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cse15, *cse60*, and *csk22* Are New Members of Mother-Cell-Specific Sporulation Regulons in *Bacillus subtilis*

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We report on the characterization of three new transcription units expressed during sporulation in *Bacillus subtilis*. Two of the units, *cse15* and *cse60*, were mapped at about 123° and 62° on the genetic map, respectively. Their transcription commenced around h 2 of sporulation and showed an absolute requirement for σ^E. Maximal expression of both *cse15* and *cse60* further depended on the DNA-binding protein SpoIIID. Primer extension results revealed −10 and −35 sequences upstream of the *cse15* and *cse60* coding sequences very similar to those utilized by σ^E-containing RNA polymerase. Alignment of these and other regulatory regions led to a revised consensus sequence for σ^E-dependent promoters. A third transcriptional unit, designated *csk22*, was localized at approximately 173° on the chromosome. Transcription of *csk22* was activated at h 4 of sporulation, required the late mother-cell regulator σ^K, and was repressed by the GerE protein. Sequences in the *csk22* promoter region were similar to those of other σ^K-dependent promoters. The *cse60* locus was deduced to encode an acidic product of only 60 residues. A 37.6-kDa protein apparently encoded by *cse15* was weakly related to the heavy chain of myosins, as well as to other myosin-like proteins, and is predicted to contain a central, 100 residue-long coiled-coil domain. Finally, *csk22* is inferred to encode a 18.2-kDa hydrophobic product with five possible membrane-spanning helices, which could function as a transporter.

A polar division that occurs early in the process of endospore formation in *Bacillus subtilis* creates two sporangial compartments of very different proportions. The larger one, called mother cell, and the smaller, or forespore, each of which carry a complete copy of the chromosome, define distinct cell types, exhibiting differential programs of gene expression and having distinct developmental destinies (20, 45). The prespore is engulfed by the mother cell and ultimately develops into the spore. The engulfed prespore, or forespore, is confined within a double membrane that separates it from the mother-cell cytoplasm. Soon after engulfment is concluded, the forespore is enclosed by a germ cell wall, closely opposed to its outer membrane. During later stages, the forespore is first encircled by a layer of modified peptidoglycan known as the cortex and finally wrapped into a thick coat formed by the assembly of different polypeptides (20). Lastly, after a period of maturation, the mother cell lyses, releasing the mature spore into the medium.

The processes just described are largely controlled by a cascade of four sporulation-specific RNA polymerase sigma factors, which appear in the sequence σ^F, σ^E, σ^G, σ^K (20, 45, 48). The activation of the different sigma factors is itself strongly coupled to specific points in the morphological sequence (45). σ^E, for example, is synthesized in a pro-form in the predivisional cell (32, 42), and its proteolytic activation is somehow coupled to the septation process and to the activity of σ^F, a prespore-specific sigma factor (35, 44). Conversion of pro-σ^E to its active form, σ^E, which occurs exclusively in the mother cell, inaugurates the program of gene expression in this compartment (19, 35, 44, 68). The σ^E form of RNA polymerase ($E\sigma^E$) directs the transcription of a heterogeneous group of genes controlling functions as diverse as engulfment (23, 56, 58, 62), germination (49, 70), cortex synthesis (2, 9, 14, 43, 52, 54, 60, 64), and coat morphogenesis (3, 18, 28, 49, 54, 60, 70), as well as genes that may assist in the control of the metabolic state of the mother cell (7, 8, 26, 37). $E\sigma^E$ also drives expression of at least two regulatory genes, *spoIIID* (40, 62, 68) and *sigK* (39, 68). Some genes in the σ^E regulon are negatively regulated by SpoIIID, whereas transcription of others is enhanced by SpoIIID (25). The *sigK* gene, which encodes an inactive pro-σ^K protein (13, 46), is initially transcribed by an σ^E-containing RNA polymerase, in conjunction with SpoIIID, and later by σ^K-associated polymerase (38, 39, 68). Another function of σ^E is the transcompartmental, postengulfment activation of the late forespore-specific sigma factor σ^G (12, 34, 36, 47, 61). An indirect effect of this is the triggering of a signal transduction pathway emanating from the forespore, which results in the conversion of pro-σ^K to active σ^K in the mother cell (13, 46). Among the first genes transcribed by $E\sigma^K$ is *gerE*, encoding a small DNA-binding protein (11, 68, 69). In combination with $E\sigma^K$, GerE controls the expression of the last temporal class of mother-cell gene expression, defined by genes such as *cotB* and *cotC*, encoding structural components of the spore coat (18, 69). The phenotype of *sigK* mutants indicates that $E\sigma^K$ activity is required for the late steps in cortex and coat synthesis, a process initiated under σ^E control (see above), and for spore maturation (15). In addition, $E\sigma^K$ controls the lysis of the mother cell (41) and possibly some aspects of the germination of the spore (11, 18, 67).

Although much is known about the transcriptional regulation of the process, the morphological changes that take place during sporulation cannot yet be adequately described. Hoping to gain insight into the morphogenetic mechanisms of sporulation, particularly those processes (delineated above) involving σ^E functions, an expression-based screening for σ^E-dependent genes was devised (2, 3, 28). The scheme involved probing a random library of *lacZ* transcriptional fusions in a strain carrying the gene encoding σ^E under the control of an inducible promoter. In this communication we describe the identi-

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TABLE 1. *B. subtilis* strains

Strain	Genotype and phenotype	Source or reference
MB24	*trpC2 metC3* Spo$^+$	Laboratory stock; P. Piggot
EU8702	*trpC2 pheA1* Δ*sigE*::*erm* (pDG180) Pm^r Em^r	Laboratory stock
EU101	Δ*sigE*::*erm chr*::Tn*917*ΩHU160::*P spac-spoIIGB* Pm^r Em^r	Laboratory stock
PY222	CU1050(pTV17) SPβ Tc^r Em^r	Laboratory stock; P. Youngman
EUX9451	EU101(SPβ*cse15-lacZ*) Em^r Pm^r Cm^r	This study
EUX9452	PY222(pTVE15) Cm^r	This study
EUX9543	*trpC2 metC3* (SPβ*cse15-lacZ*) Em^r Cm^r	This study
EUX9544	*trpC2 metC3* Δ*cse15*::*km* Km^r	This study
AH688	EU101(SPβ*cse60-lacZ*) Em^r Pm^r Cm^r	This study
AH689	PY222(pTVE60) Cm^r	This study
AH696	EU101(SPβ*csk22-lacZ*) Em^r Pm^r Cm^r	This study
AH697	PY22(pTVK22) Cm^r	This study
AH899	*trpC2 metC3* Δ*cse60*::*km* Km^r	This study
AH912	*trpC2 metC3* Δ*csk22*::*nm* Nm^r	This study
AH929	*trpC2 metC3* (SPβ*cse60-lacZ*) Em^r Cm^r	This study
AH937	*trpC2 metC3* (SPβ*csek22-lacZ*) Em^r Cm^r	This study

fication, cloning, mapping, and regulation of three transcriptional units whose activation in the mother-cell sporangial chamber directly or indirectly depends on σ^E activity.

