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Characterization of the Promoter Elements for the Staphylococcal Enterotoxin D Gene

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Deletion analysis of the promoter for the *Staphylococcus aureus* enterotoxin D determinant indicated that a 52-bp sequence, from −34 to +18, was sufficient for *sed* promoter function and *agr* regulation. A consensus −10 Pribnow box sequence, a less conserved −35 sequence, and a TG dinucleotide motif were present. Transcribed sequences (+1 to +18) are essential for promoter activity.

Staphylococcal food poisoning is an intoxication resulting from ingestion of foods contaminated with enterotoxin producing *Staphylococcus aureus* strains (16). The symptoms include emesis, diarrhea, abdominal cramping, and in severe cases, fever and shock (6, 30, 31). The staphylococcal enterotoxins are a group of secreted proteins that cause emesis when orally administered to primates (6). To date, a number of enterotoxins have been characterized based on their serological reactivities and designated SEA to SEJ, including subtypes SEC_1 to SEC_3 (5, 8, 24, 29, 32–34).

Though staphylococcal enterotoxins are similar in structure and biological properties (22), they differ with respect to genetic localization, amount of toxin produced, and mechanism of gene regulation. The genes for SEA and SEE are carried on prophage, some of which are defective prophage. SED and SEJ gene determinants are carried on the same penicillinase-type plasmid. The genes for SEB and SEC are chromosomal, but the nature of the genetic elements on which they reside has not been elucidated (7, 18, 19). SEB and SEC are expressed in greater quantities than the other enterotoxins, often on the order of 100 μg/ml of culture supernatant, whereas maximal production of SEA, SED, and SEE is usually less than 10 μg/ml (3). Furthermore, SEA is produced throughout the log phase of growth, while SEB, SEC, and SED are produced in greater quantities during the transition from the exponential to the stationary phases of growth (5). The latter expression pattern is characteristic of many staphylococcal exoprotein virulence factors which are under the control of the accessory gene regulator (*agr*) two-component regulatory system (25). The transcription of *seb*, *sec*, and *sed* is subject to regulation by the *agr* system. In *agr* mutant strains, mRNA steady-state levels were reduced 4-fold for *seb*, 5.5-fold for *sed*, and 2 to 3-fold for *sec* (2, 28).

Information regarding the promoter elements of the staphylococcal enterotoxin genes is very limited. The *sea* promoter has been identified by means of primer extension analysis in conjunction with deletion mutagenesis. However, detailed characterization of the promoters for the *agr*-regulated enterotoxin genes has not been reported. Promoter sequences for *seb* and *sed* were defined by mapping the transcription start site and by comparison to *Escherichia coli* promoter consensus −10 and −35 sequences. Mahmood and Khan found that a region upstream of the −35 element, at positions −93 to −58, is required for *seb* expression (21). Although primer extension studies have been carried out on *sed*, only a Pribnow box could be identified in the sequence (2). No convincing −35 element was obvious in the sequence analysis. As part of our effort to define the mechanism by which RNAIII regulates *sed* transcription, this study was undertaken to characterize the promoter and potential regulatory sequences for *sed*. In this study, the transcription start site was mapped by primer extension. The transcription start site and bases which are critical to promoter function were identified through characterization of site-specific sequence changes in the *sed* promoter.

Construction of the deletion mutants and promoter analysis. To characterize the promoter sequences for *sed*, we cloned 1.7 kb of DNA immediately 5′ to the ribosome binding site of *sed* and introduced a series of deletions into the sequence at either the 5′ or 3′ end by exonuclease III digestion (Erase-A-Base; Promega). All deletions were verified by sequencing, and nucleotide positions were numbered relative to the start site of transcription (+1). The deleted *sed* promoter region sequences were then fused with a promoterless chloramphenicol acetyltransferase (*cat*) gene carried by pMH109 (17). The *sed* promoter activity was then determined by measuring the expression level of *cat* (Fig. 1). The upstream boundary of the promoter element was defined by promoter active deletions which extended to position −34. The downstream boundary of the promoter element was identified as +24. Deletions up to this position did not appreciably affect *sed* promoter activity and the *agr*-regulatory effect, but deletions which extended beyond these endpoint sequences resulted in a loss of promoter function. The insert of plasmid pZS2688, containing *sed* sequences from −34 to +7, failed to drive measurable *cat* expression. This suggested that sequences beyond the start site of transcription were required for transcription from the *sed* promoter. Therefore, the essential promoter sequences appeared to be contained within the sequence between −34 to +24. To confirm this, plasmid pZS2074 containing *sed* promoter sequences from −34 to +24 was created. When CAT activity was measured from lysates of cells bearing this plasmid, the insert in pZS2704 retained promoter activity and the *agr*-stimulatory effect comparable to that of the 1.7-kb insert in pZS2605. Therefore, the 58-bp fragment contained in pZS2074 possessed all required promoter elements and the *cis* sequences responsible for the stimulation of transcription which occurs in *agr* wild-type hosts.

