σ^E Changed to σ^B Specificity by Amino Acid Substitutions in Its -10 Binding Region

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The association of a sigma factor (σ) with RNA polymerase in bacteria determines its specificity of promoter utilization. To identify amino acid residues in σ^E from *Bacillus subtilis* that determine the specificity of its interaction with the nucleotides at the -10 region of its cognate promoters, we tested whether base pair substitutions in the -10 region of a σ^B -dependent promoter could signal its utilization by σ^E -RNA polymerase. We found that a combination of base pair substitutions at positions -15 and -14 of the σ^B -dependent *ctc* promoter resulted in its utilization by σ^E -RNA polymerase in vivo. We also found that the combination of two amino acid substitutions at positions 119 and 120 in σ^E changed its specificity for promoter utilization, resulting in a sigma factor that directed transcription from the σ^B -dependent *ctc* promoter, but not from σ^E -dependent promoters. These results suggest that amino acid residues at positions 119 and 120 determine, at least in part, the specificity of interactions between σ^E and the nucleotides in the -10 region of its cognate promoters.

The association of a sigma factor (σ) with RNA polymerase in bacteria is essential for utilization of specific promoters. Many bacterial species contain multiple sigma factors which recognize unique sets of promoters (reviewed in references 9 and 10). The specificity of promoter utilization by primary sigma factors, σ^{70} homologs, is directed by two regions of the σ subunit that make sequence-specific contacts with the -10 and the -35 regions of the promoter. The two regions that interact with the -10 and -35 regions of promoters, respectively, have been identified by genetic studies in which the deleterious effect of specific base pair substitutions in the -10 or -35 recognition region was suppressed by single amino acid substitutions in specific regions (called 2.4 and 4.2) of the sigma factor, (7, 19, 27). The model is also supported by biochemical studies in which fragments of σ^{70} have been shown to bind specifically to promoter DNA (6).

The amino acid sequences of regions 2.4 and 4.2 are most highly conserved in the primary sigma factors, while the amino acid sequences in these regions of most secondary sigma factors contain several highly conserved residues separated by nonconserved amino acids (9). These secondary sigma factors probably interact with their cognate promoters in a manner similar to that of the primary sigma factors, but the variable amino acids in regions 2.4 and 4.2 of the secondary sigma factors appear to be responsible for the unique promoter recognition specificity of each sigma factor. To test the role of these variable sequences, we have examined the effects of amino acid substitutions in σ^E , a secondary sigma factor that is essential for sporulation in *Bacillus subtilis* (10). This analysis showed that the glutamine at position 217 and the tyrosine at 219 in region 4.2 of σ^E interact with base pairs at two positions in the -35 region of its cognate promoters (25). Similar genetic studies indicated that the methionine at position 124 in region 2.4 of σ^E closely contacts the highly conserved CG base pair at the upstream end of the -10 region in the *cotEPI*, *spoIID*, and *spoIIID* promoters (5, 21). However, other base

pairs in the -10 region of σ^E -dependent promoters are important for promoter utilization (5, 21), and some of these may be closely contacted by σ^E .

We have used two approaches to identify additional amino acid residues in σ^E that determine its sequence-specific interactions with the -10 region of promoters. First, we examined the effects of single amino acid substitutions, substitutions of alanine for the amino acyl residue located at positions 113 to 123 in σ^E (constructed previously [11]), on the use of mutant promoters containing single base pair substitutions in their -10 regions (also constructed previously [5, 21]). None of these amino acid substitutions affected the specificity of promoter utilization to a detectable degree (data not shown). Therefore, in a second attempt to identify additional amino acyl residues in σ^E that determine the specificity of its interaction with the -10 region of its promoters, we examined the effects of amino acid substitutions in region 2.4 of σ^E that make the sequence of its -10 recognition region more similar to that of another sigma factor, σ^B , which plays a role in stationary phase and general stress responses in *B. subtilis* (2, 3, 26). We chose to make σ^E similar to σ^B because previous results from in vitro transcription assays demonstrated that σ^E could weakly direct transcription from a σ^B -dependent promoter, the *ctc* promoter (22, 24). Furthermore, two base pair substitutions in the -10 region of the *ctc* promoter increased the efficiency of its utilization by σ^E -RNA polymerase (22). This result suggested that the use of this σ^B -dependent promoter by σ^E -RNA polymerase was limited by the interaction of the polymerase with the -10 region of the promoter. Therefore, we predicted that amino acid substitutions in σ^E that affected the specificity of its interaction with base pairs in the -10 region would enhance its use of the *ctc* promoter. We found that the combination of amino acid substitutions at positions 119 and 120 of σ^E resulted in a sigma factor that directed transcription efficiently from the *ctc* promoter.