MATERIALS AND METHODS

Bacterial strains, media, and general techniques. The *B. subtilis* strains used in the present work are listed in Table 1 or otherwise described in the text. Strains AH98, AH99, AH1175, AH100, and AH95 were the sources of the Δ*spoIIGB*::*sp*, Δ*spoIIID*::*sp*, Δ*spoIIIG*::*sp*, Δ*spoIVCB*::*sp*, and *gerE36* mutations. They are described elsewhere (27). The phenotypic analysis of the *cse15*, *cse60*, and *csk22* loci was performed in congenic derivatives of the Spo$^+$ strain MB24 (Table 1). *Escherichia coli* DH5α (Bethesda Research Laboratories [BRL]) was the recipient for plasmid construction and amplification, and its derivative DH5αF′IQ (BRL) hosted phages M13mp18 and M13mp19 and their descendants. Culture media, chromogenic substrates for β-galactosidase, isopropyl-β-D-thiogalactopyranoside (IPTG), and antibiotics were used as described before (28).

Cloning of the *cse15*, *cse60*, and *csk22* loci. The general strategy devised to detect and clone *B. subtilis* genes whose expression is under the control of the sporulation transcription factor σ^E has been described previously (2, 3, 28). A random library of *B. subtilis* chromosomal DNA partially digested with *Sau*3AI was made in the temperate bacteriophage SPβ (provided by P. Zuber), and a high-titer lysate prepared from a *c2* strain was used to transduce cells of the σ^E conditional mutant EU101 (2) (Table 1) to Cm^r. The Cm^r transductants were then screened for an inducer-dependent Lac$^+$ phenotype. The SPβ*cse15*, SPβ*cse60*, and SPβ*csk22* lysogens EUX9451, AH688, and AH696, carrying fusions to the *cse15*, *cse60*, and *csk22* genes, respectively, were identified in this way. It should be emphasized that induction of σ^E production during growth of EU101 restores sporulation to some extent to the otherwise *spo* mutant. The insert present in the different prophages was cloned in plasmid pTV17 (3) by in vivo recombination using donor chromosomal DNAs from strains EUX9451, AH688, and AH696 (Table 1). The pTV17 derivatives carrying the whole *B. subtilis* insert in the original SPβ*cse15*, SPβ*cse60*, or SPβ*csk22* phage were accordingly named pTVE15 (strain EUX9452), pTVE60 (strain AH689), and pTVK22 (strain AH697).

An insert of approximately 2 kb was released from pTVE15 by digestion with *Sal*I and inserted at the corresponding site of pUK19 (provided by W. Haldenwang), thereby forming pUK19E15 (Fig. 1A). Plasmid pUK19E15 was utilized as a vehicle in a chromosome-walking step aimed at obtaining sequences downstream of the library fusion site. First, the plasmid was transferred to the region of homology in the host chromosome by transformation of competent cells of a wild-type strain to Km^r. Integration occurred by a Campbell-type mechanism (single reciprocal crossover). Second, total genomic DNA was prepared from the resulting strain, digested to completion with *Kpn*I, and religated at low DNA concentration, and the ligation mix was used to transform *E. coli* to Ap^r. Finally, plasmids were prepared from different Ap^r colonies, characterized, and shown to contain an additional short piece of 5 kb downstream of the *Sau*3AI site at the left end of the initial insert (Fig. 1A). A plasmid with this structure was chosen and named pE15K (Fig. 1A). Plasmid pE15K carries the intact *cse15* open

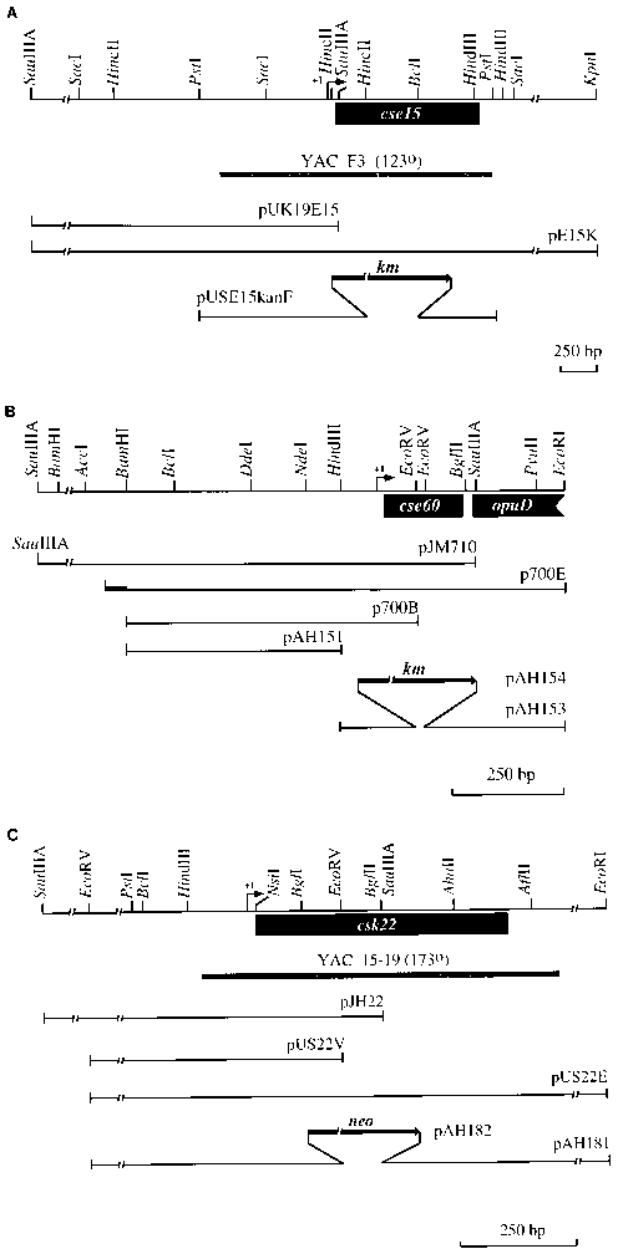


FIG. 1. *cse15*, *cse60*, and *csk22* regions of the chromosome. The positions and lengths of the *cse15*, *cse60*, and *csk22* ORFs are indicated by the solid boxes below a partial restriction map of the corresponding regions. Inserts in the indicated plasmids are represented by the lines below the partial restriction map. The positions of promoter sequences and transcription initiation sites are shown by arrows and a +1 sign. (A) *cse15* region of the *B. subtilis* chromosome, initially cloned as a *lacZ* fusion to the *Sau*3AI site represented at the beginning of the *cse15* ORF. Plasmid pE15K produced the template to replace wild-type sequences delimited by *Hin*cII and *Hin*dIII within the *cse15* ORF with a Km^r determinant. Primers OM14 and OM15 produced a 850-bp PCR product (the boundaries of which are shown by a thick line) on the YAC F3 template (1), a result that places *cse15* at about 123° on the genetic map. (B) The *cse60* ORF is followed by a *Sau*3AI site which was the original library fusion site. A Km^r cassette, inserted between the two *Eco*RV sites in pAH153 to create plasmid pAH154, was used to disrupt the *cse60* ORF. The thick line to the left of the first *Bam*HI in p700E indicates that this DNA is not contiguous with the rest of the insert. Also represented is the 3′ end of the *opuD* gene. (C) *csk22* locus initially identified as a fusion to the *Sau*3AI site in the middle of a 321-codon ORF. An Nm^r cassette was inserted between the *Eco*RV and *Bgl*II sites in pAH181, as indicated, and the resulting plasmid, pAH182, used to create a Δ*csk22*::*nm* insertion and deletion mutant. Primers OM89 and OM91 generated a 770-bp PCR fragment (thick line) with YAC templates 15-19 (1), placing the *csk22* locus in the central region of the insert in this clone. Only restriction sites present in *B. subtilis* chromosomal DNA are represented.

reading frame (ORF), preceded by about 2 kb of DNA and followed by some 5 kb of additional sequences.