Mapping the *sed* transcription start site. To confirm that the 58-bp promoter fragment accurately reflected the *sed* pro-

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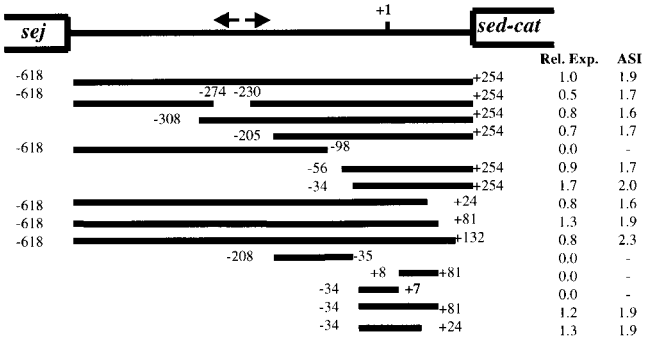

FIG. 1. Deletion analysis of the *sed* promoter. Arrows indicate the inverted repeat sequence located within the intergenic region of *sed* and *sej*. The transcription start site of *sed* is indicated as +1. Lines represent the remaining part of the *sed* promoter which were fused with a promoterless *cat* gene in pMH109. Deletions are numbered according to distance from the transcription start site. Promoter activities were analyzed by measuring CAT expression levels in both KSI2054 (Agr^+) and ISP546 (Agr^-) host strains (33). CAT values were converted to relative expression (Rel. Exp.), which is the value obtained with the deletion mutant divided by the value obtained with the wild-type *sed* promoter in strain KSI2054. The *agr* stimulation index (ASI) is the CAT value obtained with KSI2054 divided by the value obtained from ISP546 cells bearing the same plasmid.

moter activity, the transcription start site was mapped. First, we mapped the transcription start site when the intact upstream sequences were present. RNA was isolated (14) from *S. aureus* strains KSI2054 (agr^+) and ISP546 (*agr*) harboring plasmid pZS2607 (carrying 1.7 kb of DNA immediately 5′ to the *sed* ribosome binding site [34]). Primer extension assays (9) revealed a reverse-transcribed DNA fragment at a position corresponding to the T residue located 265 bp upstream of the translation start site (S. Zhang and G. C. Stewart, unpublished data). This is in agreement with the results of Bayles and Iandolo (2). To map the transcription start site from the 58-bp promoter, RNA was isolated from KSI2054 and IPS546 harboring pZS2704. The same start site of transcription was identified with the 58-bp promoter fragment as was seen with the intact upstream sequence (Fig. 2). The signal is stronger with the RNA isolated from the agr^+ host (compare lane 4 to lane 3), consistent with the results obtained with the CAT assays. The primer extension assay was also carried out with RNA isolated from cells bearing the *lac-sed* hybrid promoter. This promoter has the −35 and spacer sequences from the *lac* promoter and the Pribnow box and downstream sequences from the *sed* promoter. This promoter is stronger than the wild-type *sed* promoter (Fig. 3, pZS2743), but the start site of transcription is unchanged (Fig. 2, lanes 1 and 2).

Five bases upstream of the +1 position in the *sed* promoter there is a sequence, TATAAT, that matches the consensus Pribnow box of bacterial promoters. A less conserved −35 (ATGAAA) is located 17-bp upstream from the −10 element. The *sed* promoter also contains a TG dinucleotide at position −14/−13. This dinucleotide is an important element of procaryotic promoters with poor −35 elements (1, 27).

