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Regulation, Initiation, and Termination of the *cenA* and *cex* Transcripts of *Cellulomonas fimi*

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We characterized the in vivo transcripts of two *Cellulomonas fimi* genes, the *cenA* gene, which encodes an extracellular endo-β-1,4-glucanase (EC 3.2.1.4) and the *cex* gene, which encodes an extracellular exo-β-1,4-glucanase (EC 3.2.1.91). By Northern blot analysis, *cenA* mRNA was detected in *C. fimi* RNA preparations from glycerol- and carboxymethyl cellulose-grown cells but not from glucose-grown cells. In contrast, *cex* mRNA was detected only in the preparations from carboxymethyl cellulose-grown cells. Therefore, the transcription of these genes is subject to regulation by the carbon source provided to *C. fimi*. By nuclease S1 protection studies with unique 5′-labeled DNA probes and *C. fimi* RNA isolated in vivo, 5′ termini were found 51 and 62 bases before the *cenA* translational initiation codon and 28 bases before the *cex* translational initiation codon. S1 mapping with unlabeled DNA probes and *C. fimi* RNA which had been isolated in vivo but which had been 5′ labeled in vitro with guanylyltransferase and [α-^{32}P]GTP confirmed that true transcription initiation sites for *cenA* and *cex* mRNA had been identified. Comparative analysis of the DNA sequences immediately upstream of the initiation sites of the *cenA* and *cex* mRNAs revealed a 30-base-pair region where these two sequences display at least 66% homology. S1 mapping was also used to locate the 3′ termini of the *cenA* and *cex* transcripts. Three 3′ termini were found for *cenA* messages, whereas only one 3′ terminus was identified for *cex* mRNA. The transcripts of both genes terminate in regions where their corresponding DNA sequences contain inverted repeats.

Cellulomonas fimi is a gram-positive, nonsporeforming facultative anaerobe which grows best at 30°C (3, 25, 42). An interesting feature of *C. fimi* DNA is its G+C content of 72 mol% (42). At least three classes of β-1,4-glucanases are produced by *C. fimi* under appropriate physiological conditions: β-1,4-endoglucanases (EC 3.2.1.4) (1, 15, 28, 44, 52) and at least one β-1,4-exoglucanase (EC 3.2.1.91) (17, 36, 50) which can act synergistically to hydrolyze carboxymethyl cellulose (CMC) (17) and a β-glucosidase (EC 3.2.1.21) (48) which hydrolyzes cellobiose to glucose.

We previously reported the molecular cloning of two *C. fimi* genes in *Escherichia coli*: the *cenA* gene, which encodes an extracellular endo-β-1,4,-glucanase (Eng) (15, 52), and the *cex* gene, which encodes an extracellular exo-β-1,4-glucanase (Exg) (15, 36, 50). Although these cloned genes have been well characterized, very little information is available on the molecular mechanisms which govern their expression in *C. fimi*. In this study we used Northern blotting to investigate the in vivo regulation of *cenA* and *cex* transcription and nuclease S1 protection analysis to map the initiation and termination sites of the *cenA* and *cex* transcripts. DNA probes derived from the cloned genes in *E. coli* were used in the analysis. To our knowledge, this is the first characterization of transcription in *C. fimi*, and *cenA* and *cex* are the first cellulase-encoding genes that have been shown directly to be regulated at the transcriptional level.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *C. fimi* ATCC 484 and *E. coli* JM101 (31) and JM83 (46). Plasmids pBR322 (9), pUC12, pUC13 (46), and pUC18 (46, 53) and their derivatives (as described below) were propagated in *E. coli* JM83 or JM101.

Plasmid pcEC2 is a derivative of pBR322 that contains a 2.2-kilobase-pair *Bam*HI-*Sma*I fragment (Fig. 1A) carrying the *cenA* gene of *C. fimi* (52). Plasmid pUC12A25 is a derivative of pUC12 which contains a 2.6-kilobase-pair *Bam*HI-*Sal*I fragment (Fig. 1B) carrying the *cex* gene of *C. fimi* (36).