Digestion of pTV60 with *Sal*I released a fragment of approximately 3 kb which was inserted at the corresponding site of pJM103 (51). In the resulting plasmid, named pJM710, the *lacZ*-proximal *Sau*3AI site (at the 3′ end of the insert) is closer to the *cm* gene of the vector. Digestion of pJM710 with *Bam*HI released a fragment of about 2.2 kb internal to the original insert. Religation of the vector yielded plasmid p700B, in which two noncontiguous sections of DNA were brought together, as a consequence of deletion of the intervening *Bam*HI fragment. These are a short segment of about 200 bp between the *lacZ*-distal *Sau*3AI site and the rightmost *Bam*HI site of the pJM710 insert and a piece of about 800 bp from the second *Bam*HI site to the *lacZ* junction end. Plasmid p700B was utilized in a chromosome-walking step as described above for pUK19E, except that the chromosomal DNA was cut with *Eco*RI. All the plasmids resulting from this maneuver were shown to contain an additional short piece of 200 bp downstream of the *Sau*3AI/*Bgl*II sites close to the putative terminator of the *cse60* gene (Fig. 1B). One was chosen and named p700E (Fig. 1B). Plasmid p700E carries the intact *cse60* ORF, preceded by about 400 bp of DNA and followed by a DNA segment of 200 bp.

Incubation of pTVK22 with *Sal*I liberated a chromosomal insert of approximately 4 kb, which was inserted at the *Sal*I site of pJH101 (21). This inactivated the tetracycline resistance marker of the vector (21). The resulting plasmid, pJH22 (whose insert is represented in Fig. 1C), carries the σ^E-dependent transcriptional unit in the same orientation as the interrupted tetracycline resistance gene. To create a plasmid carrying an intact *csk22* locus, we first constructed plasmid pUS22V by inserting a 850-bp *Eco*RV fragment obtained from pJH22 into the *Sma*I site of pUS19 (4). In pUS22V the rightmost end of the *Eco*RV insert, carrying *csk22* sequences, is close to the *sp* gene in the vector. *B. subtilis* Sp^r transformants resulting from Campbell integration of pUS22V were used in chromosome walking (as described above), after digestion of the recombinant chromosome with *Eco*RI. Plasmid pUS22E resulted from this cross. It carries a genomic insert of 1.6 kb (encompassing an intact *csk22* transcriptional unit), 700 bp of which consists of newly cloned sequences downstream of the leftmost *Eco*RV site in pUS22V (Fig. 1C).

Insertional inactivation of *cse15*, *cse60*, and *csk22*. The inactivation of the *cse15* locus was accomplished in two steps. First, a 2-kb *Pst*I fragment encompassing the *cse15* ORF was released from pE15K and introduced into the *Pst*I site of pUS19, yielding pUSE15. Second, pUSE15 was digested with *Hin*cII and *Bcl*I, and a 1.5-kb Km^r cassette (65) was released from pKD102 (obtained from W. Haldenwang) with *Sma*I and *Bam*HI, inserted between the vector arms. Linearization of the resulting plasmid, pUSE15kanF, with *Sca*I produced a transforming molecule capable of converting MB24 to Km^r (but not to Sp^r), consequently originating a *cse15* insertion and deletion mutant, which we named EUX9544 (Fig. 1A and Table 1).

Disruption of the *cse60* locus was accomplished by replacing the 22-bp *Eco*RV fragment internal to the predicted *cse60* ORF in pAH153 (Fig. 1B) with a *km* resistance cassette (65), obtained as a *Sma*I fragment from pKD102 (pAH153 carries the *Hin*dIII-to-*Eco*RI segment from p700E between the corresponding sites of pUS19). The resulting plasmid, pAH154, was subsequently linearized with *Sca*I and used to transform *B. subtilis* MB24 to Km^r. Strain AH899 (Sp^s) (Table 1) results from this cross, which produced the recombinant chromosome depicted in Fig. 1B, the structure of which was verified by PCR. Plasmid pAH151 is a pUS19 derivative carrying the indicated fragment obtained from p700E between the corresponding sites of the vector (Fig. 1B).

A fragment of about 1.6 kb was obtained by digesting pUS22E with *Eco*RI and *Sal*I. The *Sal*I site in pUS22E (Fig. 1C) originates from the pUS19 polylinker, as the result of a subcloning step (see above). This fragment was inserted between the *Eco*RI and *Sal*I sites of pUS19 to create pAH181. Plasmid pAH181 was then digested with *Bgl*II and *Eco*RV to release a 400-bp fragment internal to the *csk22* coding sequence, which was replaced by an Nm^r gene obtained by digestion of pBEST501 (31) with *Bam*HI and *Sma*I. The resulting plasmid was called pAH182. To create a *csk22* insertional mutant, plasmid pAH182 was linearized at its unique *Sca*I site and the DNA was used to convert *B. subtilis* MB24 (Table 1) to Nm^r. Transformants (Nm^r/Sp^s) were obtained as the result of a double crossover event that transferred the in vitro-generated insertional mutation into the *csk22* locus. The presence of the *nm* gene in the postulated configuration with respect to *csk22* sequences was confirmed by colony PCR (27) of several of the recombinant clones, one of which was selected and named AH912 (Table 1).

Physical mapping of the *cse15*, *cse60*, and *csk22* loci. Total yeast genomic DNA was prepared from the *B. subtilis* YAC library strains (1), and samples (approximately 50 ng) were used in PCR with *cse15*, *cse60*, or *csk22* sequence-specific primers. The locations of the different loci on the *B. subtilis* physical map were then inferred from the approximate location of the YAC clone that produced a positive result. The primer pairs used were as follows: for *cse15*, OM14, 5′-d(GCTGTTCAGTATTTGCATATTG)-3′, and OM15, 5′-GGTGACATCTTCAACGGATA-3′; for the *cse60* gene, OM57, 5′-GTATACAAACTTGCTGTGGCTG-3′, and OM60, 5′-ATATCTATCAGCTGCTCCTCCG-3′; and for *csk22*, OM89, 5′-d(CCATCGCTAACAAACGTTCCAG)-3′, and OM91, 5′-d(GCAAGGA**AT**TCTTTTTCTTTATTGTAT)-3′ (a TG pair in the original sequence was replaced by the boldfaced **AT** doublet, a change that creates an *Eco*RI site). The expected sizes of PCR products were 850, 857, and 770 bp for *cse15*, *cse60*, and *csk22*, respectively. Reaction mixes, containing 0.05 U of *Taq* DNA polymerase (Boehringer Mannheim) per μl, were subjected to 30 cycles of incubation at 94°C for 60 s, 55°C for 90 s, and 72°C for 120 s.

