

Identification and Characterization of Sporulation-Dependent Promoters Upstream of the Enterotoxin Gene (*cpe*) of *Clostridium perfringens*

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Received 8 August 1997/Accepted 19 October 1997

Three promoter sites (P1, P2, and P3) responsible for the sporulation-associated synthesis of *Clostridium perfringens* enterotoxin, a common cause of food poisoning in humans and animals, were identified. Nested and internal deletions of the *cpe* promoter region were made to narrow down the location of promoter elements. To measure the effects of the deletions on the expression of *cpe*, translational fusions containing the promoter deletions were made with the *gusA* gene of *Escherichia coli*, which codes for β -glucuronidase; *E. coli*-*C. perfringens* shuttle vectors carrying the fusions were introduced into *C. perfringens* by electroporation. In addition, in vitro transcription assays were performed with the *cpe* promoter region as the DNA template for extracts made from sporulating cells. DNA sequences upstream of P1 were similar to consensus SigK-dependent promoters, while P2 and P3 were similar to consensus SigE-dependent promoters. SigE and SigK are sporulation-associated sigma factors known to be active in the mother cell compartment of sporulating cells of *Bacillus subtilis*, the same compartment in which enterotoxin is synthesized in *C. perfringens*.

Clostridium perfringens is a common source of food poisoning in humans, being responsible for 10% of the outbreaks of known etiology in the United States alone (17). After ingestion of contaminated food containing vegetative cells, food poisoning symptoms are caused by the production of a potent enterotoxin (CPE) protein made by sporulating cells in the gastrointestinal tract (20). The enterotoxin binds to receptors on the surface of the brush border membranes of intestinal epithelial cells (18, 27). The receptor-CPE complex then seems to form a pore, leading to the loss of solutes and eventual cell death (19).

Sporulation and enterotoxin synthesis are genetically and temporally linked. Duncan (9) performed a kinetic analysis of sporulation and enterotoxin synthesis during the growth and sporulation cycles of *C. perfringens* NCTC 8798 and showed that entry into the stationary phase (i.e., onset of sporulation) and enterotoxin synthesis are closely linked. In 1972, Duncan et al. (10) provided genetic evidence linking sporulation and CPE synthesis in CPE-positive (CPE⁺) *C. perfringens* NCTC 8798. Mutants blocked at stage 0 of sporulation were unable to express CPE, while mutants blocked at stages III, IV, and V continued to do so, although in reduced amounts (10). This pattern of CPE expression indicates that CPE synthesis is dependent on one or more functions that are also needed to reach stage I or II of the sporulation cycle. Electron microscopy and immunological labeling studies of thin sections of sporulating cells indicate that the vast majority of CPE protein is made in the mother cell compartment, rather than in the forespore (24). This result implies compartment-specific regulation of *cpe* expression. However, CPE synthesis is probably not required to produce a normal spore, since many CPE-negative (CPE⁻) *C. perfringens* strains are capable of sporulating (21).

Enterotoxin expression is controlled at the level of transcription during the sporulation cycle (7, 22). When *C. perfringens* NCTC 10240 was grown in sporulation medium, only very low levels of *cpe* mRNA and no CPE protein could be detected in the exponential growth phase (0 to 4 h of growth) (22). However, the levels of both *cpe* mRNA and CPE protein rose rapidly and in synchrony about 1 h after the cells entered the stationary phase, indicating that the increase in CPE expression as cells initiate sporulation results from increased transcription of the *cpe* gene. In addition, Czeczulin et al. (7), using Northern blot analyses, recently showed that only sporulating cells and not vegetative cells produce the monocistronic, 1.6-kb *cpe* mRNA, confirming that *cpe* is regulated at the level of transcription during the sporulation cycle. When several natural CPE⁻ strains of *C. perfringens* were transformed by electroporation with a *C. perfringens*-*Escherichia coli* shuttle vector carrying the *cpe* gene and ~0.4 kb of upstream DNA, the synthesis of CPE was still sporulation dependent, indicating that general sporulation-associated factors were responsible for *cpe* regulation (7).

When the regions immediately upstream of the *cpe* gene in six CPE⁺ *C. perfringens* strains were analyzed, a 45-bp insertion (located about 265 nucleotides upstream of the start of the *cpe* coding sequence) was detected in three of these strains; this result indicates that there are two populations of upstream regions in *cpe*⁺ isolates, designated types A and B (22). Translational fusions made between the *E. coli gusA* gene (encoding β -glucuronidase [16]) and DNA containing the putative *cpe* promoter region and N-terminal coding sequence from strains NCTC 8798 (promoter type A) and NCTC 10240 (promoter type B) were cloned in the *C. perfringens*-*E. coli* shuttle vector pJIR750 (22). When introduced into strain NCTC 8798 by electroporation, they showed the same pattern of induction as did the direct measurements of *cpe* mRNA and CPE protein in strain NCTC 10240 (as described above); this result indicates that all of the necessary regulatory elements were present on the promoter regions tested.