Plasmids pNG101 and pNG102 are derivatives of pUC18 which contain the 636-base-pair (bp) *Bam*HI-*Sal*I and 939-bp *Sma*I fragments, respectively, of pcEC2. Plasmid pNG201 is a derivative of pUC18, from which the 216-bp *Sma*I-*Pvu*II pUC18 fragment was deleted, and carries the 138-bp *Pst*I-*Ban*I fragment of pUC12A25. Plasmid pNG202 is a derivative of pUC18 carrying the 253-bp *Sau*3A1-*Sal*I fragment of pUC12A25. Plasmids pNG101, -102, -201, and -202 were constructed to facilitate the preparation of high-specific-activity hybridization probes by minimizing the number of unwanted fragments which could compete for label in end-labeling reactions. Plasmid pUC13Bam31 carries the 397-bp *Sau*3A1 fragment of pUC12A25 in the *Bam*HI site of pUC13 and was kindly provided by G. P. O'Neill.

Enzymes and reagents. Restriction endonucleases *Bam*HI, *Bgl*II, *Sau*3A1, and *Sma*I, S1 nuclease, T4 polynucleotide kinase, guanylyltransferase, yeast tRNA, and redistilled phenol were from Bethesda Research Laboratories, Inc. Enzymes *Ban*I, *Hin*dIII, *Pst*I, and *Sal*I, dextran sulfate, and DNA-grade Sephadex G-50 were from Pharmacia P.-L. *Nar*I and *Sty*I were from New England BioLabs, Inc. Diethylpyrocarbonate, MOPS (morpholinepropanesulfonic acid), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and Trizma base were from Sigma Chemical Co. Formamide was from BDH. Radionuclides were from New England Nuclear Corp. All other chemicals were of reagent grade or higher and were purchased from commercial suppliers.

Media and growth conditions. *C. fimi* was grown in basal medium (43) supplemented with either 0.2% (wt/vol) glyc-

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erol, 0.2% (wt/vol) glucose, or 1% (wt/vol) CMC (Sigma; low viscosity) as a carbon source. *E. coli* strains were grown in 2 × YT medium (31). All strains were grown at 30°C. When 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^{-1} in liquid or solid medium.

RNase-free work. Chemicals and reagents used for RNA work were purchased solely for this purpose and were kept separate from regular laboratory supplies. All glassware used for RNA work was either baked at 300°C for 3 h or was bought as disposable labware. When appropriate, solutions were treated with 0.2% (vol/vol) diethylpyrocarbonate as described previously (12, 29). All plastics (pipette tips and microfuge tubes) were sterilized by autoclaving without further pretreatment.

RNA extraction. *C. fimi* RNA was prepared by a modification of the procedures of Miller et al. (32) and Kennell and Bicknell (26). Cultures (up to 100 ml) in the late log phase were rapidly chilled on ice and centrifuged for 5 min at 6,000 × *g*. Cells were washed with 1/10 volume of ice-cold 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA (TE), transferred to polycarbonate tubes (Oak Ridge type), and centrifuged for 5 min at 6,000 × *g*. The cells were then suspended in 1/25 volume of 50 mM Tris hydrochloride (pH 6.8)–2 mM EDTA–1% sodium dodecyl sulfate (SDS), and the tubes were placed immediately into a boiling water bath for 90 s. The tubes were chilled on ice for 5 min, and an equal volume of ice-cold 5 M NaCl was added and mixed briefly on a vortex mixer. After 5 min on ice, the resultant slurry was centrifuged for 10 min at 30,000 × *g*, and the cleared supernatant fluid was carefully decanted into a 30-ml Corex (Corning Glass Works) glass tube. The nucleic acids were precipitated with 2.5 volumes of 95% ethanol at −20°C for 12 to 16 h and recovered by centrifugation for 20 min at 10,000 × *g*. The pellets were washed with 70% ethanol at −20°C and redissolved in 2 ml of 10 mM Tris hydrochloride (pH 7.5)–5 mM $MgCl_2$. Samples were treated with 5 U of RQ1 DNase I (Promega) for 15 min at 37°C, EDTA was added to 5 mM, and the mixture was extracted twice with phenol-chloroform (1:1) and once with chloroform. The organic phases were combined and back extracted with 1 ml of TE (pH 7.5). The aqueous phases were pooled, and RNA was recovered by precipitation with 3 volumes of 95% ethanol and centrifugation for 10 min at 10,000 × *g*. The pellets were washed with 70% ethanol and redissolved in 20 mM $NaPO_4$ (pH 6.5)–1 mM EDTA (RNA storage buffer). The RNA preparations were compared for similar banding patterns after analytical electrophoresis on agarose gels and subsequent staining with ethidium bromide. RNA concentrations were determined by A_{260}, and samples were divided into aliquots and stored at −70°C.