Growth, sporulation, and germination of *B. subtilis*. Growth and sporulation of *B. subtilis* cultures were essentially as previously described (28). The extent of sporulation was estimated by measuring the titer of heat, chloroform, or lysozyme CFU per ml (27). Purification of spores on Renografin gradients, as well as plate assays for germination using 2,3,5-triphenyltetrazolium chloride (Tzm), and the extraction and electrophoretic resolution of spore coat polypeptides were as described earlier (28).

Isolation and primer extension analysis of mRNA. Total *B. subtilis* RNA was prepared at the indicated times, from cells grown in DSM medium as previously described (2, 3). Primer extension reactions were carried out with primers complementary to the indicated positions in the sequences shown in Fig. 2: a 29-mer, 5′-GCTTATGGCACCTTGAAAGCTCGGATTTT-3′, is complementary to the region of the *cse15* sequence delimited by positions 315 to 343 (Fig. 2A); the 18-mer 5′-GTCTGGGTCGCACGCCGC-3′ hybridizes to nucleotides 695 to 712 of the *cse60* sequence (Fig. 2B); finally, the 19-mer 5′-CGGCTGGTATCCGATGGAC-3′ anneals to nucleotides 362 to 380 of the *csk22* sequence (Fig. 2C). Sequencing and primer extension reactions were as described elsewhere (2, 3). Appropriate double-stranded templates were used, so that the initiation nucleotide in the mRNA species could be directly determined from the autoradiographs.

Enzyme assays. The activity of β-galactosidase was determined with the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and expressed in Miller units as described elsewhere (28).

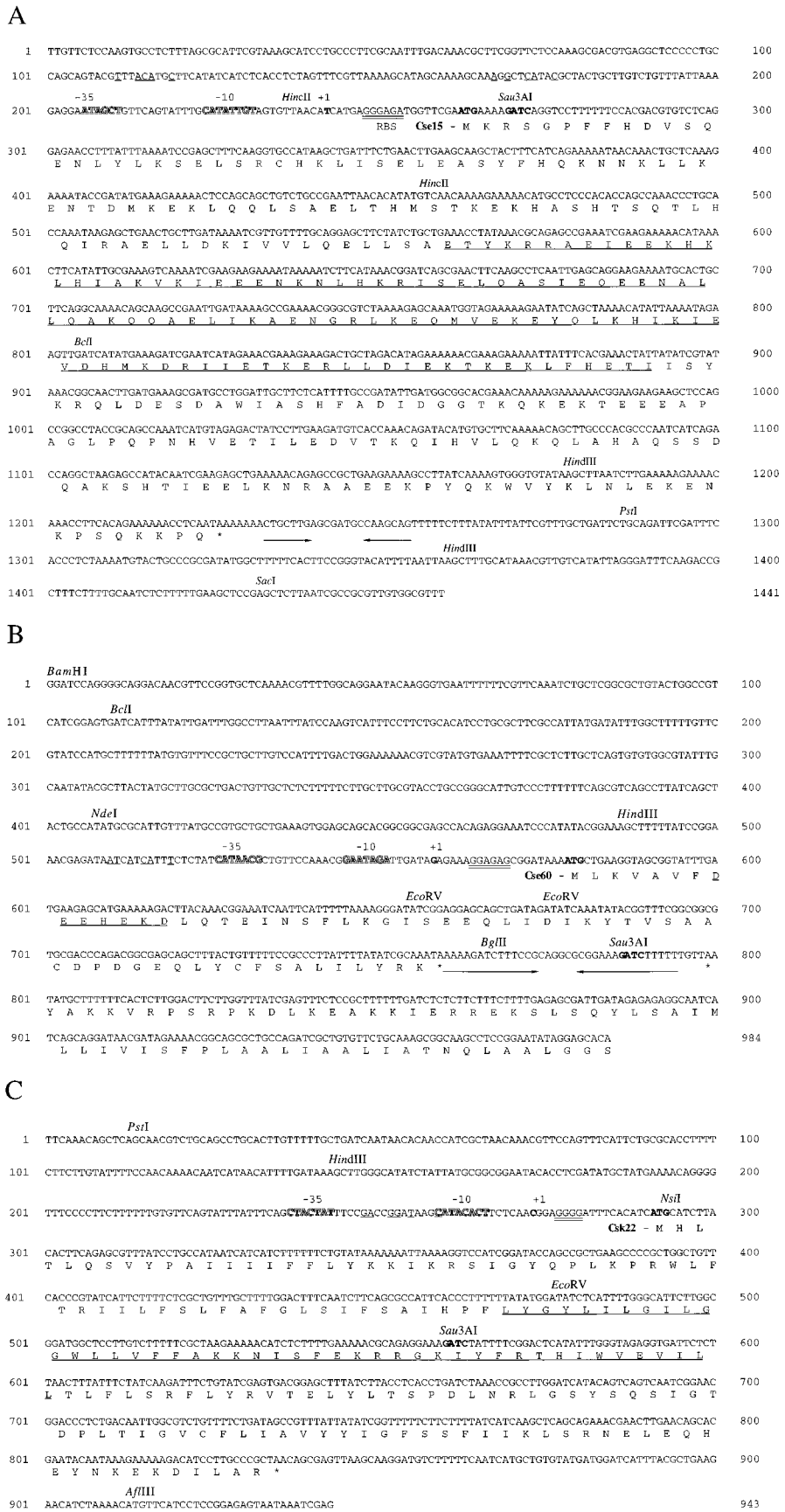
DNA sequencing. The sequence across the library fusion site in plasmids pTVE15, pTVE60, and pTVK22 was determined by using the Sequenase (U.S. Biochemical Corporation) protocol for double-stranded DNA and the *lacZ* primer, 5′-GCGCTATGTCGTAGGGTCT-3′. Inserts in M13 vectors encompassing the *cse15*, *cse60*, and *csk22* regions were sequenced in both strands by the dideoxy chain termination method using Sequenase. In general, sequences were analyzed by using the programs in the Genetics Computer Group (GCG) package (24); the PAIRCOIL program (5) was used on line after the Massachusetts Institute of Technology server was accessed.

Nucleotide sequence accession numbers. The nucleotide sequences shown in Fig. 2A (*cse15*), 2B (*cse60*), and 2C (*csk22*) were deposited in GenBank and assigned accession numbers U72072, U70041, and U70042, respectively.

RESULTS

Cloning and nucleotide sequence analysis of the *cse15*, *cse60*, and *csk22* regions of the chromosome. The chromosomal inserts in phages SPβE15, SPβE60, and SPβK22 were first cloned into pTV17 by in vivo homologous recombination (2, 3, 28). The sequence obtained across the library junction in plasmids pTVE15, pTVE60, and pTVK22 did not correspond to any previously known sporulation genes, and the clones were studied further. The integrative plasmids pUK19E15, pJM710, and pJH22, derived from the corresponding pTV17 prototypes (see Materials and Methods), were then used in a chromosome-walking step aimed at cloning sequences downstream of the library junction point. This approach produced plasmids pE15K (Fig. 1A), p700E (Fig. 1B), and pK22E (Fig. 1C), carrying the intact *cse15*, *cse60*, or *csk22* loci. Sequence analysis of these plasmids (and of appropriate DNA fragments derived from them) revealed the following features.

