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Regulation and Initiation of *cenB* Transcripts of *Cellulomonas fimi*†

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We characterized the in vivo transcription of the *Cellulomonas fimi cenB* gene, which encodes an extracellular endo-β-1,4-glucanase (EC 3.2.1.4). By Northern blot (RNA blot) analysis, *cenB* mRNA was detected in *C. fimi* RNA preparations from glycerol-, glucose-, and carboxymethyl cellulose (CMC)-grown cells. The relative abundance of the specific mRNAs in these preparations appeared to depend on the carbon source provided, with the preparations from CMC-grown cells having the greatest amount of *cenB* transcripts, followed by glycerol- and glucose-grown cells. Therefore, the transcription of this gene could be regulated by the carbon source provided to *C. fimi*. High-resolution nuclease S1 protection studies were used to map *cenB* mRNA 5′ termini with a unique 5′-labeled DNA probe and *C. fimi* RNA isolated in vivo. With this procedure, three 5′ termini were found in abundance upstream of the translational initiation ATG codon in RNA preparations from *C. fimi* grown on CMC, while less-abundant 5′ termini were found 52 bases closer to the ATG codon in RNA prepared from *C. fimi* grown on any one of the three substrates. These results are indicative of a tandem promoter arrangement, with the ATG-proximal promoter directing constitutive low-level *cenB* transcription and the more distal promoter directing higher levels of transcription under the inducing effects of the cellulosic substrate. The corresponding transcripts were not detected in S1 mapping experiments with RNA isolated in vivo from *Escherichia coli* clones harboring recombinant plasmids carrying *C. fimi* genomic inserts. Comparative analysis of the 5′-flanking DNA sequences of the *cenB* gene and the *cenA* and *cex* genes of *C. fimi* (N. M. Greenberg, R. A. J. Warren, D. G. Kilburn, and R. C. Miller, Jr., J. Bacteriol. 169:646–653, 1987) revealed a region of 50 bases in which these sequences displayed at least 64% homology.

We are studying the regulation of cellulase gene expression in *Cellulomonas fimi*, a gram-positive, nonsporeforming facultative anaerobe which grows best at 30°C (5, 6, 9). We have previously reported a transcriptional analysis of two *C. fimi* genes: the *cenA* gene, which encodes an extracellular endo-β-1,4-glucanase (16), and the *cex* gene, which encodes an extracellular exo-β-1,4-glucanase (13). These genes are regulated at the level of transcription by the carbon source provided to *C. fimi* during growth in basal medium. High levels of *cenA* and *cex* mRNAs are detected when *C. fimi* is grown on a soluble cellulosic substrate such as carboxymethyl cellulose (CMC). When *C. fimi* is grown on glycerol as the carbon and energy source, *cenA* but not *cex* mRNA is detected in Northern blots (RNA blots), implying that the *cex* gene is more stringently regulated than the *cenA* gene. When *C. fimi* is grown on glucose as the carbon and energy source, the transcription of both *cenA* and *cex* is dramatically reduced, suggesting catabolite repression (8, 14) as one mechanism regulating gene expression in this organism.

A second endoglucanase gene (*cenB*) from *C. fimi* has been cloned in *Escherichia coli* (5). The *cenB* gene encodes an extracellular endo-β-1,4-glucanase with an M_r of 110,000 (6; J. Owolabi, personal communication). In this study we used Northern blot and nuclease S1 protection analysis to investigate the in vivo regulation and initiation of *cenB* transcription in *C. fimi*. We demonstrate that the *cenB* gene is transcribed as a monocistronic mRNA. We also show that the transcription of *cenB* is controlled by two tandemly oriented promoters: the distal *cenBp1* promoter, which is responsible for the cellulose-dependent transcription of the gene, and the proximal *cenBp2* promoter, which directs the constitutive transcription of the gene. Constitutive *cenB* expression may serve to generate low-molecular-weight cellulose-specific degradation products which can act, presumably, as true inducers for cellulase synthesis once a suitable substrate is encountered (for a review, see reference 3). This is the first demonstration at the transcriptional level of constitutive expression of a cellulase-encoding gene in *C. fimi*.