Although primer extension analysis identified several appar-

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TABLE 1. Bacterial strains used in this study

Strain	Genotype or phenotype	Source or reference
<i>E. coli</i>		
JM107	<i>endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lac-proAB)</i> (F' <i>traD36 proAB lacI^q lacZΔM15</i>)	29
INVαF'	F' <i>endA recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 gyrA96 relA1 φ80lacZΔM15 Δ(lacZYA-argF)U169 λ⁻</i>	Invitrogen
<i>C. perfringens</i>		
NCTC 10240	CPE ⁺	C. Duncan
NCTC 8798	CPE ⁺	C. Duncan
SM101	Derivative of NCTC 8798; high efficiency of electroporation	This study

ent mRNA 5' ends in the *cpe* promoter region (22), the promoters responsible for sporulation-dependent regulation have not yet been identified. In order to determine if these 5' ends represent actual promoter elements, we undertook a series of mutational and biochemical analyses of transcription in this region. The results of these studies are reported here.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 1. *E. coli* strains were cultured in Luria-Bertani medium. *C. perfringens* strains were grown routinely in PGY medium (22); for experiments involving sporulation, DSSM medium (22) was used. For anaerobic growth on plates, *C. perfringens* strains were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.). Anaerobic medium used to measure *cpe-gusA* expression in *C. perfringens* during sporulation was prepared by adding 75 ml of DSSM to 100-ml serum bottles fitted with butyl rubber stoppers. A needle was inserted through the stopper, and the bottles were autoclaved and then allowed to cool to room temperature in the Coy anaerobic chamber. Electroporation of *E. coli* and *C. perfringens* strains was carried out as previously described (22).

Electroporation experiments with *C. perfringens* NCTC 8798 typically resulted in 8 to 10 transformants per μg of DNA (23). Two chloramphenicol-resistant (Cm^r) transformants of strain NCTC 8798 containing the *E. coli*-*C. perfringens* shuttle vector pJIR750 were cured of the plasmid by growth in PGY medium lacking chloramphenicol. One of the Cm^r isolates, strain SM101, was then subjected to electroporation with plasmid pJIR750. It showed a 1,000-fold increase in electroporation efficiency, yielding ~1 × 10⁴ transformants/μg of DNA. Additionally, this strain showed no other changes in its growth, sporulation, or *cpe* regulatory characteristics (23).

Plasmid constructs and DNA manipulations. The plasmids used in this study are listed in Table 2. Nested deletions of the NCTC 10240 *cpe* promoter were made with PCR methods, except for plasmid pSM145 (see below). Primers containing an *SphI* cleavage site at the 5' end and primer OSM6, which contains an *XbaI* cleavage site and anneals to the N-terminal coding region of the *cpe* structural gene (22), were used to amplify the desired region with pSM100 (NCTC 10240 *cpe* promoter) as a template. The PCR products were ligated to vector pCRII (Invitrogen Corp.), and the plasmids were then digested with *SphI* and *XbaI*. The small fragments containing the deletions in the *cpe* promoter were ligated to the large fragment of pSM104, which had been digested with *SphI* and *XbaI*, thereby replacing the full-length *cpe* promoter with the deletion constructs fused to *gusA*. Plasmid pSM145 was made by partial digestion of pSM104 with *HindIII*. There is a *HindIII* site in the *cpe* promoter region at position -183 (Fig. 1), a second *HindIII* site in the polylinker region upstream of the *cpe* promoter inserts of pSM104 and pSM105, and a third *HindIII* site immediately downstream of the *gusA* gene. After partial digestion with *HindIII*, self-ligation, and transformation of *E. coli*, plasmid pSM145 was isolated.

Plasmid pSM170 was made in two steps. First, two *BglII* sites were introduced into pSM104 by sequential PCR mutagenesis (6) at positions -56 to -61 and -122 to -127 (Fig. 1). The resulting double mutant, pSM164, was tested for *cpe-gusA* expression during sporulation and showed very little difference from that seen with pSM104. After digestion with *BglII*, the large fragment was self-ligated, yielding pSM165, in which promoter sites P1 and P2 are deleted. Second, pSM165 was subjected to partial *HindIII* digestion to delete the region upstream of the *HindIII* site in the *cpe* promoter (Fig. 1), as described above for pSM145, creating pSM170, in which only the P3 region of the *cpe* promoter is intact. pSM183 was also made by sequential PCR mutagenesis. After *BglII* sites were introduced at positions -56 to -61 and -177 to -182, the intervening region was deleted by digestion with *BglII* and self-ligation. All mutations were confirmed by DNA sequence analysis (26).

Plasmids pSM154 and pSM155, used as templates for in vitro transcription,

TABLE 2. Plasmids used in this study

Plasmid	Parent	Relevant genotype (<i>cpe</i> promoter region present)	Source or reference
pCRII			Invitrogen
pBS-			Stratagene
pBluescript SK-			Stratagene
pSM100	pBluescript SK-	NCTC 10240 <i>cpe</i> promoter	22
pSM101	pBluescript SK-	NCTC 8798 <i>cpe</i> promoter	22
pJIR750			J. Rood (2)
pSM104	pJIR750	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>) (-481 to +41)	22
pSM123	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>) (-296 to +41)	This study
pSM127	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>) (-121 to +41)	This study
pSM129	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>) (-56 to +41)	This study
pSM145	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>) (-183 to +41)	This study
pSM151	pBS-	<i>cpe</i> terminator region	This study
pSM154	pBS-	NCTC 8798 <i>cpe</i> promoter; in vitro transcription vector	This study
pSM155	pBS-	NCTC 10240 <i>cpe</i> promoter; in vitro transcription vector	This study
pSM164	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>)	This study
pSM165	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>)	This study
pSM170	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>)	This study
pSM183	pSM104	φ(<i>cpe</i> _{NCTC 10240} - <i>gusA</i>)	This study

