Effects of Amino Acid Substitutions in the -10 Binding Region of σ^E from *Bacillus subtilis*

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The sigma subunit of bacterial RNA polymerase is required for specific binding to promoters. One region in most sigma factors makes sequence-specific contacts at the -10 region of its cognate promoters. To test the role of the amino acids in this -10 binding region, we examined the effects of 49 single-amino-acid substitutions in σ^E from *Bacillus subtilis*. We assayed the effect of each amino acid substitution on spore formation because σ^E is essential for endospore formation in *B. subtilis*. Our results showed that substitutions at several positions, including the highly conserved aromatic amino acid at position 102, had little or no detectable effect. Substitutions at another position, position 117, produced dominant negative mutations; we suggest that these mutations allow RNA polymerase containing the mutant sigma factor to bind specifically to promoters but prevent transcription initiation. Of the recessive defective alleles, those that produced substitutions at positions 113, 115, and 120 produced the most defective sigma factors. These results suggest that the residues at or near these positions in wild-type σ^E play important roles in σ^E function.

The sigma subunit of the bacterial RNA polymerase holoenzyme governs the specificity of promoter binding (reviewed in references 11 and 23). Many species of bacteria contain multiple RNA polymerase sigma factors, which direct the utilization of different sets of promoters. The specificity of promoter utilization is determined by two regions in most sigma factors (excluding σ^{54} and its homologs [26]). One region of each sigma factor makes sequence-specific contacts with the promoter about 35 bp upstream from the starting point of transcription, while the other region of each sigma factor makes sequence-specific contacts about 10 bp upstream from the starting point of transcription (the -35 and -10 regions, respectively). This model is based primarily on the results of genetic studies in which the deleterious effects of single-base-pair substitutions in promoters were suppressed by single-amino-acid substitutions in sigma factors (4, 8, 17, 18, 29, 31, 34, 35). In each case the specificity of the suppression was used to argue that the amino acid residue in the sigma factor that was changed by the suppressor substitution closely contacted the position in the promoter at which the effect of a base substitution was suppressed.

Amino acid substitutions in four sigma factors have been shown to specifically suppress the effects of base pair substitutions in the -10 regions of promoters (4, 6, 8, 17, 18, 29, 31, 34, 35). These substitutions lie within a region of amino acid sequences that is highly conserved in most sigma factors (11) (Fig. 1). Therefore, most sigma factors may use a similar motif to interact with the -10 regions of their cognate promoters. However, only a few positions in these -10 binding regions of sigma factors may determine the specificity of these interactions.

Promoter utilization involves several steps after the initial binding of RNA polymerase to the promoter. These steps include DNA melting, binding of the initial ribonucleoside triphosphates, and release of the sigma factor prior to

elongation of the transcript (reviewed in reference 2). The sigma subunit may participate directly in some of these steps. Recently, we found that RNA polymerase containing a mutant form of σ^E from *Bacillus subtilis* was able to bind specifically to a promoter but was not able to initiate transcription (14). The amino acid substitution in this σ^E may have inactivated a function of the sigma factor that is required for a step in transcription initiation that follows the initial binding of the polymerase to the promoter. This amino acid substitution, an arginine for cysteine at position 117, is located within the highly conserved -10 binding region, only seven residues from the methionine residue at position 124 (Fig. 1), which has been implicated in genetic suppression experiments to closely contact the base pairs at positions -13 and -12 of σ^E -dependent promoters (6, 31). Additional amino acid residues in the -10 binding regions of sigma factors may also be directly involved in transcription initiation. Helman and Chamberlin (11) pointed out that the sequence of amino acids in this region is similar to the sequence found in single-stranded nucleic acid-binding proteins and suggested that the highly conserved aromatic amino acids in this region may participate directly in DNA melting.