DNA extraction and purification. Plasmid DNA was isolated by a modification of the alkaline lysis procedure of Birnboim and Doly (8). When required for the preparation of high-specific-activity probes, DNA was further purified by centrifugation to equilibrium in CsCl density gradients containing ethidium bromide (29).

Preparation of ^{32}P end-labeled DNA. To end label DNA fragments, plasmid DNA was digested with restriction enzyme for 1 h at 37°C, extracted twice with phenol-chloroform (1:1) and once with chloroform, and precipitated with ethanol. For 5′ labeling, fragments were treated with calf intestinal alkaline phosphatase and labeled with [γ-^{32}P]ATP (3,000 Ci $mmol^{-1}$) and T4 polynucleotide kinase as described previously (29). The 3′ ends were labeled with [α-^{32}P]dGTP (3,000 Ci $mmol^{-1}$) and the Klenow fragment of DNA polymerase I as described previously (29). Incorporation of label was monitored by liquid scintillation spectrophotometry in an ISOCAP-300.

Preparation of hybridization probes. ^{32}P-end-labeled DNA was digested wih an appropriate restriction endonuclease to liberate fragments uniquely labeled at one end. The digestions were routinely performed under conditions recommended by the suppliers. The hybridization probes were purified by electrophoresis in 5% polyacrylamide gels, electroeluted, and precipitated from ethanol, as described previously (29). In some instances, yeast tRNA (20 μg ml^{-1}) was added as a carrier in the final precipitation. Pellets were washed with 70% ethanol, dried briefly in air, and redissolved in TE (pH 7.5). Samples were removed for quantitation by liquid scintillation counting. Typical specific activities were 1 × 10^4 to 4 × 10^4 dpm ng of DNA $probe^{-1}$.

Single-stranded M13 molecular weight standards. To prepare single-stranded DNA molecular weight standards, M13mp11 single-stranded DNA was digested with *Hae*III (Bethesda Research) by the procedure of von Gabain et al. (47) and then 5′ end labeled as described above. Labeled *Hae*III fragments were recovered by centrifugation through small columns of Sephadex G-50 (29). Fragments were ethanol precipitated with yeast tRNA carrier, redissolved in TE (pH 7.5), and stored at −20°C. Fragment sizes were determined from the complete nucleotide sequence of M13mp11 (45, 53). The 525-base fragment arises from partial digestion (N. M. Greenberg, unpublished observations).

Northern blot analysis of *C. fimi* RNA. For Northern blotting, 20 μg of *C. fimi* RNA was precipitated with ethanol, redissolved in 10 μl of 20 mM MOPS–1 mM EDTA–5 mM sodium acetate (running buffer [pH 7]) with 50% formamide and 2.2 M formaldehyde, heated for 5 min at 68°C, and cooled briefly on ice. Loading dye was added (to give 3% [wt/vol] Ficoll [Pharmacia] and 0.02% [wt/vol] bromphenol blue and xylene cyanol), and samples were electrophoresed alongside single-stranded M13 molecular weight markers on 1.0% agarose–6.6% formaldehyde gels at 20 to 40 mA with recirculation of running buffer. Nucleic acids were blotted to Biotrans membranes (Pall, Inc.) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 12 to 16 h (41), and the membranes were baked at 80°C for 1 h. Prehybridizations and hybridizations were performed essentially by the protocols supplied with the membranes. Briefly, prehybridizations were done in 5× SSC–50% formamide–4 mM PP_i–5 × Denhardt buffer (29) 10% dextran sulfate–250 μg of heat-denatured (100°C for 5 min) salmon sperm DNA ml^{-1}. Incubations were for 1 to 2 h at 42°C with constant agitation. For hybridizations, the probe DNA (8 × 10^5 to 10 × 10^5 dpm ml^{-1}) and carrier were denatured together by heating. The probes were allowed to hybridize to filters for 12 to 16 h at 42°C with constant agitation. Blots were washed at 20°C with three changes of 2× SSC–0.1% SDS and then at 60°C with three changes of 0.1× SSC–0.1% SDS and exposed to XAR-2 film (Eastman Kodak Co.) at −70°C with intensifying screens.