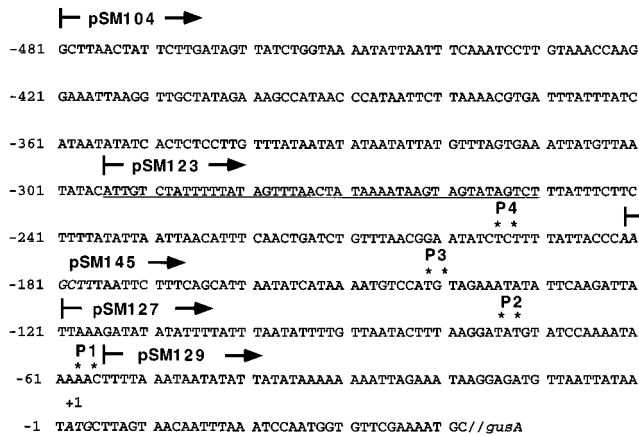


FIG. 1. Location of *cpe* promoter constructs (arrows) and putative promoter elements (asterisks) in the NCTC 10240 *cpe* type B promoter. The numbering is relative to the A of the initiator Met codon (italics) of the *cpe* structural gene. The *Hind*III site described in the text is also shown in italics. A 45-bp insertion present in type B *cpe* promoters but absent in type A promoters is underlined.

were made in several steps. First, a PCR product of 129 bp, containing 28 bp upstream of the thymidine in the *cpe* gene stop codon (TAA) and 100 bp downstream of the stop codon, was made with chromosomal DNA from strain NCTC 8798 and cloned in vector pCRII. This region contains the proposed *cpe* terminator (8). An *Eco*RI fragment containing the terminator region was then cloned in the *Eco*RI site of plasmid pBS- (Stratagene Corp.). A plasmid with the desired orientation, determined by sequencing, was named pSM151. Next, *Xba*I-*Pst*I fragments containing the *cpe* promoters from strains NCTC 10240 and NCTC 8798 were cut from plasmids pSM100 and pSM101, respectively, and cloned between the *Xba*I and *Pst*I sites of the polylinker in plasmid pSM151 to make pSM155 and pSM154, respectively.

Primer extension and β -glucuronidase assays. For experiments analyzing *cpe* promoter induction with primer extension, 300 ml of anaerobic DSSM was inoculated with 3 ml of an overnight culture of the appropriate strain. Growth was monitored by measuring the optical density of the culture at 600 nm. At various times, 10 ml of culture was removed, and total RNA was extracted from the sample with Trizol reagent (Gibco/BRL) as described previously (22). Primer extension was then carried out by previously described techniques for *C. perfringens* mRNA (22).

Measurements of β -glucuronidase activity in *C. perfringens* cells expressing *cpe-gusA* fusions were carried out as previously described (22).

In vitro transcription assays. Extracts from sporulating cells used in in vitro transcription assays were prepared as follows. Five hundred milliliters of anaerobic DSSM in a round-bottom flask was inoculated with 5 ml of an overnight culture of *C. perfringens* cells grown in PGY medium. Growth in DSSM was monitored by measuring the optical density of the culture at 600 nm. The cells were harvested by centrifugation 1.5 h after entry into the stationary phase ($T_{1.5}$) and washed once in buffer containing 20 mM NaHPO₄ (pH 7.0); the pellet was stored at -80°C . The frozen pellet was thawed and resuspended in buffer A (13), which contained 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 400 mg of phenylmethylsulfonyl fluoride per liter, 100 mM KCl, and 10% glycerol. The cells were then disrupted by sonication, and cell debris was removed by centrifugation at $15,000 \times g$ for 10 min at 4°C . The cell extract was then subjected to ultracentrifugation at $100,000 \times g$ for 1 h at 5°C . Solid ammonium sulfate was added to the supernatant from the centrifugation step just to saturation, and the protein precipitate was retrieved by centrifugation. The pellet was then resuspended in 1 to 2 ml of buffer A and dialyzed against 2 liters of buffer A at 4°C . After dialysis, the extracts were stored in 100- μ l aliquots at -80°C . Protein was measured with a protein assay kit (Bio-Rad Laboratories).

The in vitro transcription assays were carried out in microcentrifuge tubes with 40- μ l volumes containing (13) 40 mM Tris (pH 8.0), 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, ATP, GTP, and CTP at 125 μM each, 12.5 μM UTP, 12.5 μCi of [α -³²P]UTP, 1.5 to 2.2 μg of plasmid template DNA, and 5% glycerol. The reaction was started by the addition of 17 μg of protein from the cell extracts, the reaction mixture was incubated for 10 min at 37°C , and the reaction was stopped by phenol extraction and ethanol precipitation. mRNA was electrophoresed on 5% polyacrylamide-7.5 M urea gels along with RNA Century size markers synthesized according to the manufacturer's instructions (Ambion, Inc.).

RESULTS

Nested deletions of *cpe* promoter regions. In a previous report (22), primer extension experiments with *cpe* mRNA identified four potential 5' ends corresponding to mRNAs induced during sporulation. These apparent 5' termini were at positions -58 , -73 , -142 , and -196 (termed P1, P2, P3, and P4, respectively), relative to the start of translation of the *cpe* structural gene (Fig. 1). However, RNase T₂ protection experiments could only confirm the presence of P1, P2, and P3 (30). We sought to determine whether these 5' ends represent actual transcription start sites or are processing sites for mRNA originating from a single, upstream promoter. To address this question, we performed a series of deletion mutagenesis experiments with the *cpe* promoter region.

The locations of a series of nested deletions in the NCTC 10240 (type B) *cpe* promoters are shown in Fig. 1. Each of the *cpe-gusA* fusion constructs shown in Fig. 1 was cloned in the *C. perfringens-E. coli* shuttle vector pJIR750 and used to transform *C. perfringens* SM101 by electroporation (22). Strain SM101 is a derivative of NCTC 8798 selected for high transformation efficiency (see Materials and Methods). Strain NCTC 10240 cannot be transformed by electroporation (23), so expression from the NCTC 10240 *cpe* promoter was studied in the heterologous strain, SM101. The transformants were grown in sporulation medium DSSM, and samples were removed for measurements of β -glucuronidase activity during growth and the sporulation cycles as previously described (22).