To test the role of the amino acids in the -10 binding region of a sigma factor, we examined the effects of single- and double-amino-acid substitutions in this region of σ^E in *B. subtilis* (Fig. 1). σ^E is not required for viability of *B. subtilis*; therefore, mutations that prevent the function of σ^E are not lethal. However, σ^E is required for endospore formation; therefore, sporulation can be used as an assay of σ^E function. Our results showed that substitutions at some positions, including some positions occupied by highly conserved amino acids, evidently had little effect on σ^E function. In contrast, other single-amino-acid substitutions at positions 113, 115, 117, and 120 severely limited σ^E function.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* JH642 was provided by J. Hoch (7). Strain EU8701, the strain bearing *sigE* Δ 1, was

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FIG. 1. Comparison of the primary sequences of the -10 recognition regions of several sigma factors and the effects of single-amino-acid substitutions in the -10 region of σ^E . The sigma factor sequences shown are from *B. subtilis* (σ^A through σ^K) and *E. coli* (σ^{70} and σ^{32}). The areas of conservation of the primary sequence are shaded. The number at the left of each sequence indicates the position in the sigma factor of the amino-terminal residue present in each sequence. The circled residues in σ^H , σ^{70} , σ^A , and σ^E have been shown by suppression studies to interact with base pairs in the -10 region of cognate promoters. The sequence alignment is the alignment described by Stragier et al. (30). At the bottom the effects on sporulation of single-amino-acid substitutions in the -10 recognition region of σ^E are shown. The arrows indicate the severity of the defect caused by the indicated amino acid substitution relative to the level of sporulation when strain EU8701 (*sigE* Δ 1) is complemented by pJ8903 (wild-type σ^E). For example, the NA100 substitution resulted in wild-type sporulation, whereas the TP101 substitution resulted in only 10^{-5} of the spores formed by the strain with the wild-type *sigE* allele. The CS117 substitution was tested only in combination with a silent substitution, PT104.

isolated by T. Kenney (16). The *Escherichia coli* strains which we used were strains NM522 (19), RY2504 (a gift from G. Churchward), and DH5 α (33).

Plasmid construction. Shuttle plasmid pJ89 was created by cloning the 2.1-kb *Bgl*I DNA fragment of pUCf1 (Pharmacia) after the ends were filled in into *Bam*HI-cleaved pUB110 (10). pJ8900 was created by cloning the 1.1-kb *Pst*I fragment bearing *sigE* into the unique *Pst*I site of pJ89. DNA sequencing was used to determine that the 1.1-kb *Pst*I fragment was in the correct orientation so that transcription of *sigE* was directed by P_{lac} . Oligonucleotide-directed mutagenesis (33) was used to change the *lac* promoter of pUCf1 to a consensus σ^A -directed promoter (12), as described below. Using pJ8900 as a template, we used oligonucleotide 5'CCA CACATTATACGAGC3' to change both position -8 and position -9 in the *lac* promoter to adenine, creating pJ8902. Oligonucleotide 5'TAAAGTGTCAGCCTGG3' was used to change position -34 to guanine in pJ8902 to create pJ8903 (Fig. 2). The resulting promoter, P_{lacBS} , has been shown previously to be used efficiently in *B. subtilis* (12, 17).

Mutagenesis. Oligonucleotide 29RM2.3 (5'TTCTATACACCGGGAGGCATAGGTAGCAAGCTTGATTTCCTT TTCTGGATTAAATGTATT3') was synthesized (Synthecell) with each nucleotide contaminated at a level of 1.1% per cycle to give an average of two substitutions per strand. This degenerate oligonucleotide was then used in oligonucleotide-directed mutagenesis as described previously (33), with the following modifications. pJ8903 was used to transform *E. coli* RY2504 (*dut ung*) to a bleomycin (Sigma)-resistant phenotype. A bleomycin-resistant transformant was grown in Luria broth supplemented with 7 μ g of bleomycin per ml and 30 μ g of chloramphenicol per ml to an optical density at 660 nm of 0.5, and then uridine (0.25 μ g/ml) and phage M13K07 (multiplicity of infection, 10) were added. The infection was allowed to proceed for 1 h with vigorous shaking, and then the culture was diluted 25-fold into Luria broth supplemented with bleomycin (7 μ g/ml) and kanamycin (70 μ g/ml). Single-stranded DNA was annealed

to the degenerate oligonucleotide by incubating the preparation at 72°C for 2 min and then cooling it slowly to room temperature over a 3-h period. Sequenase (United States Biochemical [USB]) was used to generate the replicative form, the efficiency of which was checked by agarose gel electrophoresis before transformation into strain NM522. Bleomycin-resistant colonies were chosen and a plasmid was isolated by using the CTAB miniprep procedure (5). Plasmid sequences were determined with the *sigE*-specific oligonu-

FIG. 2. σ^E expression plasmid pJ8903. This shuttle plasmid is a fusion of pUCf1, which replicates autonomously in *E. coli*, and pUB110, which replicates autonomously in *B. subtilis*. The σ^E structural gene, *sigE*, is contained on a *Pst*I fragment, which is located downstream from promoter P_{lacBS} . The arrow indicates the direction of transcription from P_{lacBS} .