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† Dedicated to the memory of Esther Levine, who passed away 18 November 1986.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *C. fimi* ATCC 484 and *E. coli* JM83 (15) and JM101 (12). Plasmids pBR322 (2), pUC13 (15), and pUC19 (17) and their derivatives (as described below) were propagated in *E. coli* JM83 or JM101.

Plasmid pEC3 is a derivative of pBR322 which contains a 5.6-kilobase-pair (kbp) *Bam*HI fragment (see Fig. 1) carrying the *cenB* gene of *C. fimi* (5). Plasmid pNG301 carries a 400-base-pair (bp) *Bam*HI-*Pst*I fragment of pEC3. Plasmid pUC19C3PS is a derivative of pUC19 which contains a 2.0-kbp *Pst*I-*Sma*I fragment of pEC3; it was kindly provided by J. Owolabi. Plasmids pNG301 and pUC19C3PS were constructed to facilitate the preparation of high-specific-activity probes for use in hybridization reactions. Plasmid pNG303 is a derivative of pUC19 which carries a 2.4-kbp *Bam*HI-*Sma*I fragment of pEC3; it was constructed to test the ability of *E. coli* RNA polymerase to utilize *C. fimi* promoter sequences without interference from the strong *lac* promoter of pUC19.

Enzymes and reagents. Restriction endonucleases *Bam*HI, *Pst*I, and *Sma*I were from Pharmacia P-L Biochemicals. Nuclease S1, T4 polynucleotide kinase, T4 DNA ligase, DNA polymerase I, yeast tRNA, and redistilled phenol were from Bethesda Research Laboratories, Inc. Radionuclides were from New England Nuclear Corp. All other chemicals were of reagent grade or higher and were purchased from commercial suppliers.

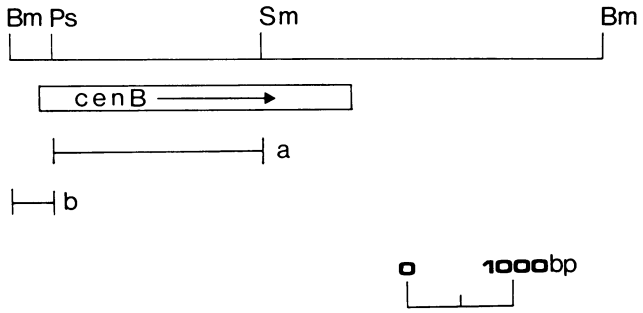

FIG. 1. Representation of the cloned 5.6-kbp *Bam*HI-*Bam*HI fragment of *C. fimi* DNA containing the *cenB* gene on plasmid pEC3 (5). The structural gene is shown as a boxed region with the 3′ end approximated from the Northern blot data (this work). Translation is from left to right. (a) *Pst*I-*Sma*I Northern blot probe; (b) *Bam*HI-*Pst*I 5′ S1 probe. The restriction endonucleases are abbreviated as follows: Bm, *Bam*HI; Ps, *Pst*I; Sm, *Sma*I.

Media and growth conditions. *C. fimi* was grown in basal medium (6) supplemented with either 0.2% (wt/vol) glycerol, 0.2% (wt/vol) glucose, or 1% (wt/vol) CMC (low viscosity; Sigma Chemical Co.) as a carbon source. *E. coli* strains were grown in 2× YT medium (12). All strains were grown at 30°C. When a solid medium was required, agar (Difco Laboratories) was added to 1.5% (wt/vol), except for basal medium containing CMC, in which 1.0% agar was used. When appropriate, ampicillin (Sigma) was added to 100 μg/ml to liquid or solid medium.

RNA extraction. RNA was prepared from *C. fimi* and *E. coli* as previously described (6).

DNA preparation. The preparation of ^{32}P-end-labeled and nick-translated DNA hybridization probes from plasmid DNA was as previously described (6, 10).

Northern blot analysis and nuclease S1 transcript mapping. The analysis of RNA prepared from *E. coli* or *C. fimi* was as previously described (6). For Northern blotting, 20 μg of RNA was used per lane. For nuclease S1 transcript analysis, 30 μg of RNA and an excess of 5′-end-labeled probe (see Fig. 1b) were dissolved in 30 μl of hybridization buffer (0.4 M NaCl, 0.04 M sodium phosphate [pH 6.5], 0.4 mM EDTA, 80% formamide), heated for 15 min at 85°C, and kept at 60°C for 3 h. Reactions were terminated by rapid 10-fold dilution into cold S1 buffer (30 mM sodium acetate [pH 4.5], 28 mM NaCl, 4.5 mM $ZnSO_4$) containing 1,000 U of nuclease S1 (1, 6, 10).