The activities of *gusA* fusions containing nested deletions of the NCTC 10240 *cpe* promoter (type B) are shown in Fig. 2. In comparison to the longest *cpe* construct, pSM104, the nested deletions encompassing the region between pSM104 and pSM127 showed activity levels 80 to 100% that seen with pSM104. However, the level of expression for pSM129, which lacks all the potential transcription start sites, was only 20% the level shown by pSM104 (Fig. 2). With all of these *cpe-gusA* fusions, the induction of β -glucuronidase activity did not occur until the cells entered the stationary phase, consistent with our previous results (22). These results suggest that most *cpe* transcription in NCTC 10240 is derived from P1 and P2.

Separation of the *cpe* promoters from strain NCTC 10240.

The nested deletion experiments with the *cpe* promoter region described above are useful for locating a single promoter but are less effective in locating multiple promoter elements that may not have simple, additive levels of expression. In order to determine if P1, P2, P3, and P4 reflect independent promoters, we made deletions in the *cpe* promoter region to isolate the region corresponding to each potential start site and to test its expression during growth and sporulation. At the present time, we do not have sufficient information to differentiate potential promoters for P1 and P2, since they are separated by only 15 bp and their proposed -10 and -35 elements would overlap to a considerable extent (Fig. 1). Constructs that carry only P3 (pSM170), P4 (pSM183), or P1 and P2 are shown in Fig. 3A. As shown in Fig. 3B and C, the complete promoter region (in pSM104) and pSM127, which contains both P1 and P2 (Fig. 3A), had similar activities. pSM170, containing the P3 promoter alone, exhibited about 50% the stationary-phase-induced expression seen with pSM104. However, the construct containing P4 alone (pSM183) exhibited only $\sim 7\%$ the level of expression observed during the stationary phase with pSM104. These results indicate that P1 and P2 together provide nearly wild-type levels of expression, while P3 can also function as an independent promoter. The apparent P4 transcription start site is probably an artifact of primer extension.

Similar results were obtained with nested deletions of the

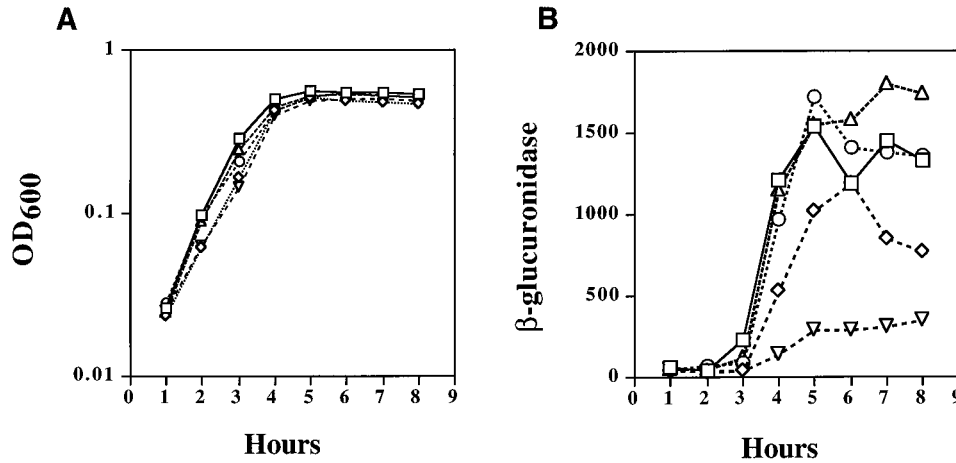


FIG. 2. Growth (A) and *cpe* expression (B) of the NCTC 10240 *cpe-gusA* fusion constructs shown in Fig. 1 and grown in DSSM sporulation medium. Symbols: □, pSM104; ○, pSM123; △, pSM145; ◇, pSM127; ▽, pSM129. OD₆₀₀, optical density at 600 nm.

type A *cpe* promoter from NCTC 8798 (30). In addition, a 1-kb region of DNA upstream of the *cpe* gene from NCTC 8798 (made by PCR amplification using previously published sequences [5]) fused to *gusA* resulted in no significant increase in expression from the clone containing only 439 bp (22, 30),

indicating there are probably no other promoters upstream of P3.

In vitro transcription of *cpe* promoters by use of extracts from sporulating cells. In order to confirm our results from the *cpe-gusA* fusions, we wished to obtain direct biochemical evi-