***cse15*.** The library junction site was found to interrupt a 321-codon ORF, predicted to encode a 37.6-kDa polypeptide with a pI of 7.37. The ORF was preceded by a ribosome binding site (5′-GGGAGA-3′, positions 248 to 253), separated from the initiation codon (ATG), by 8 bp (Fig. 2A). A possible transcription terminator, which includes a stretch of U's, is detected 5 bp downstream of the *cse15* ocher codon (positions 1225 to 1227). The sequence of the deduced Cse15 polypeptide was compared to other sequences in the databases by using the FASTA algorithm. Cse15 was found to share a 20 to 22% degree of sequence identity to the heavy chain of myosin and to a group of myosin-related proteins from a variety of sources (not shown). Some examples are the yeast NIP80 (GenBank entry P33420), NUF1 (P32380), and STU1 proteins (P38198), the last one being a component of the mitotic spindle (50); human myosin heavy chain (P13535); fruit fly paramyosin (P35415) and non-muscle myosin heavy chain (Q99323); and


A
B
C
HincII
Sau3AI
RBS
Cse15
BclI
HindIII
PstI
SacI
BamHI
NdeI
-35
-10
+1
Cse60
EcoRV
BglII
Csk22
NsiI
AflIII

sea urchin kinesin heavy chain (P35978). In all cases, the region of overlap was 200 to 280 residues. Other proteins related to Cse15 found in the FASTA search were either myosins (from different species) or related proteins, although the identity levels were lower (in the 16 to 19% range; not shown). Because all the proteins pulled in the FASTA search are known or presumed to be coiled coils, we analyzed the Cse15 primary structure using the PAIRCOIL program (5). This program detects a heptad repeat of the type $(abcdef)_n$, in which positions a and d are occupied by hydrophobic residues. This pattern is typical of coiled-coil proteins (5). Interestingly, the PAIRCOIL program unequivocally predicted a coiled-coil domain for the central 100 amino acids of the Cse15 protein (Fig. 1A), strongly suggesting that the *B. subtilis* protein consists of a central coiled-coil domain, flanked by N- and C-terminal extensions of about 100 residues.

cse60. A ribosome binding site sequence (5′-GGAGAG-3′, positions 564 to 569) (Fig. 2B), followed by an ATG codon 8 bp downstream, defines the translation initiation signals of a 60-codon ORF. The putative product of this ORF, tentatively designated Cse60, is acidic (pI 3.4) and has a predicted mass of 6.9 kDa. The *cse60* gene is just downstream and convergently oriented with respect to the recently characterized *opuD* gene (33). An inverted repeat sequence found between the two genes could form a stem-loop structure possibly acting as a factor-independent bifunctional transcription terminator (Fig. 2B). The sequence of the *cse60* gene was independently determined as part of the *opuD* locus (33) (accession number U50082) and ends at the *Hin*dIII site just upstream of the −35 region of the *cse60* promoter (Fig. 2B). For the region of overlap, the two sequences agree completely. Cse60 is not noticeably similar to any other protein in the databases. However, we note that the presence of at least one lysine residue in close proximity to several acidic residues (DEE-EKD) (Fig. 2B) is reminiscent of similar motifs in some well-characterized lysine-rich coat proteins, such as CotW (DEEKE) (67).

csk22. The library junction site is in the middle of an ORF capable of encoding a 182-residue polypeptide of 21.3 kDa. This ORF is preceded by a plausible ribosome binding site (5′-GGGG-3′, positions 277 to 280). No convincing terminator-like structures were seen at the 3′ end of *csk22* (Fig. 2C). The Kyte and Doolittle algorithm of hydropathy analysis (GCG package, window of 11) revealed the hydrophobic nature of Csk22, in which five helices with the potential to span the membrane are predicted (not shown). A central domain of Csk22, including transmembrane helix 3 and a hydrophilic region between hydrophobic helices 3 and 4 (underlined in Fig. 1C), shows an identity level of about 33% (some 63% similarity) to a segment of a putative chloride channel from several species (not shown). Among the sequences that can be aligned to Csk22 are residues 332 to 376 of a human and murine voltage-gated chloride channel (GenBank accession numbers X78520 and X78874) (6). Interestingly, this segment in several transporters may be phosphorylated to regulate the activity of the channel (6).

Map positions on the *cse15*, *cse60*, and *csk22* loci. YAC clone F3, but not any other in the YAC library (1), produced a positive signal of 850 bp with the *cse15*-specific primers. The annealing positions of the primers relative to the *cse15* ORF, as well as the size and extent of the PCR product, are represented in Fig. 1A. Clone F3 carries an insert of about 60 kb, centered at approximately 123° on the *B. subtilis* genetic map, to the left of the *pts* marker, which is carried by both clones 15-115 and F3. The 123° coordinate is assigned to the *cse15* locus. The *cse60*-specific primers produced a faint product only with YAC clone 10-201, centered at about 62°, a position that we only tentatively attribute to the locus. Finally, the *csk22* primers gave a strong and unique band of 770 bp with the 15-19 template and not any other YAC template (gray line in Fig. 1C). This places the *csk22* locus in the center region of the insert in YAC 15-19, at about 173°. Other markers assigned to YAC 15-19 are *terC*, *gltA*, *odhA*/*B*, and *sspC* (1).

Insertional inactivation of *cse15*, *cse60*, and *csk22*. Spores produced by the *cse15*, *cse60*, and *csk22* insertion and deletion mutants EUX9544, AH899, and AH912 (Table 1) were purified through Renografin gradients (28). These, as well as samples of sporulating cultures collected at T_8 or T_{18} (that is 8 or 18 h after the onset of sporulation), were subjected to heat, chloroform, and lysozyme resistance tests, as well as to plate germination tests. In addition, we examined the pattern of electrophoretically resolved proteins extracted from the coats of *cse15* and *cse60* mutant spores. In all the functions investigated, the resistant CFU titer was always close to that of the wild-type strain (1×10^8 to 2×10^8), and no difference in the germination rate or coat protein profile was detected. In the case of certain *cot* genes, even mutations that cause no detectable decrease in spore resistance properties can cause a significant coat morphological alteration (27). For this reason, purified spores of the three mutants (and of a *cse15*/*cse60* double mutant) were also analyzed by electron microscopy. No significant distinction between wild-type and the mutant spores was found. In addition to the insertion and deletion mutants referred to above, strains bearing Campbell insertions of plasmids p700E, p700B, and pAH151 for the *cse60* locus (Fig. 1B) or pUS22V for the *csk22* locus (Fig. 1C) were also studied and found not to bear any distinctive phenotype.