TABLE 1. Oligonucleotides used for site-directed mutagenesis

Mutant allele	Sequence (5' to 3')
<i>sigE113YC</i>	TGCTACCTGTGCCTCCG
<i>sigE110LF</i>	AGGTAGCAAACTTGATT
<i>sigE109KA</i>	TAGCAAGCGCGATTTC
<i>sigE113YA</i>	GGGAGGCAGCGGTAGCA
<i>sigE124MT</i>	TTCTTAAATATGTCAAGATTTC
<i>sigE124MA</i>	TCCTTAAATAGCCAGGATTTC
<i>sigE120NA</i>	AGGATTTCAAGCTTCTATACA
<i>sigE117CA</i>	TTTTCTATAGCCGGGAGGC
<i>sigE117CM</i>	TTTTCTATCATCCGGGAGGC
<i>sigE117CW</i>	TTTTCTATCCACGGGAGGC
<i>sigE117CR</i>	ATTTTCTATCCGCGGGAGGCA
<i>sigE100NA</i>	TAAATGTAGCAACAGCT
<i>sigE102FA</i>	CTGGATTAGCTGTATTA
<i>sigE112TA</i>	AGGCATAAGCAGCAAGC
<i>sigE116RA</i>	CTATACAAGCGGAGGCA
<i>sigE115SP</i>	TACACCGGGGGCAGTAC
<i>sigE114AT</i>	ACCGGGATGTATAGGTA
<i>sigE101TA</i>	GATTAAAAGCATTAACT
<i>sigE108LA</i>	AGCAAGCTTAGCTTTCTTTCT
<i>sigE117CK</i>	ATTTTCTATTTTCGGGAGGCA
<i>sigE123LA</i>	AATACATCGCGATTTCATT
<i>sigE119EA</i>	TTTCATTTCGTATACAC
<i>sigE121EA</i>	TCAGGATTGCATTTTCT
<i>sigE122LA</i>	TACATCAGGGCTTCATTTC
<i>sigE125YA</i>	CTTCTTAAAGGCATCAGGAT

cleotide 5'GAACGGATTTTGTATT3', which was used to identify mutations by the dideoxy-terminated sequencing protocol with Sequenase (USB). Additional site-directed mutants were made with the specific oligonucleotides shown in Table 1.

Sporulation assays. Strain EU8701 preparations containing pJ8903 derivatives bearing substitutions in *sigE* were grown in DS medium (27) supplemented with 0.5 μ g of phleomycin (Cayla) per ml for 20 h with vigorous shaking. A 1-ml portion of each culture was transferred to a glass tube, and the cultures were incubated for 20 min at 80°C. Dilutions of each heated sample were plated, and the number of resulting colonies was counted to determine the number of heat-resistant spores.

Primer extension analysis. Total RNAs were isolated from strains that were grown in DS medium 2 h after the onset of sporulation. RNA was purified as described previously (15). The following specific primers were used to analyze levels of transcription: for *spoIID*, 5'CACATAGTACGGATAGT3'; and for *spoIIID*, 5'GCTTCTTCGTTTTGTATGG3'. A primer extension analysis was performed by using 200 μ g of total RNA, as previously described (15).

Isolation of revertants. Ethyl methanesulfonate mutagenesis was used to select for sporulation-proficient revertants of noncomplementing σ^E substitutions in the strain EU8701 background. To do this, we used a procedure described by Olmedo et al. (24). Strains were grown to the mid-logarithmic growth phase in Luria broth supplemented with 0.5 μ g of phleomycin per ml and were plated onto solid DS medium supplemented with 0.5 μ g of phleomycin per ml. A glass microfiber filter (Whatman) was placed on the center of each plate and saturated with ethyl methanesulfonate (Sigma). The plates were incubated for 48 h at 42°C and then inverted over chloroform for 20 min to select for sporulation-proficient revertants. Survivors generally appeared over a 2-day period following chloroform treatment. Plasmid DNA was isolated from survivors, and the reversion was identified by DNA sequencing.

