvested at various growth stages. /?-Galactosidase activity of each culture was assayed by the method of Miller *(28).*

RESULTS

Strategies for Construction of the Mutant sigA Genes—The proposed promoter —10 binding helix of *B. subtilis* σ^A factor contains 12 amino acids (amino acids 195 to 206) (Fig. lc) as predicted by the Chou and Fasman method *(10).* The relative positions of amino acids in this helix were analyzed by placing them on a helical wheel in accordance with their sequence order with a 100° angular difference between each amino acid (Fig. la) and by placing them on a cylindrical plot (Fig. lb). Since σ^A is essential to the vegetative growth of *B. subtilis,* a drastic change in the character of this helix could be lethal to the cell. To avoid such a lethal effect, proline and glycine residues, which prefer a-helix N- and C-Cap positions *(20, 21),* were introduced into positions on the edge and near the N-terminus of the proposed promoter —10 binding helix. This strategy should minimize the change of helicity of the promoter —10 binding helix *(29, 30)* and generate a new helix which is still long enough to be functional. Alanine at position 197 and threonine at position 199 were our first

two choices for this purpose (Fig. 1). The mutant *sigA* genes were prepared accordingly and integrated into *B. subtilis* DB2 (see "MATERIALS AND METHODS"). Since the helix breakers would disrupt the packing interaction between the hydrophobic face of the promoter —10 binding helix and certain amino acid(s) in the hydrophobic core region of σ^A and thus affect the stability and function of σ^A, we expected that the *sigA* mutants would show distinct growth phenotypes. Indeed, a mutant *sigA* strain that grew slowly at elevated temperature was obtained at a frequency of 50% when pBY2 was transformed into *B. subtilis* DB2. The colony size of this mutant was smaller than that of DB2 on a 2xSG plate at 55°C (data not shown). The mutant was named *B. subtilis* DB1002.

Since no mutant with pBYl integrated into the DB2 chromosome was obtained, we constructed another integration plasmid (pBYlT) in which only 71 bp of DNA sequence, including the ribosome binding sequence and the first 13 amino acid codons of the *sigA* gene, were deleted. This construction increased the length (66 bp) of the flanking DNA sequence 5' to the mutated codon (proline), which would in turn enhance the effective recombinational frequency. After this, about 3% of the chloramphenicol-resistant transformants were found to be temperature-sensitive at 52°C on a 2 X SG agar plate when pBYlT was integrated into *B. subtilis* DB2. This type of *sigA* mutant was designated as *B. subtilis* DB1001.

Effects of Temperature on Growth of B. subtilis sigA Mutants—In order to distinguish clearly the effect of temperature elevation on the growth of the *sigA* mutants, the growth potentials of *B. subtilis* DB2, DB1001, and DB1002 in liquid glucose minimal medium were compared (Fig. 2). No significant difference was observed in the initial growth among the three strains of *B. subtilis* at 37°C. However, the effects of temperature elevation (37 to 49°C) on DB2, DB1001, and DB1002 were different. Upshift of temperature exerted no significant effect on the growth of DB2, whereas it had a drastic impact on that of DB1001. The growth of DB1001 stopped after the temperature upshift, but resumed 2 h after the downshift of temperature. The effect of temperature elevation on growth of *B. subtilis* DB1002 was not as significant as that observed for DB1001; however, the maximal cell density of DB1002 was always lower than that of DB2 after the upshift of temperature (Fig. 2).