MATERIALS AND METHODS

Mutagenesis, cloning, and transfer of the *ctc* promoter to the SPB prophage. Oligonucleotide-directed mutagenesis was utilized previously to make substitu-

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tions at both positions -15 and -14 (-15/-14CA) and a single base pair substitution at position -12 (-12AT) in the *ctc* promoter (22). Mutations at positions -16 and -36 in the *ctc* promoter were created earlier by site-specific bisulfite mutagenesis (23).

A 145-bp *EcoRI*-*HindIII* DNA fragment from pUC8-31 containing the *ctc* promoter region or its mutant derivatives from positions -48 to +76 was cloned between the *EcoRI* and the *HindIII* site of the vector pTKlac (13). This construction places the *ctc* promoter and its mutant derivatives in front of the promoterless derivative of the *lacZ* gene and adjacent to the *trpA* terminator in order to prevent expression from upstream promoters. These plasmids were sequenced to ensure that the proper mutation was present.

The pKT-*ctc* derivatives were linearized by cleavage with *MroI* and crossed into an SP β prophage. The SP β phage was induced from the chromosome of each strain by heat, generating phage lysates of the wild-type and mutant derivatives. These phage lysates were designated SP β trpActc-*lacZ* for a wild-type lysate and SP β trpActc(-12AT)-*lacZ* for a lysate with a mutation at position -12 in the *ctc* promoter, where adenine has been replaced with a thymine. The *ctcCA* promoter contains two base pair substitutions, cytosine at position -15 and adenine at position -14 (nontranscribed strand).

Mutagenesis and expression of *sigE*. A 1.1-kb *PstI* fragment containing the *sigE* gene was cloned into the phagemid pJ8903, which places the *sigE* gene under the control of the *PlacBS* promoter (11). Single-stranded phagemid DNA was isolated, and mutations in *sigE* were constructed by the oligonucleotide-directed mutagenesis procedure of Kunkel et al. (15). The mutations in the *sigE* gene that resulted in substitution of amino acids in σ^E at position 119 (from glutamic acid to isoleucine) and at position 120 (from asparagine to glycine) were generated by using the oligonucleotide 5'-CATCAGGATTTCACCTATTATACACGGGA-3'. This mutant derivative of σ^E is called σ^E (ENIG). Single amino acid substitutions in *sigE*, changing amino acids at positions 119, 120, 121, 122, 123, 124, and 125 to alanine, have been reported previously (11). To ensure that the proper mutation was made and that no further mutations were introduced, the entire *sigE* gene was sequenced by utilizing primers internal to the *sigE* gene.

B. subtilis strains containing *sigE* alleles under *Pspac* promoter control were constructed in three stages (21). First, the *sigE* alleles were cloned downstream from the *Pspac* promoter in pAG58bleo-1. Second, a *B. subtilis* strain containing a resident pTV21 Δ 2 plasmid was transformed with a ligated mixture which contains the *Pspac-sigE* allele in between two arms of homology from the pTV21 Δ 2 plasmid. The homology from pTV21 Δ 2 adjacent to the *Pspac-sigE* region allowed recombination with the resident pTV21 Δ 2, generating the recombinant plasmids pTVSPIGMA*sigE*, and mutant derivatives. Third, these plasmids were recombined into the chromosome of KT1100, which contained a region homologous to the Tn917 sequences in the plasmids, a deletion of the *sigE* chromosomal locus replaced with erythromycin, and a null mutation in the sigma B structural gene.