TABLE 2. Effects of *sigE* alleles in merodiploids

Amino acid substitution in σ^E	Sporulation efficiency (no. of spores/ml) with the following chromosomal <i>sigE</i> allele:	
	<i>sigEΔ1</i>	<i>sigE</i> ⁺ ^a
SigE ⁺	2 $\times$ 10 ⁸	2 $\times$ 10 ⁸
YC113	<10	2 $\times$ 10 ⁸
YA113	<10	2 $\times$ 10 ⁸
SP115	<10	2 $\times$ 10 ⁸
TP101	1 $\times$ 10 ³	2 $\times$ 10 ⁸
NA120	<10	2 $\times$ 10 ⁸
CA117	6 $\times$ 10 ⁶	2 $\times$ 10 ⁸
CK117	<10	5 $\times$ 10 ⁶
CR117	<10	4 $\times$ 10 ⁴
CR117/NA120	<10	2 $\times$ 10 ⁸
CR117/YA113	<10	4 $\times$ 10 ⁴
CW117	<10	8 $\times$ 10 ⁵
CW117/YC113	<10	2 $\times$ 10 ⁸
CW117/YA113	<10	2 $\times$ 10 ⁸
CW117/TP101	<10	2 $\times$ 10 ⁸
PT104	2 $\times$ 10 ⁸	ND ^b
CS117/PT104	<10	2 $\times$ 10 ⁶
CM117	<10	4 $\times$ 10 ⁶
CM117/SP115	<10	2 $\times$ 10 ⁸

^a The wild-type strain which we used was strain JH642.

^b ND, not done.

Western blot analysis. Cells were grown in DSM medium at 37°C and were harvested 2 h after the end of the exponential growth phase. Crude extracts were prepared as described by Trempey et al. (32), except that we omitted the ammonium sulfate precipitation step. Equal amounts of protein from the different extracts (50 to 100 μ g of total protein, as assayed by the Bio-Rad protocol) were applied to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis gels. A Western blot (immunoblot) analysis was performed as described by Blake et al. (1).

RESULTS

Collection of single-amino-acid substitutions in the -10 binding region of σ^E . We constructed a shuttle plasmid, pJ8903, in order to facilitate the mutagenesis and expression of *sigE*, the structural gene for σ^E (Fig. 2). The essential features of this plasmid include its ability to replicate as a plasmid in both *E. coli* and *B. subtilis* and as a single-stranded phage when it is complemented with *E. coli* helper phage M13K07. The single-stranded DNA form of the plasmid facilitated oligonucleotide-directed mutagenesis of *sigE*. Transcription of *sigE* on pJ8903 is directed from a consensus σ^A promoter that was derived from the *lac* promoter from *E. coli*. Expression of *sigE* on pJ8903 was sufficient to complement the sporulation phenotype of *B. subtilis* EU8701, which carries a deletion allele of *sigE* (Table 2).

Oligonucleotide-directed mutagenesis with a 60-mer degenerate oligonucleotide (see Materials and Methods) and several specific oligonucleotides (Table 1) was used to produce a collection of single-amino-acid substitutions in the -10 binding region of σ^E . Nucleotide sequencing was used to determine that the *sigE* allele carried on the pJ8903 derivatives resulted in a single-amino-acid substitution. The effect of each substitution was assayed by testing whether the mutant *sigE* allele on each pJ8903 derivative complemented the sporulation defect of strain EU8701, the *sigE* deletion strain. The sporulation proficiencies of the strain

FIG. 3. Western blot analysis of σ^E alleles. Portions (100 μ g) of crude lysates from strains bearing derivatives of σ^E were subjected to 12% polyacrylamide gel electrophoresis, transferred to nitrocellulose, exposed to anti- σ^E monoclonal antibody, and developed with an alkaline phosphatase-conjugated goat α -rabbit monoclonal antibody. Lane a contained a crude extract from strain JH642 (wild type [wt]). Lanes b through j contained extracts from strain EU8701 (*sigE* Δ I) bearing different σ^E alleles expressed from pJ8903 derivative plasmids.