B. subtilis DB1001 and DB1002 a^A Proteins Were Unstable Even at the Permissive Temperature—It is generally believed that replacement of certain amino acids with proline or glycine in an *a*-helix of a protein will change the helical structure and destabilize the protein *{29-31).* The temperature sensitivity of *B. subtilis* DB1001 was most probably due to destabilization of the mutant σ^A protein, which might in turn lead to the degradation of σ^A or the inability of σ^A to sustain a proper functional structure. To see which is the case, the degradation rates of mutant σ^A proteins *in vivo* were first analyzed. After pulse-labeling of the total cellular proteins of *B. subtilis* with ^{35}S-methionine and immunoprecipitation with anti-σ^A and anti-GroEL at designated time points, radioactivities of a^k and GroEL were read with a radioactivity image reader after SDS-PAGE. The percentages of labeled σ^A and GroEL remaining in the cells at different time points were then used for the calculation of degradation rates. The degradation rates and half-lives of GroEL in DB2, DB1001, and DB1002 were used as internal controls and they were similar in all the tested strains at 37°C (Fig. 3b and Table I). The degradation rates of wild-type and mutant σ^A factors at 37°C are depicted in Fig. 3a. The half-lives, calculated according to

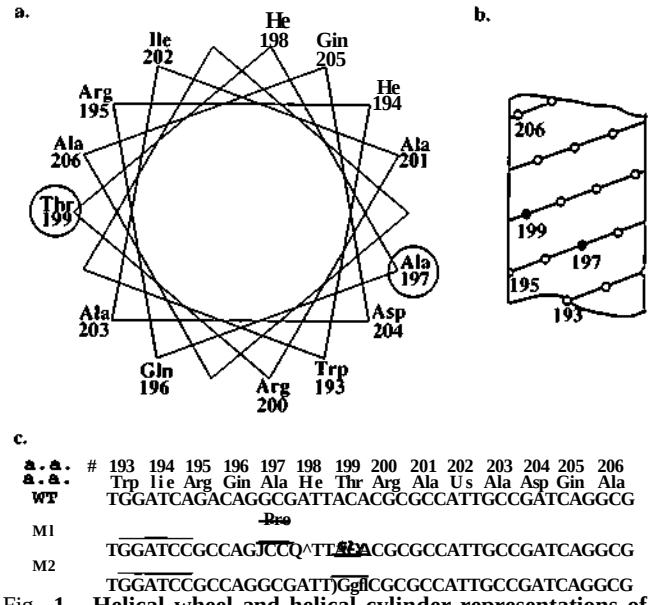

Fig. **1. Helical wheel and helical cylinder representations of the proposed promoter —10 binding helix of *B. subtilis* 6^K factor,** a: Amphiphilic *a*-helix of the promoter —10 binding region of σ^A. Amino acid residues (Ala-197 and Thr-199) investigated in this study are circled, b: Cylindrical plot of the a-helix in the promoter —10 binding region of σ^A. The amino acid residues (Ala-197 and Thr-199) are indicated by solid circles, c: Amino acid substitutions in the <*-helix of the promoter —10 binding region of σ^A. The top two lines indicate the amino acid sequence of the promoter —10 binding helix of the wild-type a^A. The bottom three lines indicate the sense-strand DNA sequences of the wild-type (WT) and mutated *sigA* genes (Ml and M2). The site of amino acid substitution for each mutant *sigA* gene is boxed. The underlined six bases (GGATCC) make up the *BaniHl* site created in the Ml and M2 *sigA* genes.

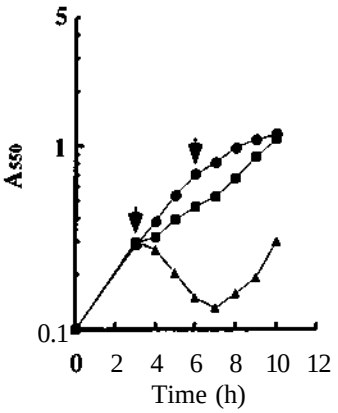

Fig. **2. Effects of temperature upshift and downshift on growth of *B. subtilis.*** Shown are growth curves of DB2 (•), DB1001 (A), and DB1002 (•) in glucose minimal medium. The three strains of *B. subtilis* were all grown at 37'C to an optical density (A_{550}) of about 0.3 before being transferred to 49"C (indicated by the left arrowhead). The cultures were shifted back to 37°C 3 h later (indicated by the right arrowhead).