Promoter distal sequences are required for *sed* promoter function. The 58-bp fragment, encompassing nucleotide positions −34 to +24, displayed comparable promoter activity to that of the undeleted *sed* promoter. However, the fragment containing sequences from −34 to +7 failed to drive *cat* gene expression. Sequences downstream of +1 appear to be required for *sed* promoter function. To test this possibility, we created a series of *sed-lac* hybrid promoters by fusing the 5′ portion of the *sed* promoter with the 3′ portion of the *S. aureus* lactose operon (*lac*) promoter sequences (26). This approach permitted a study of the role of the downstream sequence without interrupting the distance between the transcription and the translation start sites. Then the *cat* expression levels of these hybrid constructs were analyzed (Fig. 3A). The results showed that when the *sed* sequences from −12 to +24 were replaced with *lac* sequences (pZS2746, sed/lac), the promoter function was abolished. This hybrid promoter still had the −35 of *sed*, the −10 sequences are the same in the two promoters, and the spacer region was very similar to that of the *sed* (16 identical bases out of 17). Thus, the lack of promoter activity was most likely the result of the substitution of *lac* for *sed* sequences distal to the +1 position. When the *sed* sequence was extended to +7 and +14 to generate pZS2771 (M11) and pZS2781 (M12), these hybrid promoters had only barely detectable activity. To determine the 3′ boundary of the *sed* promoter, additional hybrid promoters (pZS2832, pZS2833, pZS2834, and pZS2835) were generated by replacing the last eight, six, four, and two bases of the 58-bp fragment with the corresponding *lac* promoter sequences. The CAT assay results indicated that the eight-base replacement (+17 to +24) abolished the *sed* promoter function, but a six-base replacement (+19 to +24) resulted in a 2.5-fold increase in promoter activity, relative to that obtained with the wild-type 58-bp sequence. The CAT values for pZS2834 (four-base replacement, +21 to +24) and pZS2835 (two-base replacement, +23 to +24) had essentially no effect on *sed* promoter activity. To further define the 3′ terminus of the *sed* promoter, plasmid pZS2852 was created by replacing the last seven bases of the 58-bp *sed* promoter (T_{+18} to C_{+24}) with *lac* sequences. This hybrid promoter was active, although it resulted in a CAT expression level lower than obtained with the 58-bp *sed* pro-

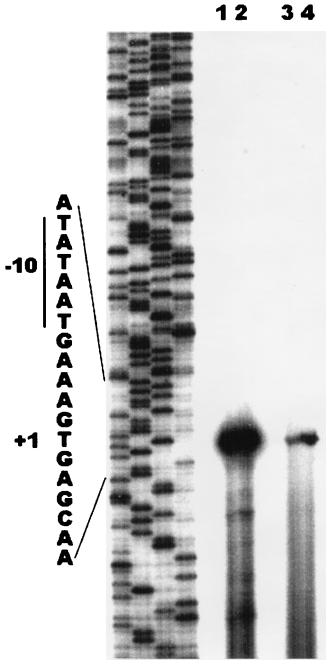

FIG. 2. Primer extension mapping of the transcription start site. RNA was isolated from *S. aureus* ISP546 (lanes 1 and 3) and KSI2054 (lanes 2 and 4) harboring plasmid pZS2704 (lanes 3 and 4) or pZS2743 (lanes 1 and 2) at an optical density at 540 nm of 1.2. Reverse transcription was carried out using the primer cat3, which anneals to a site near the 5′ end of the *cat* gene. The samples were loaded into adjacent lanes of a sequencing gel, and the lanes between the two sets were left blank. The sequence ladder was generated using the same primer and pZS2704 as the template. The transcription start site is indicated as +1.