EU8701 derivatives containing the pJ8903 derivatives are shown in Fig. 1 and Table 2. Most of the *sigE* alleles on the pJ8903 derivatives were able to complement (at least to 10% of the wild-type sporulation efficiency) the sporulation defect of the *sigE* deletion strain. These complementing alleles of *sigE* included the alleles that resulted in substitutions at nonconserved positions (e.g., positions 100, 101, 103, 104, 105, 107, 110, 111, 116, 119, 123, and 125). More surprising was the observation that the complementing alleles included some alleles that resulted in substitutions of amino acids at highly conserved positions (for example, substitution of isoleucine for phenylalanine at position 102, abbreviated F1102 [allele *sigE102FI*] (Fig. 1). Other substitutions at highly conserved positions that had surprisingly little effect included KT106, IS108, KA109, TA112, AT114, IS118, and IA122 (Fig. 1).

Some of the alleles of *sigE* were unable to complement the sporulation defect of the *sigE* deletion strain. Since strains containing these *sigE* alleles on pJ8903 sporulated from 10^3 - to 10^7 -fold less efficiently than the strain containing the wild-type allele of *sigE* did (Table 2 and Fig. 1), we concluded that these alleles of *sigE* resulted in amino acid substitutions in σ^E that inactivated this sigma factor. The deleterious effects of these amino acid substitutions on σ^E function could have occurred for several reasons. These substitutions may have replaced amino acids that participate directly in specific aspects of σ^E function, or they may have had indirect effects (for example, by producing proteins that failed to accumulate, possibly because they did not fold properly and therefore were degraded). Furthermore, σ^E is initially produced as a transcriptionally inactive protein precursor, pro- σ^E or P31 (20). Although it seems unlikely, some of the amino acid substitutions described above may have prevented the proteolytic processing of the mutant pro- σ^E . Therefore, all of the mutants that exhibited reduced efficiency in complementation of strain EU8701 were checked for accumulation of σ^E and proper processing of the protein by Western blot analysis, using anti- σ^E monoclonal antibody (Fig. 3). Our results showed that expression of the wild-type allele of *sigE* on pJ8903 resulted in accumulation of an amount of σ^E that was approximately equal to the amount found in a wild-type strain. The strains carrying the mutant alleles of *sigE* also accumulated approximately wild-type levels of σ^E (Fig. 3) (data not shown). There was some variability in the levels of σ^E that accumulated in these strains; however, the levels were probably not different

TABLE 3. Reversion analysis of noncomplementing alleles of σ^E

σ^E substitution	Mutation	Revertant	Sporulation (no. of spores/ml)
YC113	TAT $\rightarrow$ TGT	TAT	2×10^8
CW117	TGT $\rightarrow$ TGG	TGT	2×10^8
CR117	TGT $\rightarrow$ CGT	TGT	2×10^8
	TGT $\rightarrow$ CGG	CCG (Pro)	5×10^5
CM117	TGT $\rightarrow$ ATG	GTG (Val)	5×10^7
		ACG (Thr)	8×10^6
		ATC (Ile)	1×10^7

enough from wild-type levels to account for the 10^3 - to 10^7 -fold effects on sporulation efficiency.

Identification of dominant negative alleles. The deleterious effect of an amino acid substitution in a protein does not necessarily lead to the identification of a position in the protein that plays a specific role in its function. Amino acid substitutions may have indirect effects by affecting protein folding. Although it was clear from the Western blot analysis that the defective σ^E derivatives accumulated at near-wild-type levels, it was not known whether these sigma factors retained any of their normal functions. For example, it was not known whether they bound to the core subunits of RNA polymerase or whether they formed holoenzymes that were capable of binding to promoters. We previously described the effect of a substitution of cysteine for arginine at position 117 (CR117). The sporulation-defective phenotype caused by this allele of *sigE*, *sigE117CR*, was dominant to the wild-type allele. The dominant negative character of a mutant allele can be an indication that the mutant product retains some activity. Therefore, we screened the mutant *sigE* alleles that failed to efficiently complement the sporulation defect in strain EU8701 for a dominant phenotype after transformation of the plasmids bearing the mutant alleles into strain JH642, a strain containing a wild-type chromosomal allele of *sigE*. Of the mutants tested, only substitutions at the cysteine at position 117 (CR117, CW117, CK117, CM117, and CS117) inhibited sporulation in the merodiploid strains (Table 2). We showed that these sporulation-defective merodiploid strains contained the wild-type allele by curing the merodiploid strains of the plasmids bearing the position-117-cysteine-substituted *sigE* alleles. The cured strains were sporulation proficient (data not shown). All of the other mutant alleles that failed to complement strain EU8701 were recessive to the wild-type *sigE* allele.