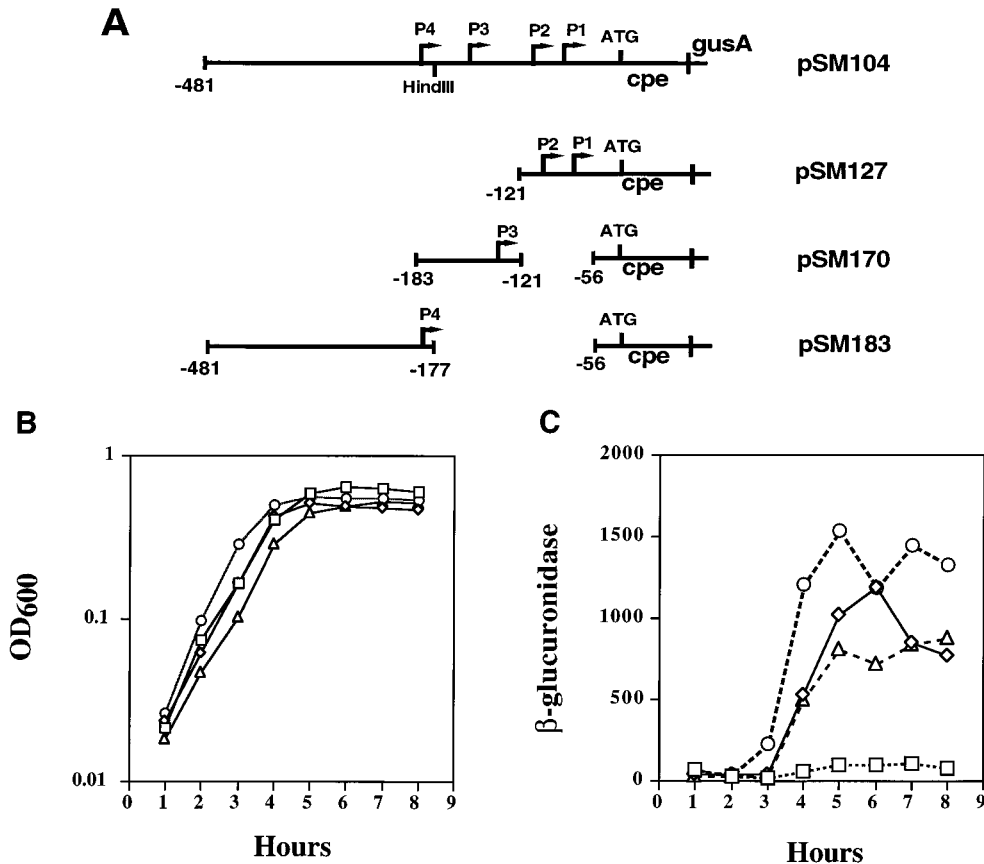


FIG. 3. (A) Schematic diagram showing the extent of internal deletions in the NCTC 10240 *cpe* promoter region. Growth (B) and *cpe* expression (C) of the NCTC 10240 *cpe-gusA* fusion constructs shown in Fig. 3A and grown in DSSM sporulation medium. Symbols: ○, pSM104, ◇, pSM127; △, pSM170; □, pSM183. OD₆₀₀, optical density at 600 nm.

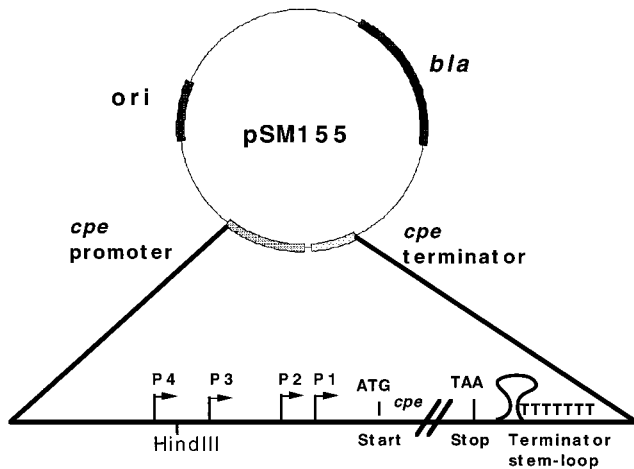


FIG. 4. Schematic diagram showing the relevant features of the plasmids used as DNA templates in in vitro transcription assays.

dence of transcription from the *cpe* promoters using extracts from sporulating cells. Presumably, sporulating cell extracts contain all the factors necessary for transcription of the *cpe* promoters. Previous reports of in vitro transcription assays with purified *C. perfringens* RNA polymerase and extracts from sporulating cells strongly indicated that linear DNA does not function efficiently as a template when *C. perfringens* RNA polymerase is used to initiate transcription (13, 22). To overcome this problem, we constructed specialized vectors to use as supercoiled templates for in vitro transcription assays. A schematic diagram illustrating the structures of these vectors is shown in Fig. 4. The relevant parts of the plasmids consist basically of the *cpe* promoter regions from NCTC 8798 (pSM154) and NCTC 10240 (pSM155) connected by a short polylinker region to the proposed terminator region of the *cpe* structural gene (8). The terminator region of *cpe* contains a potential stem-loop structure followed by seven consecutive thymidine residues (8), features common to rho-independent terminators in *Clostridium* and other bacteria (12–14). The plasmids were designed so that transcription initiating from the *cpe* promoters will terminate at the run of thymidines downstream of the stem-loop. The length of the transcript can then be used to estimate the point at which transcription was initiated in the *cpe* promoter region.

We obtained cell extracts from sporulating cells of strains NCTC 8798 and NCTC 10240 harvested at $T_{1.5}$ (see Materials and Methods) and used them to perform in vitro transcription assays with pSM154 and pSM155 as templates. The results are shown in Fig. 5. If transcription does terminate at the first thymidine in the run of seven thymidines in the *cpe* terminator region, we predict that a transcript initiating at P1 should be 228 bases long; those initiating at P2, P3, and P4 should be 243, 317, and 372 bases long, respectively. *cpe*-specific transcripts of 217 and 240 bases, estimated from the RNA size markers, were seen in Fig. 5, lanes 3 to 6. These corresponded quite well with the predicted sizes of transcripts from P1 and P2. With NCTC 8798 extracts, however, a transcript of 276 bases was observed with pSM154 as a template (Fig. 5, lane 3), but a transcript of 320 bases was seen with pSM155 as a template (lane 4). Since P3 is predicted to be 317 bases long, it most closely matches the upper band in lane 4 (320 bases). No additional transcripts corresponding in length to P3 were seen when NCTC 10240 extracts were used (Fig. 5, lanes 5 and 6), but transcripts longer than 400 bases were seen in these lanes. Transcripts of these