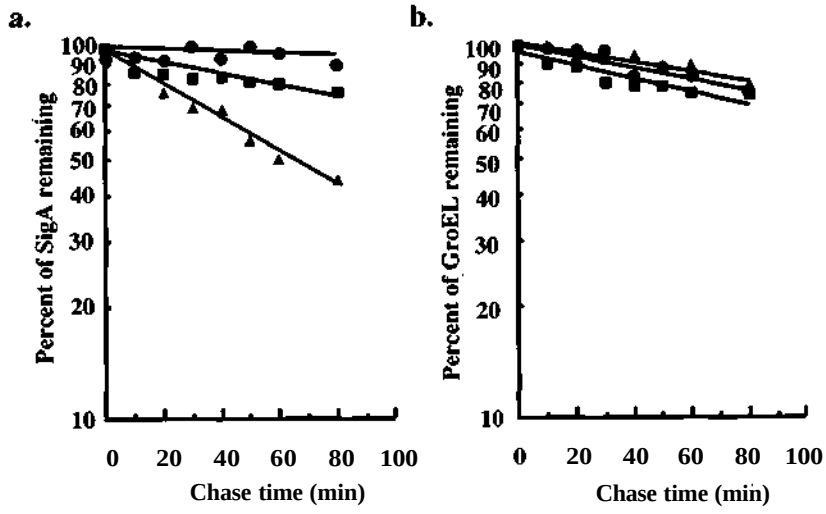

Fig. 3. **Degradation of σ^A and GroEL proteins at 37°C.** a: The regression lines of the average of triplicate determinations of σ^A remaining in the cells, b: The regression lines of the average of triplicate determinations of GroEL remaining in the cells. The standard deviation was about 15% of the average. Each point represents the ratio of cpm of σ^A or GroEL at each time point/cpm of σ^A or GroEL at time zero. Each degradation was repeated at least twice and the results were reproducible. The symbol • represents degradation in DB2, • represents degradation in DB1001, and • represents degradation in DB1002.