Plasmid	Sequence	Cat value
A. *sed-lac* fusion promoters		
pZS2704 (wt)	atgaaatggatcaaatatattgatataatgaaagtgagcaagttggatagattgcggc	10.4± 1.6
pZS2746(sed/lac)	----------ttataatttaaacgtaatcaaattgaaagccttttc	0.0 ± 0.0
pZS2771(M11)	----------aaattgaaagccttttc	0.3 ± 0.5
pZS2781(M12)	----------aagccttttc	0.9 ± 0.1
pZS2832(M21)	----------gccttttc	0.0 ± 0.0
pZS2833(M22)	----------cttttc	25.6 ± 3.1
pZS2834(M23)	----------tttc	10.3 ± 1.2
pZS2835(M24)	----------tc	11.7 ± 1.3
pZS2852(M27)	----------cctttc	6.7 ± 1.6
pZS2743(lac/sed)	ttgatttattgtttgtttatgt----------	33.4 ± 6.0
pZS2785(lac)	ttgatttattgtttgtttatgtttataatttaaacgtaatcaaattgaaagccttttc	22.4 ± 4.0
B. *sed* mutant promoters		
pZS2704 (wt)	atgaaatggatcaaatatattgatataatgaaagtgagcaagttggatagattgcggc	10.4 ± 1.6
pZS2688(Δ+8 to +24)	----------	0.0 ± 0.0
pZS2719(M1)	-----g----------	6.2 ± 1.7
pZS2782(Δ-34 to -30)	----------	0.8 ± 0.2
pZS2783(Δ-34 to -25)	----------	0.5 ± 0.3
pZS2784 (Δ-34 to -25)	----------	0.9 ± 0.1
pZS2836(M25)	t---c----------	50.6 ± 4.2
pZS2837(M25')	t---c----------	48.8 ± 8.3
pZS2853(Δ+19 to +24)	----------	23.7 ± 3.4
pZS2720(M2)	----------c----------	7.0 ± 1.9
pZS2721(M3)	----------g----------	7.3 ± 2.0
pZS2748(M7)	----------t----------	8.5 ± 1.5
pZS2749(M8)	----------c----------	7.6 ± 1.1
pZS2750(M9)	----------t----------	25.8 ± 2.1
pZS2751(M10)	----------c----------	21.6 ± 6.9
pZS2772(M13)	----------t----------	0.3 ± 0.5
pZS2773(M14)	-------t----------	10.1 ± 0.6
pZS2774(M15)	----------t----------	30.7 ± 3.2
pZS2775(M16)	----------t----------	14.5 ± 1.1
pZS2874(M30)	----------t----------	2.9 ± 1.0
PZS2875(M31)	----------c----------	3.0 ± 1.1
pZS2876(M32)	----------c----------	2.6 ± 1.1
pZS2877(M33)	----------tt----------	0.0 ± 0.0

FIG. 3. (A) The promoter sequences of *sed* and *lac* are presented in the top and bottom lines, respectively. The −35, −10, and +1 sequences of the *sed* promoter are underlined. For the hybrid promoters, dashed lines indicate *sed* sequences and the indicated bases signify replacement by *lac* promoter sequences. (B) Activity of mutant forms of the *sed* promoter. Dashed lines indicate *sed* sequences, bases indicate base substitutions, and blank spaces indicate deleted bases. CAT assays were conducted with the wild-type (wt) strain KSI2054 harboring the corresponding plasmids. CAT values are expressed as nanomoles of chloramphenicol acetylated per minute per milligram (dry weight) of cells. Average values from three independent determinations and standard deviations are given.

moter. Thus, the downstream boundary of the *sed* promoter is nucleotide position +17.

To confirm the activity of the −34 to +18 fragment, plasmid pZS2853 was created by PCR amplification of the −34 to +18 DNA fragment, which was then cloned into pMH109 to create a promoter which differed from that of pZS2852 in that it lacked the +19 to +24 *lac* sequences. The CAT assay results showed that the promoter strength of this 52-bp fragment was comparable to that of pZS2833 (six-base replacement) and was about 2.3-fold higher than that of the 58-bp *sed* promoter (Fig. 3B).

Contribution of the −35 sequences to *sed* promoter function. To determine the role of the putative −35 sequence in *sed* promoter function, the 5′ end of the 58-bp fragment was either deleted or mutated. The promoter activity of the mutants were measured (Fig. 3B). Deletion of the first 5, 10, and 15 bases (pZS2782, pZS2783, and pZS2784) destroyed the promoter function completely. Two mutant promoters (pZS2836 and pZS2837) with the putative −35 element altered to give a consensus sequence (TTGACA) were created by oligonucleotide-mediated site-directed mutagenesis (34). The promoter of pZS2836 (M25) consists of bases −34 to +24, whereas pZS2837 (M25′) includes only bases −34 to +7. The promoter strengths of both pZS2836 and pZS2837 were dramatically increased. The *cat* expression level was increased almost fivefold for pZS2836 relative to that exhibited by the 58-bp wild-type sequence. The level of expression was 4.7-fold higher for pZS2837. More significantly, the activity exhibited by the pZS2837 insert indicated that the requirement for the +8 to +18 sequence to produce an active promoter was eliminated with the introduction of the improved −35 element sequence. However, introduction of the consensus −35 sequences caused instability of the plasmids. When pZS2836 and pZS2837 were transduced into *S. aureus* KSI2054, the majority of the transductants did not produce chloramphenicol resistance. Subsequent sequence analysis indicated that deletions occurred either within the promoter region or the *cat* structure gene. In addition, a strain bearing a point mutation (A_{-29} to T;

pZS2719) within the −35 sequence was isolated. This mutation resulted in a 1.7-fold reduction in promoter activity.