RESULTS

Regulation by carbon source and approximate length of the *cenB* transcripts. The lengths of the specific *cenB* transcripts and the effects of the carbon sources provided during growth in cultures on the relative mRNA levels were determined by Northern blot analysis. The intragenic *cenB* probe (Fig. 1a) hybridized strongly to a species of *C. fimi* RNA that was approximately 3,200 bases long and that was isolated from CMC-grown cells (Fig. 2, lane 3). Less abundant hybrids of about the same size were detected in RNA that was isolated from glycerol- and glucose-grown cells (Fig. 2, lanes 1 and 2). These results indicate that the carbon source provided during growth can regulate the levels of the *cenB* gene transcripts and that transcription is incompletely repressed in the presence of glucose substrate.

Mapping the *cenB* transcription start sites with nuclease S1. To confirm the direction of *cenB* transcription and to identify

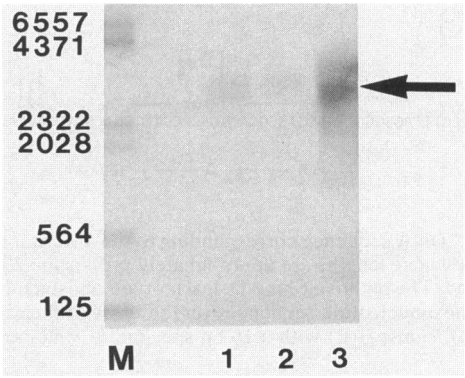

FIG. 2. Northern blot analysis of *cenB*-specific transcripts. RNA was extracted from *C. fimi* cultures grown in basal medium supplemented with glycerol (lane 1), glucose (lane 2), or CMC (lane 3). It was denatured with formaldehyde, fractionated on a formaldehyde gel containing 1% (wt/vol) agarose, and transferred to a Biotrans membrane (Pall, Inc.). Hybridization was done with the nick-translated intragenic *Pst*I-*Sma*I probe (Fig. 1a) (specific activity, 10^7 dpm/μg). M, ^{32}P-labeled *Hin*dIII restriction fragments of lambda DNA, with sizes in base pairs indicated on the left. The arrow indicates the major hybrids.

the 5′ ends of *cenB* mRNA, we analyzed transcripts synthesized in vivo by high-resolution nuclease S1 mapping with the 5′-end-labeled *Pst*I-*Bam*HI probe (labeled at the *Pst*I site) (Fig. 1b). When RNA isolated from CMC-grown cultures was used in mapping experiments, three prominent species (Fig. 3, lane 5, +1, +2, and +3) were resolved upstream of the *cenB* translation initiation codon, 201 bp from the labeled *Pst*I site. A fourth, weaker species (Fig. 3,

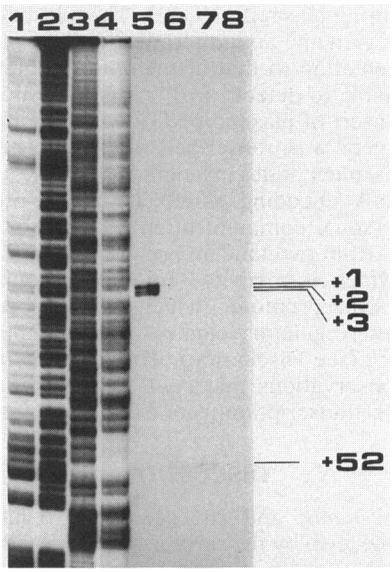

FIG. 3. Mapping the 5′ end of *cenB* mRNA. After hybridization with RNA from CMC (lane 5)-, glucose (lane 6)-, or glycerol (lane 7)-grown *C. fimi*, the *cenB*-specific ^{32}P-labeled *Bam*HI-*Pst*I probe (Fig. 1b) (labeled at the *Pst*I end) was treated with nuclease S1 and analyzed on an 8% polyacrylamide–7 M urea sequencing gel alongside the probe sequenced by the base-specific chemical cleavage method of Maxam and Gilbert (11). Lanes 1 through 4 contain the sequencing ladders G>A, G+A, T+C, and C>T, respectively. Lane 8 shows negative control hybridization with yeast tRNA. The numbers on the right identify the species of protected probes.