KT1100 was constructed in three stages. First, PY852(chr::Tn917HU160), which contains a silent transposon Tn917 insertion, was transformed to chloramphenicol resistance with linearized pTV21 Δ 2, generating EU9000 (21). The wild-type *sigE* allele was replaced in EU9000 with the gene for erythromycin resistance by transformation with chromosomal DNA from strain EU8701 and selection for growth on Luria-Bertani agar supplemented with 1 μ g of erythromycin per ml and 25 μ g of lincomycin per ml. A single transformant (EU100) which was resistant to erythromycin at 1 μ g/ml, lincomycin at 25 μ g/ml, and chloramphenicol at 5 μ g/ml and formed less than 10 heat-resistant spores per ml was chosen. The *sigB* gene which codes for σ^B was disrupted in EU100 with the gene for neomycin resistance by transformation with chromosomal DNA from strain PM430 (16) and selection for growth on Luria-Bertani agar containing neomycin at 5 μ g/ml. A single transformant (KT1100) which was resistant to erythromycin at 1 μ g/ml, chloramphenicol at 5 μ g/ml, and neomycin at 5 μ g/ml was chosen for further study.

B. subtilis KT1100 was transformed with pTVSPIGMA*sigE*, or mutant derivative pTVSPIGMA*sigE*ENIG, at the nonpermissive temperature for plasmid replication and selected for growth on phleomycin at 1.0 μ g/ml with sensitivity to chloramphenicol, creating KT1101 and KT1102, respectively. To ensure that the strains contained the desired *sigE* allele, chromosomal DNA was isolated from each strain. Utilizing two oligonucleotides internal to the *sigE* gene, an 852-bp fragment of the *sigE* gene was amplified by PCR with *Taq* polymerase from Perkin-Elmer. The PCR products were cycle sequenced with an oligonucleotide complementary to region 1.2 (5'-GGAGCAGGTTTGTGAATGAAGCTCCC-3') by using the fmol DNA sequencing system kit from Promega. Cycle sequencing of the PCR products demonstrated that all the chloramphenicol-sensitive strains had acquired the appropriate *sigE* allele.

The abilities of the strains to sporulate when induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) were assayed by counting CFU after heating sporulating cultures at 80°C for 10 min. The strain containing the wild-type *sigE* allele, KT1101, was sporulation proficient, generating 10^8 spores per ml. The strain harboring the double mutant, KT1102, sporulated 10^7 -fold less efficiently than the wild-type strain.

β -Galactosidase assays. *B. subtilis* KT1101 and KT1102 were lysogenized with the SP β derivatives that contained wild-type and mutant *ctc* promoters fused to *lacZ* and were selected by growth on Luria-Bertani agar supplemented with 5 μ g of chloramphenicol per ml. Only those transductants that were able to grow and to express activity on DS medium containing 5 μ g of chloramphenicol, 1 mM

FIG. 1. The effects of wild-type and mutant alleles of *sigE* on expression of promoter-*lacZ* fusions. Wild-type or mutant *sigE*(ENIG) alleles were fused to *Pspac*, and transductants that contained an SP β prophage carrying *lacZ* fusions to various promoters were isolated. These strains were grown in DS medium containing IPTG (■) or DS medium without inducer (▲). The cells were harvested at 1- or 2-h intervals after the end of exponential growth (time zero), and β -galactosidase levels were determined. The allele of *sigE* and the promoter fused to *lacZ* (*spoIID*, *ctc*, or *ctcCA*) are indicated at the top of each panel.

IPTG, and 40 μ g of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) per ml were used for further analysis in liquid medium. DS medium containing chloramphenicol was inoculated with a fresh transductant and grown until an optical density at 600 nm of 0.6 was attained, at which time the cultures were divided. To one half of the cultures, 1 mM IPTG was added. At hourly intervals, 1 ml of the cell culture was harvested and β -galactosidase assays were performed (18).