In vitro cap labeling of RNA. To characterize primary transcripts, total *C. fimi* RNA from CMC-grown cultures was labeled in vitro at the 5′ end by using the vaccinia virus capping enzyme, guanylyltransferase, as described previously (34, 51). Briefly, up to 60 μg of total *C. fimi* RNA was labeled in 0.1-ml mixtures containing 25 mM Tris hydrochloride (pH 7.5)–2 mM $MgCl_2$–1 mM dithiothreitol–250 μCi of [α-^{32}P]GTP (3,000 Ci $mmol^{-1}$)–10 to 25 U of guanylyltrans-

ferase. After 30 min at 37°C, the reaction was stopped by the addition of EDTA to 4 mM and SDS to 0.2%. The RNA was extracted twice with phenol-chloroform (1:1) and precipitated twice from ethanol in the presence of 2 M ammonium acetate. The RNA was finally recovered by ethanol precipitation from 0.3 M sodium acetate. Typical specific activities were 1×10^3 to 3×10^3 dpm ng^{-1}.

S1 nuclease transcript mapping. Analysis for 5′ and 3′ ends of *C. fimi* transcripts with labeled DNA probes was done essentially as described by Favaloro et al. (13) and Berk and Sharp (4, 5, 49). Up to 30 μg of *C. fimi* RNA was precipitated with 0.01 to 0.03 pmol of end-labeled DNA probe (1×10^4 to 4×10^4 dpm), redissolved 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 held at 60°C for 3 h. Samples were diluted with 300 μl of ice-cold S1 buffer (30 mM sodium acetate [pH 4.5], 28 mM NaCl, 4.5 mM $ZnSO_4$) and treated with 1,400 U of S1 nuclease for 30 min at 37°C. The reaction was terminated by the addition of 75 μl of stop buffer (2.5 mM ammonium acetate, 50 mM EDTA), and yeast tRNA (20 μg) was added. The mixture was precipitated with 400 μl of isopropanol and centrifuged.

When capped RNA and unlabeled DNA probes were used in the S1 mapping experiments, the procedure was modified as follows (51). Up to 50 μg of capped RNA was precipitated with up to 500 ng of unlabeled DNA probe, redissolved in hybridization buffer, heated to 85°C, held at 60°C for 3 h, and then treated with S1, as described above. After the S1 treatment, the 0.33-ml samples were treated with 25 ng of RNase A for 15 min at 22°C to reduce the background of unhybridized RNA. This reaction was terminated by the addition of SDS to 0.25% and two extractions with phenol-chloroform (1:1). Trimmed hybrids were recovered by precipitation from ethanol.

After either of these procedures, pellets were dissolved in sequencing dye buffer (80% formamide, 0.5× TBE [29], 0.02% [wt/vol] bromphenol blue and xylene cyanol), heated to 90°C for 2 min, and electrophoresed in polyacrylamide-urea gels with appropriate size markers (see figure legends).