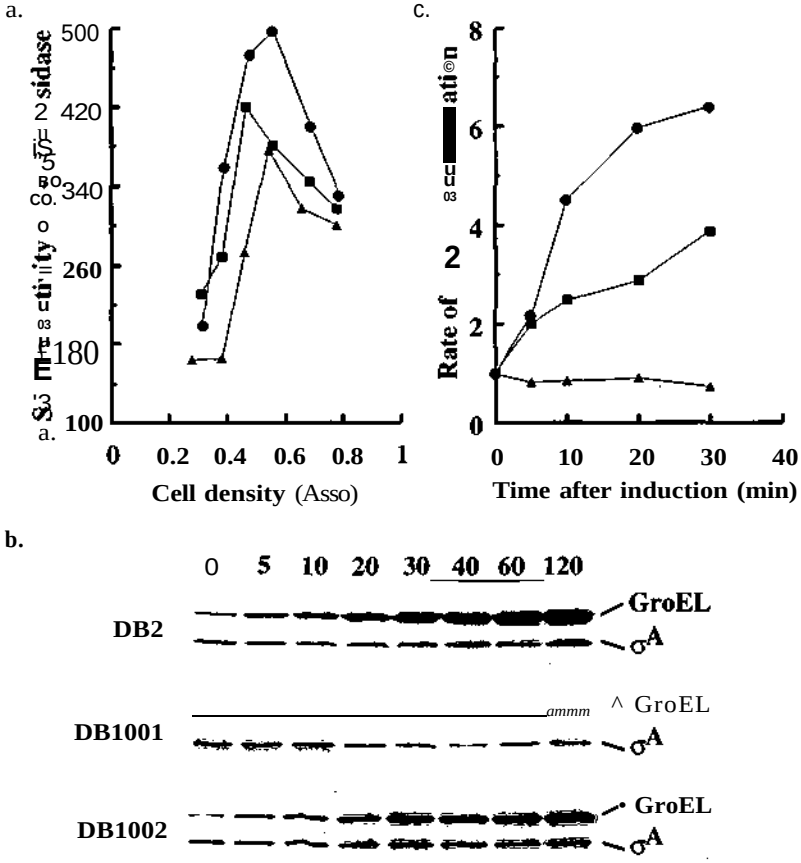

Fig. 4. **Transcription activities of the wild-type and mutant σ^A.** a: Determination of the transcription activity of σ^k at 37°C by a *lacZ* transcriptional gene fusion. *B. subtilis* DB2, DB1001, and DB1002 cells in which the pCoiZA derived plasmid (containing the wild-type *groE* promoter) has been integrated into the chromosome were grown in 2 X SG medium at 37°C with shaking. Samples were removed from each culture at designated time points and assayed for /3-galactosidase activity. The average value of three parallel samples was plotted on the graph, b: Determination of the transcription activity of σ^A at 49°C by immunological analysis of the GroEL protein. The method for the analysis was the same as that reported previously *(18).* Numbers above the panel indicate time points (in min) at which cell cultures of *B. subtilis* DB2, DB1001, and DB1002 were sampled after temperature shift from 37 to 49°C. c: Rate of GroEL induction. Band densities of GroEL protein in "b" were scanned using the Bio Image system from Millipore Corporation. The rate of GroEL induction within the first 30 min after heat shock was then calculated by dividing the band density of GroEL at 49°C over that at 37'C for each *B. subtilis* strain. Symbols: •, DB2; A, DB1001; •, DB1002.

the regression lines in Fig. 3a, are also compiled in Table I. The σ^A factor of *B. subtilis* DB2 was fairly stable at 37°C ($t_{1/2}$>600min), whereas DB1001 σ^A ($t_{1/2}$ = 63.2 min) and DB1002 σ^A ($t_{1/2}$ = 186.0 min) were relatively unstable. These results indicated that A197P and T199G substitutions had pronounced effects on the structural stability of σ^A even at 37°C. All σ^A factors, whether wild-type or mutant, became unstable with half-lives of 33.5 min (DB2), 76.4 min (DB1001), and 43.0 min (DB1002) when the incubation temperature was shifted to 49°C.

TABLE I. **Half-lives of SigA and GroEL degradation in *Bacillus subtilis* at 37°C.**

Strain	Amino acid substitution	Degradation half-lives' (min)	
		SigA	GroEL
DB2	SigA[Wt]	Stable	158.0
DB1001	A197P	63.2	148.3
DB1002	T199G	186.0	143.0

"The half-lives of SigA and GroEL degradation were determined according to the regression lines in Fig. 3. "Stable" means the half-life was greater than 600 min.

DB1001 and DB1002 a^A Were Less Active Than the Wild-Type Counterpart in Transcribing thegroE Promoter at Both 37 and 49° C—To measure the relative activities of mutant a^A factors at 37°C, the *groE* promoter, which has been proven to be of the <r^A-type (28, *32, 33)* was transcriptionally fused to *lacZ* and integrated into the chromosomes of *B. subtilis* DB2, DB1001, and DB1002. /8-Galactosidase activity of each *B. subtilis* strain cultivated at 37°C was then measured. As shown in Fig. 4a, the descending order of /?-galactosidase activity among these strains was DB2, DB1002, and DB1001, suggesting that the single amino acid substitution (A197P) of DB1001 σ^A had a more significant effect on σ^A function at 37°C.