RESULTS

Base pair substitutions in the -10 region of a σ^B -dependent promoter facilitate its use by σ^E -RNA polymerase. We examined the use of the *ctc* promoter and mutant derivatives by σ^E -RNA polymerase in vivo. We constructed a strain that contained the structural gene for σ^E (*sigE*) under the control of an inducible promoter, *Pspac*. In this strain, KT1101, the normal chromosomal copy of *sigE* was replaced with the *erm* gene, and *sigB*, which encodes σ^B , was disrupted with a neomycin cassette. This strain was lysogenized with the SP β prophage containing either the wild-type or mutant derivatives of the *ctc* promoter fused to *lacZ*. The accumulation of β -galactosidase activity in these strains was monitored in liquid sporulation media in the presence and in the absence of 1 mM IPTG. When the wild-type allele of σ^E was induced, no measurable activity from the *ctc* promoter was detected (Fig. 1). However, in the strain containing the wild-type allele of *sigE* and the *ctcCA-lacZ* promoter, β -galactosidase was detected within 2 h after the onset of sporulation (Fig. 1), which is about the time that σ^E is activated by proteolytic processing of its precursor form (10). The activity of the *ctcCA* promoter was dependent on the expression of σ^E , since little or no β -galactosidase accumulated in the absence of IPTG. The nucleotide sequence in the -10 region of the *ctcCA* promoter resembles the consensus for σ^E -dependent promoters, since the *ctcCA* promoter contains cytosine and adenine (nontemplate strand) at positions -15 and -14, respectively (Fig. 2). These results suggest that the nucleotides at position -15 and -14 in the *ctcCA* promoter facilitate recognition of the promoter by σ^E RNA polymerase in vivo.

Amino acid substitutions in σ^E change its specificity to a σ^B promoter specificity. Since two base pair substitutions in the -10 region of the *ctc* promoter enhanced its use by σ^E -RNA

FIG. 2. The -10 binding regions of sigma factors. Shown are the amino acid sequences (single-letter code) of the region from several sigma factors that interact with the -10 regions of their cognate promoters. The sigma factors with superscript numbers are from *E. coli*, and the sigma factors with superscript letters are from *B. subtilis* (see the text). The number at the end of each line indicates the position of the amino acyl residue at the end of the line. The shaded regions indicate the most highly conserved residues. The circled amino acids are those that have been shown to be involved in determining sequence-specific interactions with the -10 region of their cognate promoters. The amino acid residues in σ^E that were changed to be σ^B -like are boxed in σ^E and σ^B (see the text). Shown on the lowest line is the nucleotide sequence of part of the *ctc* promoter. The positions of nucleotides from the start point of transcription (+1) are indicated below the sequence. The consensus nucleotide sequence of σ^E -dependent promoters is indicated above the *ctc* promoter sequence. The shaded nucleotides indicate those changed in the *ctc* promoter to C and A (*ctcCA*), resulting in its use by RNA polymerase containing wild-type σ^E .

polymerase, it seemed likely that amino acid substitutions in σ^E that enable it to interact productively with the -10 region of the wild-type *ctc* promoter would increase the efficiency of *ctc* promoter utilization by σ^E -RNA polymerase. We were guided in our choice of which amino acid substitutions to make in σ^E by our previous genetic experiments that showed that the methionine at position 124 (M-124) of σ^E interacts near the conserved CG base pair at the upstream end of the -10 region (Fig. 2) (5, 21) and that alanine substitutions at positions 123 and 122 have little or no effect on σ^E activity (11). Furthermore, Q-437 in σ^{70} , Q-196 in σ^A , and R-96 in σ^H affect specific interactions of these sigma factors with the -10 regions of their cognate promoters (4, 7, 13, 14). Therefore, we made two substitutions in σ^E , resulting in a derivative of σ^E (ENIG) containing isoleucine at position 119 and glycine at position 120, which are the two amino acyl residues found in the homologous positions in σ^B (Fig. 2). This allele of *sigE* was placed under the control of the *Pspac* promoter in a strain of *B. subtilis* in which the *sigB* gene was disrupted (see Materials and Methods). Transductants of this strain that contained an SP β prophage carrying either the wild-type or mutant derivatives of the *ctc* promoter fused to *lacZ* were isolated.