The *cse15* and *cse60* promoters are utilized by $E\sigma^E$, whereas P_{csk22} is used by $E\sigma^K$. The start points of *cse15*, *cse60*, and *csk22* transcription in vivo were investigated in a time course primer extension experiment with samples of total RNA isolated from sporulating cultures of a wild-type strain. The *cse15*- and *cse60*-specific messages started to accumulate at h 2 of sporulation and persisted until at least h 6 (Fig. 3A and B). In contrast, synthesis of the major *csk22* message was detected only from h 4 of sporulation onward (Fig. 3C). The sizes of the observed extension products suggested that (i) transcription of *cse15* starts at a T residue at position 242 in the sequence in Fig. 2A, (ii) transcription of *cse60* initiates at a G residue at position 558 of the *cse60* sequence (Fig. 2B), and lastly, (iii) transcription of *csk22* begins with residue C-273 (Fig. 2C). Inspection of the DNA sequences centered 10 and 35 bp upstream of these apparent start sites revealed sequences highly similar to those of promoters utilized by the σ^E-containing RNA polymerase (*cse15* and *cse60*; Fig. 5) (3, 30, 48) or σ^K RNA polymerase (*csk22*) (55). A second, shorter extension product is seen for *csk22*. Accumulation of this product closely

FIG. 2. Nucleotide sequence and translation of the *cse15*, *cse60*, and *csk22* loci. The figure depicts the nucleotide sequence of the nontranscribed strand of a stretch of DNA containing the *cse15* (A), *cse60* (B), and *csk22* (C) loci. Promoter −10 and −35 sequences and the transcription initiation nucleotide (+1) are indicated. Potential ribosome binding sites (RBS) are doubly underlined, and initiation codons are boldfaced. Also boldfaced are the *Sau*3AI library fusion sites; other landmark restriction sites are represented above the DNA sequences. Asterisks indicate ocher stop codons, and arrows below the sequence indicate regions of dyad symmetry proposed to function as transcriptional terminators. The primary structure of the Cse15, Cse60, and Csk22 polypeptides was predicted from the corresponding DNA sequences and is shown in single-letter code. Residues that participate in motifs described in the text are underlined.

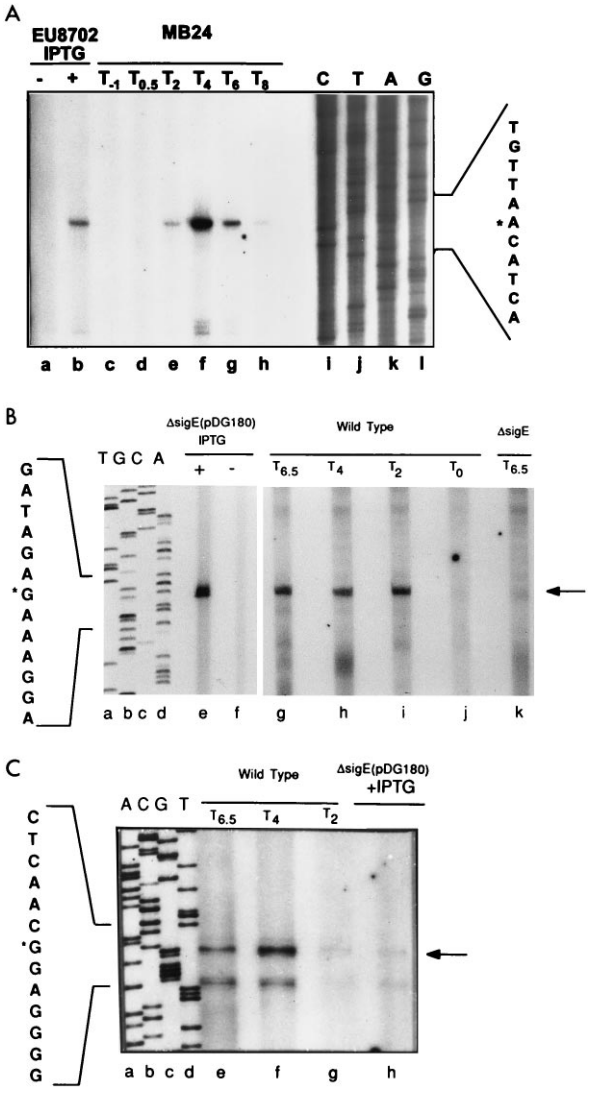


FIG. 3. Mapping of the 5′ terminus of the *cse15*, *cse60*, and *csk22* messages. Shown are autoradiographs of *cse15* (A)-, *cse60* (B)-, and *csk22* (C)-specific primer extension products after electrophoretic resolution in polyacrylamide gels. Oligonucleotides complementary to the 5′ end of the nontranscribed strand of each cistron were used to direct cDNA synthesis of total RNA purified from cultures of a wild-type Spo$^+$ strain in sporulation medium (DSM) at the indicated times (in hours) before or after T_0, defined as the onset of sporulation. In the case of *cse15* and *cse60*, RNA prepared from mid-log-phase cultures of strain EU8702 [Δ*sigE*::*erm* (pDG180)] with (+) or without (−) 1 mM IPTG added to the culture was also analyzed. Δ*sigE*::*erm* represents a strain carrying a deletion of the wild-type allele of the *sigE* gene (lane k in panel B). Plasmid pDG180 (a gift from P. Stragier) carries an allele of *sigE* encoding an active version of σ^E under the control of an IPTG-inducible promoter. In the case of *csk22*, only RNA prepared from a mid-log-phase culture of EU8702 grown in the presence of IPTG was analyzed (lane h in panel C). The same oligonucleotide used for the primer extension experiments was also used in parallel to prime sequencing reactions (labeled C, A, T, or G) from appropriate templates, so that the initiating nucleotide in the nontranscribed strand (asterisk) could be determined by direct inspection of the ladder. Arrows indicate the positions of the *cse15*-, *cse60*-, and *csk22*-specific primer extension products.

parallels that of the longer product, suggesting a similar regulatory pattern. Its deduced 5′ end lies just downstream of the proposed *csk22* ribosome binding site, and no canonical −10 and −35 sequences could be found upstream of this position. This suggests that the shorter product may result from processing of the longer one or be due to premature pausing of the transcriptase. Induction of σ^E production in vegetative cells was found to result in the accumulation of products similar in size to the *cse15*- and *cse60*-specific products detected during development (Fig. 3A and B). The *cse15* and *cse60* promoters are therefore utilized by Eσ^E, whereas *csk22* is transcribed by Eσ^K.

The *cse15*, *cse60*, and *csk22* promoters are subject to complex transcriptional regulation. As discussed above, the analysis of the 5′ end of the *cse15* and *cse60* messages led to the idea that both promoters were utilized by the σ^E-containing RNA polymerase. Expression from the *cse15* and *cse60* promoters was measured in wild-type cells lysogenic for SPβ*cse15-lacZ* or SPβ*cse60-lacZ*. Transcription of *cse15* and *cse60* was found to start at around h 2 of sporulation, consistent with the primer extension results (Fig. 4A and B). To examine the effects of various *spo* mutations on expression from both promoters, the SPβ*cse15-lacZ* and SPβ*cse60-lacZ* phages were used to lysogenize several sporulation mutants, the resulting strains were induced to sporulate, and β-galactosidase production was monitored (Fig. 4). Mutations in the *sigE* gene, encoding σ^E, totally abolished transcription of *cse15* and *cse60*, which was essentially unaffected by mutation of the *sigG* (encoding σ^G) (34, 47, 61) or *sigK* locus (encoding σ^K) (38, 39) (Fig. 4A). *cse15* and *cse60* transcription was found to be decreased in a *spoIIID* mutant (Fig. 4A and B). Putative binding sites for SpoIIID, based on the consensus sequence WWRRA CAR-Y (W stands for A or T, R stands for purine, and Y stands for pyrimidine) (25), were found in the sequences of the *cse15* and *cse60* promoters (Fig. 2A and B). Thus, in addition to σ^E dependency, a second level of control is imposed upon the *cse15* and *cse60* promoters, which in wild-type cells are further activated by SpoIIID.