Since /?-galactosidase loses its activity at 49°C, the relative transcription activities of the mutant σ^A factors at elevated temperature were examined by analyzing the rates of GroEL induction in the cells. In parallel, the levels of a^A proteins were analyzed. As shown in Fig. 4b, the induction of GroEL protein was clear in both *B. subtilis* DB2 and DB1002. The induction rates of GroEL in these two strains were significantly different from that of DB1001 (Fig. 4c). It was rapid in DB2 (1 to 6.4-fold), but gradual in DB1002 (1 to 3.9-fold) within the first 30 min after the upshift of temperature; however, no significant induction of GroEL was observed for DB1001. Evidently, the introduction of helix breakers (proline and glycine) into the promoter —10 binding region of <r^A impaired the function of σ^A, and the impairment was more pronounced with the A197P substitution at 49°C. Furthermore, a transient decrease and a gradual restoration in the amount of σ^A were observed for the three tested strains; it seems that the restoration of <r^A for DB2 and DB1002 was more rapid than that for DB1001 (Fig. 4b).

The Temperature Sensitivity of B. subtilis DB1001 Was Not Due to Insufficient a^A Concentration at the Elevated Temperature—Although disruption of the promoter —10 binding region rendered DB1001 <r^A more degradable than DB2 <r^A in the cells at the permissive temperature (Fig. 3a), both wild-type and mutant <r^A factors became relatively unstable after temperature elevation. These results suggested that the temperature sensitivity of *B. subtilis* DB1001 could not be attributed to degradation, and thus insufficient concentration of DB1001 σ^A in the cells. To confirm this, the pCX2F and pCX2Fl plasmids, which were able to express the wild-type and mutant *sigA* genes in *B. subtilis*, were introduced into *B. subtilis* DB2 and DB1001, respectively (see "MATERIALS AND METHODS"). The growth potentials and the levels of a^A in JB. *subtilis* DB2(pCX2F), DB1001(pCX2Fl) at 49°C were analyzed simultaneously. We found that *B. subtilis* DB1001(pCX2Fl) was still unable to grow at 49°C while *B. subtilis* DB2(pCX2F) grew (data not shown). We also found that the levels of <r^A in *B. subtilis* DB2(pCX2F) were relatively constant at both permissive and restrictive temperatures. In contrast, there was an induction of <r^A synthesis in *B. subtilis* DB1001-(pCX2Fl) at the restrictive temperature and the amount of σ^A was higher than those in DB2(pCX2F) at parallel time points after temperature elevation (Fig. 5). The inability of a higher σ^A concentration to sustain the growth and the induction of GroEL synthesis in DB1001 at the elevated temperature (Fig. 5) indicated that the temperature sensitivity of *B. subtilis* DB1001 was not due to insufficient <r^A concentration in the cells.

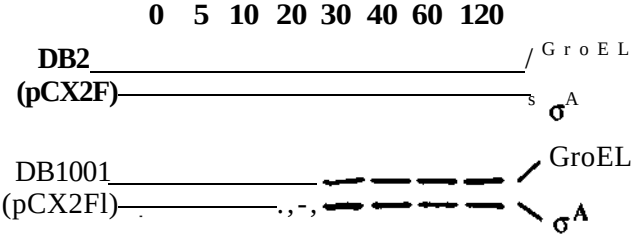

Fig. 5. Levels of G^k and GroEL in *B. subtilis* harboring the ^'-expressing plasmids. *B. subtilis* DB2(pCX2F) and DB1001-(pCX2Fl) were grown in glucose minimal medium at 37'C to a cell density *(A_{5i0})* of 0.4 (referred to as time zero) and then transferred to 49'C. Cell samples were harvested at different time points (min) after temperature elevation as indicated on the upper part of the figure. After resuspending the cell pellet with 50 //I of 0.5 x SET buffer (20% sucrose, 50 mM Tris-HCl, pH 7.6, 50 mM EDTA, 2 mM PMSF) and digestion of the cell wall with lysozyme, 50 ^1 of sample application buffer *(17)* was added to break the cells. Lysate from equal volumes of the cell cultures was used for Western blot analysis of the content of <r^A protein. The name of each tested *B. subtilis* strain is shown on the left side of the panel.