The accumulation of β -galactosidase activity in these strains was monitored in liquid sporulation media in the presence and in the absence of 1 mM IPTG. Induction of σ^E (ENIG) resulted in accumulation of β -galactosidase beginning about 2 h after the end of exponential growth (Fig. 1). This accumulation appeared to be dependent on σ^E (ENIG), since little β -galactosidase accumulated in the uninduced culture (Fig. 1). Evidently, the two amino acid substitutions in σ^E (ENIG) changed its specificity for promoter utilization. It could direct transcription from the wild-type *ctc* promoter, but it did not direct expression from the *ctcCA* promoter or from the *spoIID* pro-

motor (Fig. 1). Utilization of the *ctc* promoter by RNA polymerase containing σ^E (ENIG) required base pairs that are also required for use of the *ctc* promoter by RNA polymerase containing σ^B , as σ^E (ENIG) directed no detectable transcription from mutant derivatives of the *ctc* promoter containing single base pair substitutions at positions -12, -16, and -36 (data not shown). These base pair substitutions had been shown previously to reduce utilization of the promoter in vitro by σ^B - and σ^E -RNA polymerases (24).

We also note that substitution of isoleucine at position 119 and glycine at position 120 in σ^E not only makes this mutant sigma factor similar to σ^B , it also makes its sequence similar to σ^F in this region (Fig. 2). Efficient transcription by σ^F -RNA polymerase is correlated with promoters containing guanine residues at positions -15 and -16, a purine at position -13, and a thymine at position -7 (20). We tested whether σ^E (ENIG) could direct transcription from the σ^F -dependent promoter (*sspE2G*) and found some activity (data not shown). However, interpretation of this result is complicated by the presence of σ^F , which is required for the proteolytic activation of σ^E in the strains used in this experiment.

DISCUSSION

Our results show that the specificity of promoter utilization by RNA polymerase containing σ^E is changed by the amino acid substitutions E to I at position 119 (E119I) and N to G at position 120 (N120G) in σ^E . The sigma factor containing these two substitutions, σ^E (ENIG), directed transcription from the σ^B -dependent promoter *ctc* but not from the σ^E -dependent promoter *spoIID*. These two amino acid substitutions in σ^E probably affected its interaction with the base pairs at positions -14 and -15 of the *ctc* promoter, since the *ctcCA* promoter containing base pair substitutions at -14 and -15 was not used by σ^E (ENIG), whereas these base pair substitutions enhanced utilization of the promoter by wild-type σ^E .

Although the exact interactions between these amino acyl residues and the base pairs at positions -14 and -15 are not known, it seems likely that these amino acid residues at positions 119 and 120 of σ^E are close to the base pairs at positions -14 and -15 when σ^E -RNA polymerase is bound to the promoter. Gribskov and Burgess (8) have suggested that this section of the 2.4 region of sigma factors forms an α -helix, and we have found previously (5, 21) that amino acid substitutions at position 124 (one turn of an α -helix from position 120) of σ^E affect the specificity of its interaction with the base pairs near position -14 or -15 in promoters.

The amino acid residues at positions 124, 120, and 119 may not interact directly with DNA, but substitutions at these positions could be affecting the interactions of neighboring amino acyl groups. However, we favor the alternative interpretation, in which these residues at positions 124, 120, and 119 interact directly with the base pairs near positions -15 and -14 of the promoters. This conclusion is strengthened by the finding that amino acid substitutions in other sigma factors at positions homologous to 120 in σ^E also affect the specificity of their interaction with the -10 region of their cognate promoters (4, 7, 13, 14). We also note the possibility that more than one of these amino acids may be involved in recognition of a single base pair. Two amino acid residues have been found to cooperate in contacting a single base pair in crystallographic studies of the DNA-binding proteins, *EcoRI* (17), λ repressor (12), and phage 434 repressor (1). Moreover, amino acid substitutions at either of two positions in σ^{70} (437 or 440) affect recognition at one base pair (27).

We conclude that our results are consistent with the model

in which amino acid residues near positions 120, 119, and 124 interact with the base pairs at the 5' end of the conserved sequence found at the -10 region of σ^E -dependent promoters. It is not known which amino acids interact with the 3' end of the -10 region or even whether it is σ or a core subunit of RNA polymerase that contacts the base pairs in this region of the promoter.

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