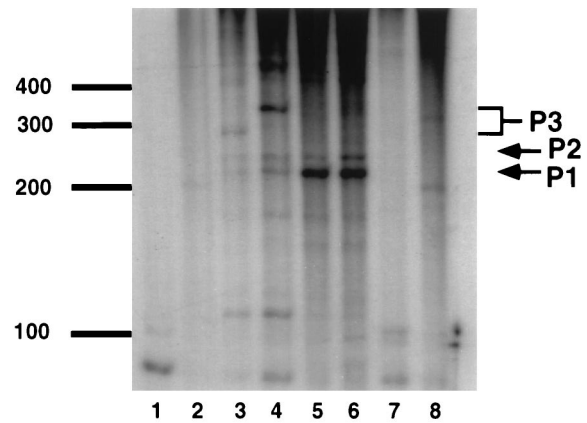


FIG. 5. In vitro transcription assays with extracts from sporulating *C. perfringens* cells. Lanes: 1, 3, 4, and 7, NCTC 8798 extract; 2, 5, 6, and 8, NCTC 10240 extract. Plasmids used were none (lanes 1 and 2), pSM154 (lanes 3 and 5), pSM155 (lanes 4 and 6), and pBS- (lanes 7 and 8). Markers on the left show the migration of RNA Century size markers (bases).

lengths could have initiated within the *cpe* promoter region (Fig. 1). Lanes 3 and 4 of Fig. 5 also contained a band of ~115 bases. This would be the length expected for a transcript originating from P3 in a region of the *cpe* mRNA that can potentially form a secondary structure (see below). This result may indicate that termination or mRNA processing occurs in cell extracts.

In addition to the complete *cpe* promoter regions present on pSM154 and pSM155, we cloned the following *cpe* internal deletion fragments into pSM151 for use as in vitro transcription templates: the *cpe* promoters from pSM127 (P1 and P2), pSM170 (P3 alone), and pSM183 (P4 alone). However, we could not generate any significant levels of transcription using these plasmids as templates (30), even though the *cpe* promoters from pSM127 and pSM170 showed significant levels of expression in vivo (Fig. 3). The reason for the differences between the in vivo and in vitro activities is unknown.

Kinetics of induction of transcription from *cpe* promoters P2 and P3. The results shown above allowed us to identify three promoter elements in the *cpe* promoter region (i.e., P1, P2, and P3). In order to determine if these *cpe* promoters are induced at similar times during the developmental cycle, we wished to determine the kinetics of promoter induction during the stationary phase. We did this by using primer extension analysis of *cpe* mRNA extracted at different times during the growth and sporulation cycles. However, the use of a primer that annealed near the N terminus of the *cpe* structural gene consistently gave a very strong termination signal near positions -35 to -37, and the 5' ends of P1, P2, and P3 could only be detected after long exposure times (22, 30). The 5' end at positions -35 to -37 may represent an additional promoter downstream of P1 but more likely is due to premature termination of the reverse transcriptase, since it corresponds to the 5' side of a potential hairpin secondary structure that can form in the *cpe* mRNA between positions -32 and -55 (30). To overcome the premature termination of the reverse transcriptase, we used a primer (OSM71) that annealed to the downstream half of the putative hairpin structure (from positions -17 to -44) to both inhibit secondary structure formation and act as a primer for the reverse transcriptase. Unfortunately, this primer annealed too closely to P1 (at position -55) for us to be able to resolve it on a polyacrylamide sequencing gel.

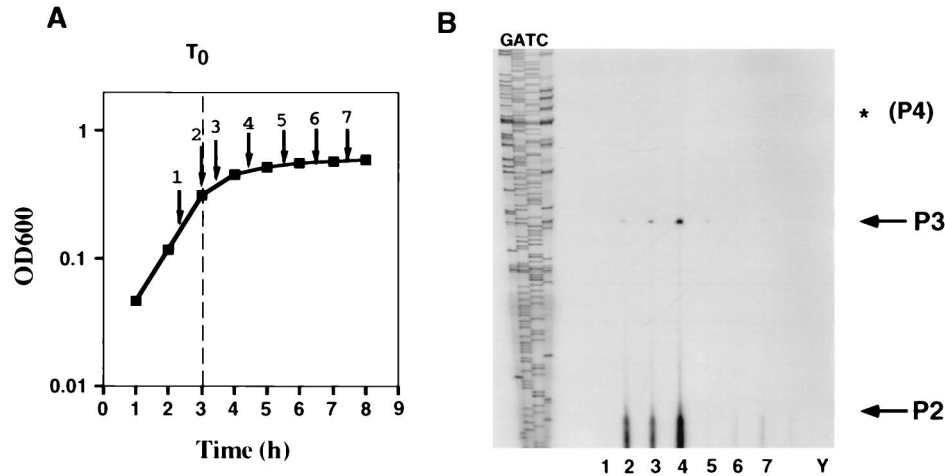


FIG. 6. Growth (A) and primer extension analysis (B) of *cpe* mRNA from strain NCTC 10240 with primer OSM71. The numbered arrows in panel A show the times sampled for RNA extraction and correspond to the lanes in panel B. Y, yeast RNA control. The asterisk denotes the location of P4, based on the sequence shown at the left. GATC lanes show the sequence of the template strand of pSM104 derived with primer OSM71. OD₆₀₀, optical density at 600 nm.

Therefore, we could only analyze the kinetics of P2, P3, and P4 induction.

The results of the primer extension analysis of *cpe* mRNA for strain NCTC 10240 are shown in Fig. 6. The cells entered the stationary phase (T_0) after about 3 h of growth. Interestingly, the intensity of the signal from P2 was much higher than that of the signal from P3, and a 5' end at P4 could not be detected at all in this experiment (Fig. 6), supporting our previous finding that little transcription originates from P4 under the conditions tested here. There was a sustained signal from P2 beginning at the entry into the stationary phase, T_0 (Fig. 6, lane 2), until $T_{1.5}$, with the signal intensity dropping sharply between $T_{1.5}$ and $T_{2.5}$. There was also induction of P3 at T_0 ; this induction was sustained at a high level through $T_{1.5}$ (lane 4) before dropping off. Although the signal from P2 was much stronger than that from P3, the promoters showed similar kinetics of induction. Quantitative densitometry analysis of these data was difficult due to the extended, smeared signal exhibited by P2.