The observation that substitutions at position 117, but not the substitutions that were tested at other positions in the -10 binding region, were associated with the dominant negative phenotype led us to test whether these alleles contained additional mutations that had not been identified during the original sequence analyses of the alleles. This possibility was eliminated because we isolated sporulation-proficient revertants of strains carrying the CR117, CW117, or CM117 substitution. Each of these revertants either was a true revertant or contained substitutions in the same codon as the original lesion (Table 3). Second-site or extragenic revertants were not found. We also isolated a true revertant of a recessive defective allele, *sigE113YC* (Table 3).

If the other dominant negative substitutions at position 117 have effects similar to that of CR117, then we expected that these alleles, like the *sigE117CR* allele, would reduce transcription of σ^E -dependent promoters during sporulation of the merodiploid strains. A primer extension analysis was

FIG. 4. Primer extension analysis of RNA levels directed from the *spoIID* and *spoIIID* promoters in merodiploid strains. RNAs isolated from strains bearing different σ^E alleles and grown for 2.5 h after the end of exponential growth were hybridized to radiolabeled oligonucleotides that were complementary to sequences downstream from the *spoIID* (A) and *spoIIID* (B) promoters and extended with avian myeloblastosis virus reverse transcriptase. A radiolabeled primer that was complementary to *spoVG* RNA was also included in the experiment shown in panel A. The extension reaction mixtures were subjected to electrophoresis in a 10% (wt/vol) acrylamide gel containing 8 M urea. Dideoxynucleotide-terminated sequencing reactions (panel B, lanes a through d) in which we used a plasmid containing the *spoIID* promoter as a template were produced with Sequenase (USB) and were primed with the same oligonucleotide that was used in the *spoIID* primer extension reactions. Wt, wild type; IIID, *spoIIID*; IID, *spoIID*; VG, *spoVG*. CR, CK, and CS indicate *sigE* alleles resulting in substitutions of R, K, and S, respectively, at position 117 of σ^E .

used to assay transcription from two well-characterized σ^E -dependent promoters (*spoIID* [25] and *spoIIID* [31]), both of which are essential for sporulation. Our results showed that transcription of *spoIIID* (Fig. 4A) and *spoIID* (Fig. 4B) was inhibited in the merodiploid strains containing the wild-type allele of *sigE* in combination with either the *sigE117CR*, *sigE117CK*, or *sigE117CS* allele. *spoVG* transcription, which is directed by σ^H , was not affected in the merodiploid strains (Fig. 4A). Since the dominant negative effects of these alleles were restricted to σ^E -dependent promoters, it is likely that these mutant forms of σ^E retained some activity. Since several substitutions at position 117 produced the dominant phenotype, it seemed unlikely that the mutations created a novel function for σ^E ; rather, these substitutions probably inactivated a normal function of the sigma and in so doing blocked the activity of wild-type sigma factor molecules.

Some defective recessive alleles may suppress dominant mutations. The most defective recessive alleles of *sigE* resulted in substitutions at positions 113, 115, and 120. It was difficult to predict whether the defective sigma factors produced by these alleles would retain any specific sigma factor functions. In fact, it is likely that many recessive defective alleles of *sigE* produce sigma factors with no function. For example, it would not be surprising if the proline substitution at position 115 drastically changed the folding of the sigma, eliminating its activity.