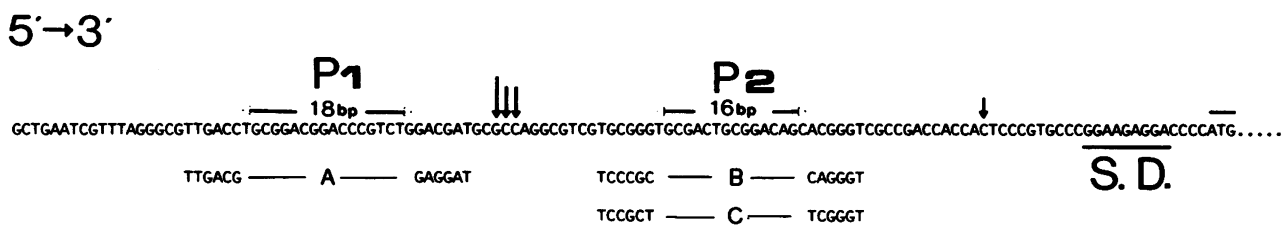

FIG. 4. DNA sequence corresponding to the 5'-terminal region of *cenB* mRNA. The 3' nucleotides of the protected fragments are denoted by arrows whose lengths are approximately proportional to the intensities of the bands in the gel shown in Fig. 3. The ATG initiation codon is overlined. The putative Shine-Dalgarno-type ribosome-binding site (S. D.) is underlined. P1 and P2 identify the putative *cenB* promoter −10 and −35 hexanucleotide sequences on the basis of their homologies to the characterized gram-positive promoters *ermp2* (A) with an 18-bp spacing (4), *cenAp1* (B) with a 16-bp spacing (6), and *cex* (C) with a 16-bp spacing (6).

lane 4, +52) was seen upon prolonged exposure of the dried gel to X-ray film (results not shown). The +1, +2, +3, and +52 species initiated at bases G, C, C, and C, respectively. When RNA isolated from glucose-grown cultures was used in mapping experiments, only the +52 species was detected (Fig. 3, lane 6). In mapping studies with RNA isolated from glycerol-grown cells, the +52 species was detected as the major species (Fig. 3, lane 7), while the +1, +2, and +3 species were detected only after prolonged exposure of the dried gel to X-ray film (results not shown). No hybrids were detected in control experiments with yeast tRNA (Fig. 3, lane 8). These results were in agreement with the results of the Northern blot analysis and showed that while transcripts could be detected in RNA prepared from glycerol-, glucose-, or CMC-grown cultures, the initiation sites of the transcripts were influenced by the substrate.

We wished to determine if the regions identified by S1 mapping with *C. fimi* RNA are recognized by *E. coli* RNA polymerase in vivo. Therefore, S1 mapping experiments were performed with RNA isolated in vivo from *E. coli* strains harboring plasmid pNG303. This plasmid carries the 5'-flanking region and amino-terminal portion of *cenB* in the opposite orientation to that of the *lac* promoter of pUC19. We were unable to detect *cenB* transcripts initiating within the *C. fimi* insert of plasmid pNG303 (results not shown).

The *cenB* gene was transcribed as a monocistronic mRNA of about 3,200 nucleotides in length. This is the expected size for an mRNA encoding a 110,000-dalton protein (6; J. Owolabi, personal communication). Transcription of *cenB* was directed from two tandem promoters: the distal *cenBp1* promoter, which is regulated by carbon source, and the proximal *cenBp2* promoter, which functions constitutively (Fig. 4). These regulatory elements did not appear to function in *E. coli* (see Discussion). This result is in agreement with early observations that *cenB* expression in *E. coli* is dependent on transcription from *E. coli* promoters.

DISCUSSION

The *cenA*, *cenB*, and *cex* genes are regulated at the transcriptional level by the carbon source provided to *C. fimi* during growth (6; this work). As with the *cenA* and *cex* genes, the most abundant *cenB*-specific transcripts detected in hybridization experiments were from CMC-grown cells. The *cenB* gene is clearly cellulose inducible.