Effect of point mutations on *sed* promoter function. To determine the role of bases outside of the −35 and −10 elements, single or double mutations were introduced into the *sed* promoter region (Fig. 3B). Mutations within the spacer region, A_{-22} to C and A_{-21} to G (pZS2720 and pZS2721), led to moderate reductions in *cat* expression levels, whereas a G_{-27}-to-T transversion mutation (pZS2773) had no effect on *sed* promoter activity. Base substitutions near the −10 sequence had profound effects on *sed* promoter function. When G_{-13} was changed to T (pZS2772), the *cat* expression level was reduced to barely detectable level. Mutations G_{-5} to T and C (pZS2874 and pZS2875) and A_{-4} to T (pZS2876) led to a three- to fourfold reduction in *cat* expression level. Double mutations $A_{-4}A_{-3}$ to TT (pZS2877) abolished *sed* promoter activity. Base changes adjacent to the transcriptional start site also influenced the promoter strength. When G_{-1} or G_{+2} was changed to T (pZS2774 or pZS2775), an increase in *cat* expression resulted. Base substitutions within the downstream region, G_{+8} to T and C (pZS2750 and pZS2751), increased expression about twofold.

The rate of transcription from constitutive promoters depends on the sequences of the −35 and −10 promoter elements (12). However, bases outside of these core promoter elements are also important for promoter function. Footprint analyses have revealed protected regions as far upstream as −70 and downstream to +20 in *E. coli* promoters (10, 11, 20). An AT-rich upstream region can enhance promoter activity by facilitating promoter bending and wrapping around the RNA polymerase. Mahmood and Khan demonstrated that the upstream region between −93 and −58 was required for *seb* expression but that sequences distal to +1 were not required (21). Bases upstream from the −35 element are not required for *sed* expression, but sequences downstream of +1 are essential for *sed* promoter function. Thus, the promoters for these two staphylococcal enterotoxin genes are fundamentally different. Hybrid promoter analysis revealed that the region downstream from +1 to +18 was required for *sed* promoter activity, and a base substitution (G_{+8} to T or C) within this region also affected the promoter strength. Studies have shown that the downstream regions of certain *E. coli* promoters can influence the transcription rate by affecting promoter clearance (13). During this process, sigma factor is released from RNA polymerase holoenzyme which allows the elongation steps to proceed. Expression of *sed* does not occur when the +1 distal sequences are derived from the staphylococcal *lac* promoter but is restored when sequences from the *seb* promoter are used (Zhang and Stewart, unpublished data). This is interesting because these sequences are not required for expression from the *seb* promoter.

When base substitutions were introduced into the *sed* promoter to create a consensus −35 element, the *cat* expression level was increased approximately fivefold and the downstream region requirement was eliminated. Based on these data, the role of the downstream region may be to compensate for the poor −35 sequence. This could be accomplished by DNA bending to enable a better interaction with RNA polymerase, rather than just playing a rate-limiting role in the late steps of transcription initiation.

A required $T_{-14}G_{-13}$ dinucleotide was found one base upstream of the Pribnow box sequence. In studies of *E. coli* promoters, it has been proposed that RNA polymerase makes an additional contact with the TG dinucleotide during transcription initiation, which lowers the thermal energy required for the strand separation (1, 27). Compilation analysis of *Bacillus subtilis* promoter sequences also indicated that this dinucleotide pair is conserved in extended −10 element promoter sequences from this organism (15). The TG motif was also shown to be a conserved feature in 26% of *Lactobacillus* promoters and is an important determinant of promoter strength (23).

In addition to the TG dinucleotide, bases immediately downstream of the Pribnow box also play a vital role in *sed* promoter function. Base substitutions within this region (G_{-5} to T or C, A_{-4} to T, $A_{-3}A_{-4}$ to TT) either abolished the promoter activity or reduced it dramatically. These findings suggest that not only the $T_{-14}G_{-13}$ dinucleotide but those bases downstream from the −10 element are involved in transcription initiation, possibly by facilitating strand separation and open complex formation.

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