Expression of a *csk22-lacZ* fusion in SPβ was also monitored in the wild-type strain MB24 and in various *spo* mutants. A time course measurement of β-galactosidase accumulation in strain MB24 revealed that the *csk22* promoter was activated at about h 4 of sporulation (Fig. 4C), a result in excellent agreement with the primer extension analysis (see above). Also, in complete support of the notion that *csk22* expression would be driven by the σ^K form of RNA polymerase (see above), we found no activity of the *csk22-lacZ* fusion in a *sigE* or *sigK* mutant (Fig. 4C). Interestingly, the *gerE36* mutation caused increased expression of *csk22-lacZ*, with unaltered timing or kinetics (Fig. 4C). Unlike in wild-type cells, in which *csk22-lacZ* expression peaks roughly 2 h after activation of the *csk22* promoter, in GerE$^-$ cells strong expression of the fusion persisted for at least 2 h more (Fig. 4C). A sequence that conforms to the proposed GerE binding site (RWWTRGGY--YY [the abbreviations are as above]) (66, 69) and that could be involved in repression is underlined in the *csk22* regulatory region (Fig. 2C). As clearly suggested by these results, GerE may act to repress transcription from the *csk22* promoter by Eσ^K.

DISCUSSION

Three *B. subtilis* transcription units designated *cse15*, *cse60*, and *csk22* were found in a search for mother-cell-specific promoters and are the subject of the present communication. The *cse15*, *cse60*, and *csk22* loci, mapped at about 123°, 62°, and 173° in the *B. subtilis* chromosome, do not correspond to any other previously characterized sporulation-associated loci, a conclusion that we also base on the sequencing data presented here (Fig. 2). We found all three units to be expressed exclusively during sporulation, albeit at different times during the process. Using both transcriptional fusions to the *lacZ* gene (Fig. 4) and primer extension analysis (Fig. 3), we showed that

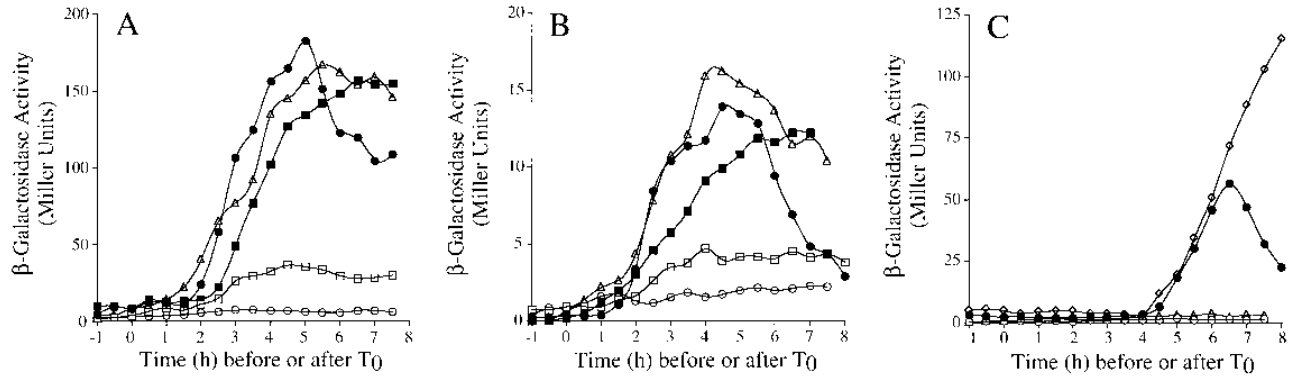


FIG. 4. Analysis of *cse15-*, *cse60-*, and *csk22-lacZ* expression. The formation of β-galactosidase was monitored throughout growth and sporulation of Spo$^+$ and congenic Spo$^-$ lysogens of SPβ*cse15-lacZ* (A), SPβ*cse60-lacZ* (B), and SPβ*csk22-lacZ* (C). The Spo$^+$ lysogens EUX9543, AH929, and AH937 (Table 1) are derivatives of strain MB24 (closed circles). Lysogens bearing various developmental mutations are indicated as follows: Δ*spoIIGB*::*sp*, open circles; ΔspoIIID::*sp*, open squares; ΔspoIIIG::*sp*, closed squares; Δ*spoIVCB*::*sp*, open triangles; *gerE36*, diamonds. The level of β-galactosidase activity measured in the Δ*spoIIGB*::*sp* strain was very close to our normal background levels (28). Samples were taken at 30-min intervals during growth and sporulation and assayed for β-galactosidase activity. The end of the exponential phase of growth, or T_0, is defined as the onset of sporulation.

transcription from the *cse15* and *cse60* promoters commenced around h 2 of sporulation whereas utilization of the *csk22* promoter was not detected before the 4th h. The temporal pattern of expression detected for *cse15* and *cse60* is that observed for many σ^E-controlled genes (20, 44, 47). σ^E is activated in the mother cell (19, 34, 43, 44) and is functional in this sporangial compartment until at least the end of the engulfment sequence (20, 45, 48). The *cse15* and *cse60* promoters are therefore active during the same period of development as σ^E (Fig. 4A and B), and deletion of the σ^E-encoding gene, *sigE* (32, 42), totally prevents *cse15* and *cse60* expression (Fig. 4A and B). In total agreement with these results, the −10 and −35 hexamers found upstream of the *cse15* and *cse60* transcriptional start sites are very similar to analogous sequences known to govern promoter utilization by Eσ^E (20, 48). Moreover, expression of σ^E in vegetative cells resulted in transcription of *cse15* and *cse60* (Fig. 3A and B). The promoter sequences of the *cse15* and *cse60* loci, together with those of the recently described *mmg* and *ssd* operons, bring to 27 the number of sequences included in the alignment of σ^E-dependent promoters shown in Fig. 5. This alignment extends and revises previous analyses, which have included 11 or 15 promoters (3, 30). The −35 sequences of the *cotE* P_2 and *sigK* promoters differ significantly from the consensus, and they were not included in the group of typical σ^E promoters (Fig. 5). The updated −35 and −10 consensus sequences for σ^E-dependent promoters are ATAHTT and CATAYAHT, respectively (H represents A, C, or T, and Y denotes C or T) (Fig. 5), separated by 13 to 14 bases, as indicated. Mutational analysis and suppression studies showed that highly conserved base pairs in the −35 region of the *spoIID* and *spoIIID* promoters or the −10 region of the *spoIID*, *spoIIID*, and *cotE* P_1 promoters were important for promoter utilization by Eσ^E (17, 53, 62, 63).