DISCUSSION

We have constructed two *B. subtilis sigA* mutants which were either Ts or low in growth potential at high temperature (Figs. 1 and 2). By the introduction of a proline residue into the edge proximal to the N-terminus of the proposed promoter —10 binding helix of <r^A, a *B. subtilis* Ts *sigA* mutant termed DB1001 was obtained. The phenotype of this mutant was similar to that of *B. subtilis* DB1005, in which two conserved hydrophobic isoleucine residues on the hydrophobic face of the promoter —10 binding helix of <r^A were replaced with alanines (9). The <r^A factors of these two Ts *sigA* mutants were easily degraded even at 37°C (Fig. 3a and Ref. *19)* and were unable to induce GroEL synthesis to the wild-type level at 49°C (Fig. 4b and Ref. *19).* These results indicate that changes of helicity and hydrophobicity of the promoter —10 binding helix destabilize σ^A, leading to its degradation even at the permissive temperature and loss of activity at the restrictive temperature. Thus, the promoter —10 binding region is essential not only to promoter recognition *(13),* but also to maintenance of a stable and functional a^A structure.

The lower transcription activity of DB1001 a^A at 49°C (Fig. 4c), as well as the failure of a higher σ^A concentration in *B. subtilis* DB1001 to rescue the Ts phenotype, demonstrates that the temperature sensitivity of *B. subtilis* DB1001 is due to altered function, rather than insufficient <r^A concentration, in the cells. This feature is similar to what is observed for *B. subtilis* DB1005 *(19).* Evidently, the nature of temperature sensitivity of both *B. subtilis* DB1001 and DB1005 is different from that of the *E. coli rpoD 800* Ts mutant which has a 14-amino-acid deletion at position 330 of the σ^{70}. The Ts phenotype of the latter is attributed to insufficient concentration, rather than altered function, of the a^{70} protein in the cells since it can be suppressed by mutations in the *Ion* protease gene *(34, 35).* More importantly, the effect of changing the hydrophobicity and helicity of the promoter —10 binding helix on both structural stability and transcription activity of <r^A suggests that there is an important packing interaction

between the hydrophobic face of promoter —10 binding helix and certain amino acid(s) in the hydrophobic core region of a^A.

The slight increase in the expression of the mutant *spoIIGHC* promoter of *B. subtilis* by the T199I substitution of cx^A and the suppression of the —12 T to C or G substitution of *lac* promoter by the substitution of an isoleucine for a threonine at the homologous position of *E. coli* a^{70} indicate that the conserved threonine residue has direct contact with certain base(s) of the wild-type promoter DNA (6, *12).* Consequently, the T199G substitution in our case might affect the contact of the promoter —10 binding helix with the promoter DNA, and therefore the transcription activity of a^A. The lower σ^A activities in *B. subtilis* DB1002 compared with those in *B. subtilis* DB2 at both 37 and 49°C might support this idea (Fig. 4). Moreover, the instability of DB1002 σ^A at 37°C (Fig. 3a) might be due to the disruption of the promoter —10 binding helix by T199G substitution. Alternatively, it could be attributed to the removal of the hydroxyl group on Thr-199, which is required for intramolecular hydrogen bonding and stabilization of σ^A.

The induction of a^A in *B. subtilis* DB1001(pCX2Fl), but not in DB2(pCX2F), within 5 min after heat shock is intriguing (Fig. 5). A similar phenomenon was observed for *B. subtilis* DB1005 *(19)* and its pseudorevertants under heat stress (Wang, W.H. and Chang, B.Y., unpublished results). This induction seems to be related to the presence of defective σ^A factors in the cells at high temperature. Mechanisms involved in the rapid σ^A induction remain unclear. It is possible that a common regulatory circuit is adopted by all the *sigA* mutants at the elevated temperature.

In conclusion, the maintenance of a proper helical structure in the promoter —10 binding region is not only essential to overall structural stability, but also important for ensuring a normal function of ff^A. The multiple roles of the promoter —10 DNA binding helix seem to be a unique property of <r factor as a member of the class of DNA-binding proteins.

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