A similar pattern of P2 and P3 induction was seen with primer extension products from strain NCTC 8798 during the growth and sporulation cycles (30); in addition, a 5' end at P4 was not detected in this strain.

DISCUSSION

In a previous report, we identified by primer extension analysis several 5' ends that may indicate the location of *cpe* sporulation-dependent promoters (22). In this study, we used nested and internal deletions of the *cpe* promoter regions to narrow down the locations where transcription can be initiated and confirmed the presence of at least three promoters, P1, P2, and P3. In vitro transcription assays done with extracts from sporulating cells provided additional biochemical evidence for transcription being initiated at these three promoters. A fourth 5' end detected by primer extension (P4) in our previous report (22) could not be detected with the techniques described here, including primer extension (Fig. 6), RNase T_2 protection (30), and in vitro transcription (Fig. 5) assays. In addition, deletion analysis in which a P4-*gusA* fusion was assayed showed very little sporulation-dependent expression (Fig. 3C). These results suggest either that P4 is a primer extension artifact or that

it requires sequences downstream of the start site of transcription for activity.

The DNA sequences immediately upstream of the apparent start sites for P1, P2, and P3 show significant similarity to consensus recognition sequences for RNA polymerase containing the sporulation-dependent sigma factors SigE and SigK. In particular, sequences upstream of P1 are similar to SigK-dependent promoters, while sequences upstream of P2 and P3 exhibit strong similarity to SigE-dependent promoters (Fig. 7). These results could indicate that the synthesis of these sigma factors during the course of sporulation is, at least in part, responsible for the sporulation-dependent synthesis of CPE. Indirect support for this regulatory model comes from the kinetic analysis of P2 and P3 induction (Fig. 6). Transcription of both P2 and P3 was initiated immediately upon entry into the stationary phase. This coinduction was consistent with P2 and P3 being regulated by a common mechanism, e.g., the synthesis and activity of SigE in the mother cell. If P1 is regulated by the synthesis and activity of SigK, it presumably would be induced at a later time in the sporulation cycle than P2 and P3. However, *C. perfringens* produces a spore in a very short time (~2.5 h) compared to *B. subtilis* (~6 to 8 h). This rapid spore production may compress the time differences

P1	AC	(16 bp)	<u>CA</u> AA ⁻¹⁰ NN ⁻³⁵ NAA
SigK	AC	(17 bp)	CATANNNTA
P2	TATTTTA	(14 bp)	<u>AA</u> TACTTT
SigE	TAATATA	(14 bp)	CATACAAT
	GC AT		C
P3	TAATTCT	(14 bp)	<u>CATA</u> AAAT
SigE	TAATATA	(14 bp)	CATACAAT
	GC AA		C

FIG. 7. Alignment of *cpe* promoters P1, P2, and P3 with consensus SigE and SigK promoter recognition sequences derived from *B. subtilis* (15). Degenerate bases in the consensus are shown below each line, the spacing between the promoter -10 and -35 regions is shown in parentheses, and the -10 base relative to the start site of transcription in the *cpe* promoters is underlined.

between which SigE- and SigK-dependent promoters are expressed.

It should be pointed out that the consensus recognition sequences shown in Fig. 7 are derived from studies with *Bacillus* spp., mostly *Bacillus subtilis* (15). In addition, *B. subtilis* does not regulate the *cpe* promoter in a sporulation-dependent fashion and does not seem to initiate transcription from any of the promoters described in this report (22). There are at least two possible reasons for this. (i) *B. subtilis* may not recognize the promoters due to the differences that exist between the proposed recognition sequences for P1, P2, and P3 and the consensus sequences of SigE- and SigK-dependent promoters. (ii) *C. perfringens* may contain additional transcriptional regulators, absent from *B. subtilis*, that allow it to recognize and initiate transcription from P1, P2, and P3.

An important point to consider is whether *C. perfringens* possesses homologs of SigE and SigK. The genes encoding these sigma factors as well as SigA and SigG homologs have recently been cloned and sequenced from *Clostridium acetobutylicum* (25, 28). In addition, we have also recently cloned and sequenced the genes encoding homologs of SigE, SigK, and SigG from *C. perfringens* (30). Therefore, these sigma factor proteins are present in *C. perfringens* and could function as potential regulators of transcription at the *cpe* promoters. Unfortunately, with *cpe*⁺ strains, it is not possible at the present time to make specific knockout mutations in the sigma factor genes to test our model directly.

Interestingly, it has been clearly shown that SigE and SigK are active only in the mother cell compartment of the developing sporangium in *B. subtilis* (see references 11 and 15 for reviews). If this is also the case with *C. perfringens*, then SigE and SigK would be active in the same compartment in which the large majority of CPE protein is made. Such compartment-specific regulation of toxin synthesis has also been seen with the *cryIA* gene from *Bacillus thuringiensis*, which encodes an insecticidal toxin and is transcriptionally regulated by SigE and SigK (1, 3, 4). If the regulation of *cpe* promoters also is found to be SigE and SigK dependent, this finding would represent a remarkable example of convergent evolution, where two toxins, with no evident sequence homology, have a similar regulatory mechanism and are active on intestinal mucosal cells but in completely different hosts (mammals versus insects).

Definitive proof of our model that transcription from P1, P2, and P3 requires SigE and SigK will come from the purification of RNA polymerase forms that can initiate transcription from these promoters. Such experiments are currently under way in our laboratory.