The SpoIIID protein modulates the expression of specific genes within the σ^E regulon (25, 68). Because transcription of the *spoIIID* gene is in part autoregulated (40, 59), genes that show a requirement for SpoIIID for transcription tend to belong to the latest temporal class of σ^E-dependent gene expression. Transcription of both the *cse15* and the *cse60* genes is enhanced by SpoIIID. Presumably, the cellular concentration of the Cse15 and Cse60 products peaks towards the end of the σ^E window of activity. Both proteins could be functionally recruited near the end of the engulfment sequence, shortly after which the first visible signs of cortex and coat synthesis are noticed. (The link between transcriptional regulation and morphogenesis is a loose one, however: the products of some σ^E-controlled genes, although present in the mother cell from an early stage, are needed only much later in the process, after σ^K becomes active.) The function of the Cse15 and Cse60 polypeptides is unknown. They could participate in either cortex or coat formation. Because the FASTA searches suggested similarity to coiled-coil proteins of the myosin type, we analyzed the Cse15 sequence with the PAIRCOIL program (5). The results clearly suggest that a central region of Cse15, between residues 100 and 200, consists of a coiled-coil domain. A two-stranded, parallel coiled coil, a structural motif that could be shared by Cse15, has been associated with several fibrous proteins, including myosin (5). A matrix formed by fibrillar proteins would be compatible with the ultrastructural data available for the coat (e.g., the long lamellae of the inner coat or the striated appearance of the outer coat; see for example references 27, 67, and 70), although this has never been shown to be the case. The fact that no differences were observed between wild-type and *cse15* mutant spores in the pattern of electrophoretically resolved soluble coat proteins could imply that Cse15 is either a very minor component or otherwise part of an insoluble, cross-linked fraction (67). We also suspect that Cse60 may be a coat component, a suggestion that we base on the pattern of lysines and acidic residues found near its N-terminal moiety.

Soon after completion of engulfment (around h 4 of sporulation), active σ^K makes its appearance in the mother cell (12, 13, 39, 46, 68). The temporal pattern and dependency of *csk22* expression are compatible with σ^K control. Moreover, the −35 and −10 regions of the *csk22* promoter conform well to those known to signal promoter utilization by Eσ^K, tACt (the consensus is mACm, where m represents A or C) and CATA---Tt (the consensus sequence is CATA---Ta), respectively (see reference 55 for a recent compilation). Transcription of the *csk22* gene is turned off about 2 h after its activation by Eσ^K, an event that requires the *gerE* gene product (Fig. 5C). Thus, *csk22* belongs to the group of genes in the first class of σ^K-dependent gene expression that are repressed by GerE. This group includes *cotA* (57), *spoVF* (15), and *sigK* (38, 39, 68, 69). *csk22* is transiently expressed under σ^K control, perhaps indicating that its product is required only soon after activation of the σ^K regulon. Because of its hydrophobic nature, Csk22 could be inserted into the forespore outer membrane just prior to the

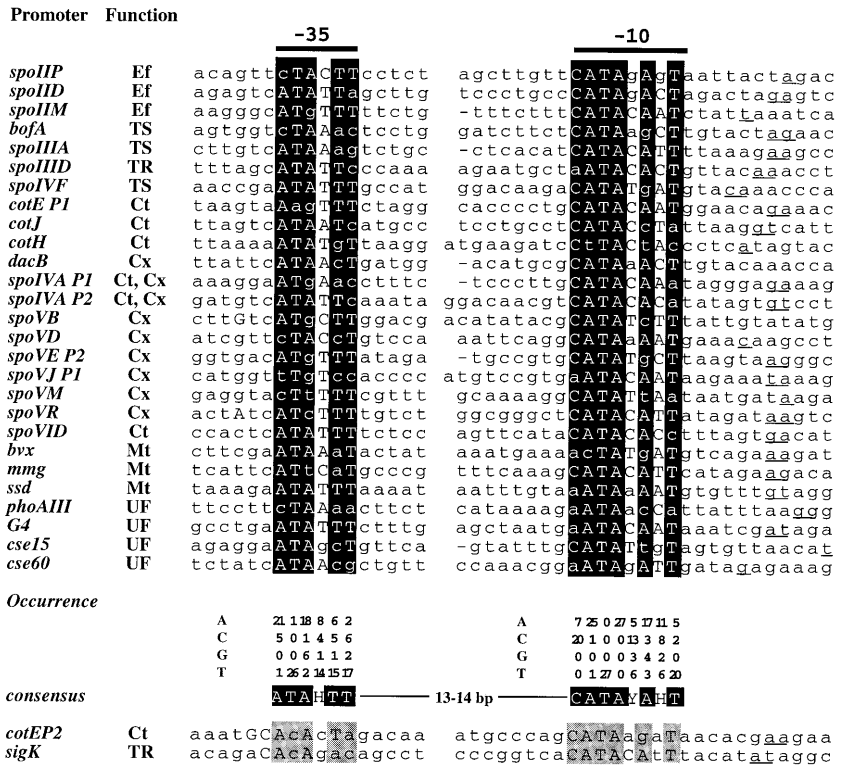


FIG. 5. Alignment of σ^E-dependent promoters. The sequences encompassing the −10 and −35 regions and the transcriptional start site of σ^E-dependent promoters were aligned, and a consensus sequence was derived, in extension of earlier work (3, 30). The alignment includes the *cse15* and *cse60* promoter sequences described in the present work, as well as the sequences of the recently described *mmg* and *ssd* promoters (7, 8). Transcriptional initiation nucleotides (if known) are underlined. The incidence of different bases at each position of the −10 and −35 regions is indicated, on which basis the proposed consensual promoter sequence was derived. Invariant or highly conserved bases (60 to 100% of the cases) are indicated against a dark background, and bases that match the consensus sequence are uppercased. An H in the consensus sequence represents base A, C, or T, and a Y represents a pyrimidine. The function controlled by each promoter is indicated as follows: Ef, engulfment; TS, transcompartmental signaling; TR, transcriptional regulator; Ct, coat synthesis; Cx, cortex synthesis; Mt, metabolism; UF, unknown function. The sequence for each promoter was obtained from the following references or other sources: *spoIIP*, 23; *spoIID*, 56; *spoIIM*, 58; *bofA*, 30; *spoIIIA*, 29; *spoIIID*, 16; *spoIVF*, 12; *cotE*, 68; *cotJ*, 28; *cotH*, 49; *dacB*, 9; *spoIVA*, 54 and 60; *spoVB*, 52; *spoVD*, 14; *spoVE*, 64; *spoVJ*, 22; *spoVM*, 43; *spoVR*, 2; *spoVID*, 3; *bvx*, 26 and 37; *mmg*, 8; *ssd*, 7; *phoAIII*, 10; G4, 53; *sigK*, 39.

initiation of cortex and coat biosynthesis or in the mother-cell membrane, where it could function as part of a transport system.

By screening for mother-cell-specific promoters, we expected to identify genes with functions in morphogenetic processes controlled mainly from this sporangial chamber. A similar screen had already resulted in the identification of loci involved in cortex and coat synthesis (2, 3, 28). The fact that no obvious phenotype could be associated with disruption of the *cse15*, *cse60*, or *csk22* loci suggests that their function is either redundant or dispensable under our laboratory conditions.

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