In theory, the effect of double-amino-acid substitutions could provide evidence that some substitutions have only localized effects and that the mutant sigma factors retain some function. We have shown previously that the dominant negative effect of the CR117 substitution requires that RNA polymerase containing the defective sigma factor bind specifically to σ^E -dependent promoters. Therefore, the dominant negative character of a substitution at position 117 (e.g., σ^{CR117}) would be eliminated by an amino acid substitution at

a second site in the sigma factor that prevents the doubly mutant sigma factor from binding to RNA polymerase or prevents the holoenzyme from binding to promoters. Therefore, we examined the effects of several doubly mutant sigma factors in merodiploids that also contained a wild-type *sigE* allele (Table 2). The addition of the NA120 substitution to the CR117 substitution eliminated the dominant negative effect, producing a defective allele that was recessive to wild-type *sigE*. The dominant negative phenotype caused by the CW117 substitution was eliminated by the YC113, YA113, or TP101 substitution, and the dominant negative phenotype caused by the CM117 substitution was eliminated by the SP115 substitution (Table 2). It is tempting to speculate that these second-site mutations, which presumably prevented the dominant negative sigma factors from forming holoenzymes that bound to promoters, also prevented the formation of holoenzymes that bound stably to promoters when they existed as single-amino-acid substitutions.

In contrast to the results described above, the dominant negative phenotype was retained in a sigma factor containing the YA113 substitution in combination with the CR117 substitution. The dominant negative effect of the doubly mutant sigma factor seemed to indicate that RNA polymerase containing this sigma factor still bound specifically to promoters. Since the YA113 substitution did not prevent promoter binding by RNA polymerase containing the two substitutions (CR117 and YA113), the single YA113 substitution probably produced a sigma factor that retained many wild-type functions, including proper folding, polymerase binding, and initial promoter binding. However, the dominant character of the CW117 substitution was eliminated by the YA113 substitution. This apparent contradiction may be explained by the possibility that the CW117 substitution caused a less severe dominant phenotype. Therefore, promoter binding by the CW117-substituted sigma factor may

have been weaker and more easily disrupted than promoter binding by the CR117-substituted sigma factor.

DISCUSSION

Our examination of the effects of amino acid substitutions in the -10 binding region of σ^E revealed substitutions that had little or no effect and substitutions that reduced sigma factor function. It was somewhat surprising that the amino acid substitutions that appeared to have little or no detectable effect on sigma factor function included some that replaced amino acids that are highly conserved in most sigma factors. We assayed the effects of amino acid substitutions in σ^E by testing whether the mutant sigma factor functioned adequately for endospore formation. However, efficiency of sporulation may not be proportional to σ^E activity for two reasons. In a previous study, expression of *sigE* on an SP8 prophage was found to fully complement the requirement for σ^E during sporulation (17) even though the level of σ^E that accumulated in this strain was substantially lower than the level found in most wild-type strains (13). Evidently, the wild-type strain produces more σ^E than is required for endospore formation under laboratory conditions. Our Western blot analyses showed that expression of σ^E from plasmid pJ8903 resulted in approximately wild-type levels of σ^E ; therefore, substitutions that reduced σ^E affinity for the core enzyme or for DNA may not have had a detectable effect on sporulation since excess σ^E was produced. In addition, substitutions in σ^E that reduced σ^E efficiency may have slowed the developmental process, but this effect might not have been detectable in our single-time-point (20-h) assay. This scenario is plausible because sporulating cells appear to utilize several checkpoints to delay development from proceeding prematurely from one developmental stage to the next (3, 21). The selective pressure for maintaining highly conserved amino acids that appeared to be nonessential in our laboratory tests may come from the requirement in natural situations for more rapid sporulation or from a requirement for more efficient σ^E function in nonlaboratory strains that accumulate lower levels of σ^E . Other conditions found in natural environments may also provide selective pressure (e.g., temperature). The conserved phenylalanine at position 102 is thought to play a role in DNA melting (11), but the phenotype of F1102 was the same whether it was assayed at 37 or 30°C (data not shown). Because of these limitations in our assay for σ^E function, we cannot eliminate the possibility that the highly conserved amino acids that were changed without effect in our assay may yet be shown to play important roles in sigma factor function. However, in contrast, the relatively small effects of some substitutions emphasize the critical defects caused by the same substitutions at other positions.