In contrast to *cenA* and *cex*, however, *cenB* is transcribed, albeit to a lesser extent, in the presence of glucose. Cellulases, most notably the endoglucanases, are expressed constitutively in many cellulolytic organisms (for a review, see reference 3). Such constitutive low-level endoglucanase expression by *C. fimi* would be preferable to producing a full complement of cellulases in the absence of an appropriate substrate. Once cellulose was encountered, it could be hydrolyzed by the constitutive enzyme to produce the true inducers for cellulase synthesis, which have yet to be defined for *C. fimi*. The inducers would then be present as long as there was sufficient substrate or until they were themselves converted to a metabolizable carbon and energy source. The exhaustion of the substrate would result in a drop in the levels of the inducers, and *C. fimi* could return to basal-level cellulase production.

The *C. fimi* promoters directing *cenB* transcription were mapped by nuclease S1 protection studies. Three major transcription start sites were found upstream of the ATG codon in CMC-grown cultures. A similar clustering of initiation sites occurs with the *cenA* and *cex* transcripts of *C. fimi* (6) and may reflect some flexibility of *C. fimi* RNA polymerase in selecting a start site. A minor transcript initiates 52 bases closer to the ATG codon in *C. fimi* grown on any one of the three substrates. Therefore, *cenB* can be transcribed from either of two promoters: the distal promoter *cenBp1*, directing transcription predominantly from position +1 (which appeared to be the strongest signal on the S1 gels), and the proximal, weaker promoter *cenBp2*, directing transcription from position +52. Promoter *cenBp1* is cellulose inducible, and promoter *cenBp2* is constitutive. This tandem arrangement of a regulated promoter and a constitutive promoter closely resembles that found for the promoters of the *Streptomyces lividans* galactose operon (4).

The DNA sequence immediately upstream of the *cenBp1* transcription start site displays significant homology with both the "−10" and "−35" regions of the *ermp2* promoter of *Streptomyces erythraeus* (Fig. 4, line A), which is not used efficiently by *E. coli* (7). The region upstream of the *cenBp2* start site has only limited −10 region homology with the *cenAp1* and *cex* promoters of *C. fimi* (Fig. 4, lines B and C). There are no strong homologies between *cenBp1* and *cenBp2*.

There are at least three possibilities as to why *cenB* transcripts were not found to initiate in *E. coli* carrying plasmid pNG303. First, *E. coli* RNA polymerase may not recognize the *C. fimi* promoters. Second, *E. coli* RNA polymerase may recognize the *C. fimi* promoters but remain incapable of initiating or elongating the transcript. Third, the resulting "hybrid" transcript in *E. coli* may be intrinsically unstable. It seems that for regulated transcription of cloned *C. fimi* genes to occur in *E. coli* we must rely, for the time being, on appropriate *E. coli* promoters.

The strength and inducibility of the *C. fimi cenBp1*, *cenAp1*, and *cex* promoters may be related to an extended −35 region (Fig. 5). This region could facilitate transcription initiation in a *cis*-dominant manner (presumably by binding a

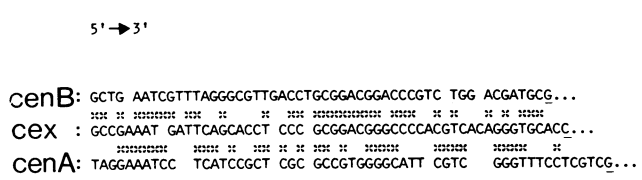

FIG. 5. Conserved *C. fimi* DNA sequences located 5′ to mapped mRNA start sites. Gaps have been introduced into the sequences to allow for best matches. Matches are denoted by asterisks. The +1 sites for *cenBp1* (top), *cex* (middle), and *cenAp1* (bottom) are underlined, and transcripts are indicated by dots.

trans-acting factor) when *C. fimi* is grown in the presence of a cellulosic substrate. Under inducing conditions, initiation at the *cenBp1* promoter might decrease the frequency of initiation at the *cenBp2* promoter. This would explain why fewer transcripts appear to initiate at the +52 site in cells grown in the presence of CMC than in cells grown in the presence of glycerol or glucose.

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