ACKNOWLEDGMENTS

This work was initiated at Tufts University School of Medicine with support from U.S. Public Health Service grant GM42219 to A. L. Sonenshein. Studies at the University of Tennessee were supported by NRICGP/USDA grant 96-01542 and by grant IN-176-E from the American Cancer Society, both awarded to S.B.M.

REFERENCES

- Adams, L. F., K. L. Brown, and H. R. Whiteley. 1991. Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. *J. Bacteriol.* **173**:3846–3854.
- Bannam, T. L., and J. I. Rood. 1993. *Clostridium perfringens*-*Escherichia coli* shuttle vectors that carry single antibiotic resistance determinants. *Plasmid* **29**:233–235.
- Brown, K. L., and H. R. Whiteley. 1988. Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing crystal protein genes. *Proc. Natl. Acad. Sci. USA* **85**:4166–4170.
- Brown, K. L., and H. R. Whiteley. 1990. Isolation of the second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. *J. Bacteriol.* **172**:6682–6688.
- Brynstad, S., L. A. Iwanejko, G. S. Stewart, and P. E. Granum. 1994. A complex array of Hpr consensus DNA recognition sequences proximal to the enterotoxin gene in *Clostridium perfringens* type A. *Microbiology* **140**:97–104.
- Cormack, B. 1994. Introduction of a point mutation by sequential PCR steps, p. 8.5.7–8.5.9. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. Greene Publishing Associates, Inc./John Wiley & Sons, Inc., New York, N.Y.
- Czczulin, J. R., R. C. Collie, and B. A. McClane. 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *C. perfringens*. *Infect. Immun.* **64**:3301–3309.
- Czczulin, J. R., P. C. Hanna, and B. A. McClane. 1993. Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. *Infect. Immun.* **61**:3429–3439.
- Duncan, C. L. 1973. Time of enterotoxin formation and release during sporulation of *Clostridium perfringens* type A. *J. Bacteriol.* **113**:932–936.
- Duncan, C. L., D. H. Strong, and M. Sebald. 1972. Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *J. Bacteriol.* **110**:378–391.
- Errington, J. 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol. Rev.* **57**:1–33.
- Garnier, T., and S. T. Cole. 1986. Characterization of a bacteriocinogenic plasmid from *Clostridium perfringens* and molecular genetic analysis of the bacteriocin-encoding gene. *J. Bacteriol.* **168**:1189–1196.
- Garnier, T., and S. T. Cole. 1988. Studies of UV-inducible promoters from *Clostridium perfringens* in vivo and in vitro. *Mol. Microbiol.* **2**:607–614.
- Graves, M. C., and J. C. Rabinowitz. 1986. In vivo and in vitro transcription of the *Clostridium pasteurianum* ferredoxin gene. *J. Biol. Chem.* **261**:11409–11415.
- Haldenwang, W. G. 1995. The sigma factors of *Bacillus subtilis*. *Microbiol. Rev.* **59**:1–30.
- Jefferson, R. A., S. M. Burgess, and D. Hirsh. 1986. β -Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. USA* **83**:8447–8451.
- Johnson, C. C. 1989. *Clostridium perfringens* food poisoning, p. 629–638. *In* S. M. Finegold and W. L. George (ed.), *Anaerobic infections in humans*. Academic Press Ltd., London, England.
- Kokai-Kun, J. F., and B. A. McClane. 1996. Evidence that a region(s) of the *Clostridium perfringens* enterotoxin molecule remains exposed on the external surface of the mammalian plasma membrane when the toxin is sequestered in small or large complexes. *Infect. Immun.* **64**:1020–1025.
- McClane, B. A. 1994. *Clostridium perfringens* enterotoxin acts by producing small molecule permeability alterations in plasma membranes. *Toxicology* **87**:43–67.
- McDonel, J. L. 1980. Mechanism of action of *Clostridium perfringens* enterotoxin. *Food Technol.* **1980**(April):91–95.
- Melville, S. B., R. E. Collie, and B. A. McClane. 1997. Regulation of enterotoxin production in *Clostridium perfringens*, p. 471–490. *In* J. I. Rood, B. A. McClane, J. G. Songer, and R. W. Titball (ed.), *The clostridia: molecular biology and pathogenesis*. Academic Press, Inc., San Diego, Calif.
- Melville, S. B., R. Labbe, and A. L. Sonenshein. 1994. Expression from the *Clostridium perfringens cpe* promoter in *C. perfringens* and *Bacillus subtilis*. *Infect. Immun.* **62**:5550–5558.
- Melville, S. B., and A. L. Sonenshein. 1995. Unpublished data.
- Roper, G., J. A. Short, and D. A. Walker. 1976. The ultrastructure of *Clostridium perfringens* spores, p. 279–296. *In* A. M. Baker, J. Wold, D. J. Ellar, G. H. Dring, and G. W. Gould (ed.), *Spore research*. Academic Press Ltd., London, England.
- Sauer, U., A. Treuner, M. Buchholz, J. D. Santangelo, and P. Durre. 1994. Sporulation and primary sigma factor genes in *Clostridium acetobutylicum*. *J. Bacteriol.* **176**:6572–6582.
- Tabor, S., and C. C. Richardson. 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA* **84**:4767–4771.
- Wieckowski, E. U., A. P. Wnek, and B. A. McClane. 1994. Evidence that an approximately 50-kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound *Clostridium perfringens* enterotoxin. *J. Biol. Chem.* **269**:10838–10848.
- Wong, J., C. Sass, and G. N. Bennett. 1995. Sequence and arrangement of genes encoding sigma factors in *Clostridium acetobutylicum* ATCC 824. *Gene* **153**:89–92.
- Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.
- Zhao, Y., and S. B. Melville. 1997. Unpublished data.