Substitutions at positions 113, 115, 117, and 120 had the most severe effects. It is usually difficult to know whether an amino acid substitution in a protein that reduces its activity interferes with a specific function or whether it disrupts the overall structure of the protein. Two kinds of mutations in sigma factors clearly fall within the former category. Several single-amino-acid substitutions in five different sigma factors have been shown to alter the specificity of promoter utilization by RNA polymerase containing these mutant sigma factors (4, 6, 8, 17, 18, 22, 28, 29, 31, 34, 35). Single-amino-acid substitutions in σ^{70} from *E. coli* and in σ^A , σ^E , and σ^H from *B. subtilis* have been found to suppress the effects of specific base substitutions in their cognate promoters. Amino acid substitutions in σ^F from *B. subtilis* have been

shown to broaden the specificity of this σ so that it can direct transcription from a σ^G -dependent promoter (28) or, in another case, a σ^B -dependent promoter (22). Clearly, these amino acid substitutions alter specific functions of these sigma factors without altering their overall structure.

A second type of example in which an amino acid substitution in a sigma factor alters a specific function is the substitution of arginine for the cysteine at position 117 of σ^E (CR117) (14). This substitution results in a σ that associates with core RNA polymerase to form a holoenzyme that binds specifically to promoters but is unable to initiate transcription. This sigma factor evidently retains many of its normal functions.

The *sigE117CR* allele, which encodes the CR117-substituted sigma factor, is dominant to the wild-type *sigE* allele (Table 2). We found that of the mutations that inactivated σ^E in our study, only those that resulted in substitutions at position 117 of σ^E were dominant to the wild-type allele. Several models might be used to explain the dominant negative effect of these alleles. However, we have previously shown that the CR117-substituted σ^E (σ^{CR117}) binds to core RNA polymerase to form a holoenzyme that binds to a promoter, forming a very stable complex in vitro, but is unable to initiate transcription (14). The dominant negative effect in vivo may result because RNA polymerase containing σ^{CR117} binds to one or more σ^E -dependent promoters but fails to initiate transcription. In merodiploids the σ^{CR117} RNA polymerase bound to a promoter may block access to that promoter by RNA polymerase containing the wild-type σ^E and thereby prevent transcription of σ^E -dependent genes during sporulation.

We found no obvious relationship (charge, hydrophobicity, size of R group) among the substitutions at the cysteine at position 117 that cause a dominant negative sporulation phenotype. Of these substitutions, methionine, tryptophan, and arginine are found at positions homologous to the position of the cysteine at position 117 in other sigma factors (Fig. 1). Evidently, the context (neighboring residues) in which these residues are found is a factor in the resulting phenotype. Furthermore, of the four substitutions that were isolated as revertants of noncomplementing substitutions of the cysteine at position 117, two were hydrophobic, one was polar, and the fourth resulted in a proline substitution (Table 3). These substitutions, along with the alanine substitution (CA117), complemented the deletion strain partially but not to wild-type levels. Gribskov and Burgess proposed that the residues in the region from positions 118 to 126 (σ^E numbering) are in an α -helical conformation (9). The proline that is amino terminal to this region in several sigma factors (σ^G , σ^F , σ^B) (Fig. 1) probably breaks this α -helical structure. It is interesting that proline is tolerated in σ^E to differing degrees at positions 116 and 117, but not at all at position 115. The amino acid residues at positions 116 and 117 in σ^E may act as a hinge or turn between the region that interacts with the base pairs in the upstream half of the -10 region, such as M124 (identified by suppression experiments in references 6 and 31), and the adjacent N-terminal proximal region of σ^E . The role of this latter region, corresponding to position 115 to position 99, has not been determined.

The substitutions at positions 113, 115, and 120 produce severely defective sigma factors and also may define positions that are important for σ^E function. Since the one substitution at position 115 was a proline and since this substitution suppressed the dominant character of a second-site mutation in a doubly mutant sigma factor, it may have affected the overall structure of the protein. It would be interesting to examine the effects of other substitutions at this position.

The effects of the alanine substitutions at positions 113 and 120 are consistent with the previously proposed roles of amino acids at these positions in DNA melting (11) and promoter recognition (31), respectively. These models should be examined further in direct biochemical tests. The YA113-substituted sigma factor probably retains some normal function, including those steps up to and including promoter binding by RNA polymerase, since this mutation fails to suppress the dominant negative effect of the CR117 substitution. This interpretation is based on the untested, but likely, assumption that the amino acid substitutions in the doubly mutant sigma factor act independently and, therefore, must be considered tentative. However, of the defective sigma factors tested in this study, the YA113-substituted sigma factor is probably the best candidate for purification and in vitro studies since it is most likely to have some activity.

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