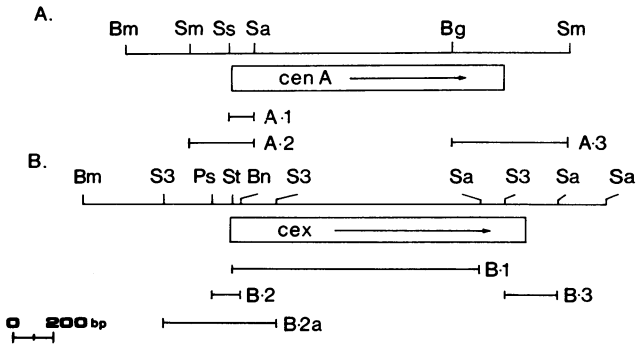

FIG. 1. Representation of cloned segments of *C. fimi* DNA containing the *cenA* (52) and *cex* (36) genes. The structural genes are shown as boxed regions. Both genes are translated from left to right. (A) The 2.2-kilobase *Bam*HI-*Sma*I fragment of pcEC2 that contains the *cenA* gene. A-1, *Sst*I-*Sal*I Northern blot probe; A-2, *Sma*I-*Sal*I 5′ S1 probe; A-3, *Bgl*II-*Sma*I 3′ S1 probe. (B) The 2.6-kilobase *Bam*HI-*Sal*I fragment of pUC12A25 that contains the *cex* gene. B-1, *Sty*I-*Sal*I Northern blot probe; B-2, *Pst*I-*Ban*I 5′ S1 probe; B-2a, *Sau*3A1 fragment used in 5′ mapping experiment with RNA labeled by guanylyltransferase and GTP; B-3, *Sau*3A1-SalI 3′ S1 probe. The restriction endonucleases are abbreviated as follows: Bn, *Ban*I; Bg, *Bgl*II; Bm, *Bam*HI; S3, *Sau*3A1; Sa, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; St, *Sty*I.

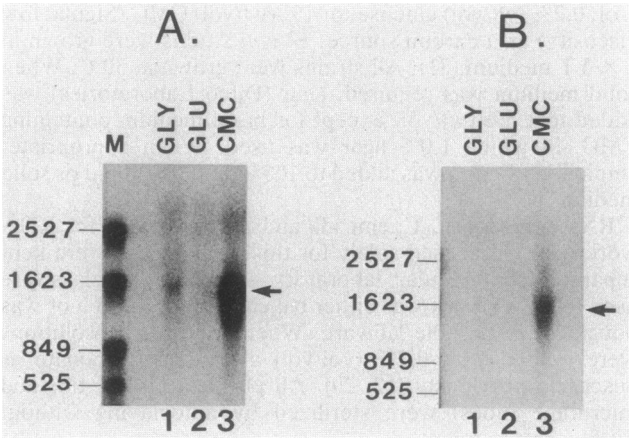

FIG. 2. Northern blot analysis of *cenA*- and *cex*-specific transcripts. RNA was extracted from *C. fimi* cultures grown on basal medium supplemented with glycerol, glucose, or CMC, denatured with formaldehyde, fractionated on formaldehyde gels containing 1.0% agarose, and transferred to Biotrans membranes. (A) Hybridization with *cenA* intragenic *Sst*I-*Sal*I (site of 5′ end labeled with ^{32}P) fragment (Fig. 1, A-1). Lanes: M, *Hae*III restriction fragments of single-stranded M13mp11 (sizes in nucleotides are indicated on the left); 1, RNA from glycerol-grown cells; 2, RNA from glucose-grown cells; 3, RNA from CMC-grown cells. (B) Hybridization with *cex* intragenic *Sty*I-*Sal*I (site of 5′ end labeled with ^{32}P) fragment (Fig. 1, B-1). Lanes are as indicated in panel A. Arrows indicate major hybrids.

The gels were dried to Whatmann 3MM filter paper and exposed to XRP-1 film (Eastman Kodak) at −70°C with intensifying screens.

RESULTS

Regulation by carbon source and approximate lengths of *cenA* and *cex* mRNA transcripts. The lengths of the specific *cenA* and *cex* transcripts and the effects of the carbon sources provided during growth in culture on the relative mRNA levels were characterized by Northern blot analysis. The intragenic *cenA* probe (Fig. 1, A-1) hybridized strongly to a species of *C. fimi* RNA approximately 1,400 bases long isolated from CMC-grown cells (Fig. 2A, lane 3). A less abundant hybrid of about the same size was detectable in RNA from glycerol-grown cells (Fig. 2A, lane 1). Hybrids between this probe and RNA isolated from glucose-grown cultures were not detected (Fig. 2A, lane 2). The intragenic *cex* probe (Fig. 1, B-1) hybridized strongly to an RNA approximately 1,500 bases long which was present only in preparations from CMC-grown cells (Fig. 2B, lane 3). These results indicate that the carbon source provided during growth can regulate the levels of both the *cenA* and *cex* gene transcripts and that the *cex* gene appears to be more stringently regulated than the *cenA* gene.

Mapping *cenA* and *cex* transcriptional start sites with S1 nuclease. To identify the 5′ ends of the *cenA* and *cex* mRNA, transcripts synthesized in vivo were analyzed by S1 nuclease mapping with the 5′-end-labeled probes A-2 (labeled at the *Sal*I site) and B-2 (labeled at the *Ban*I site) (Fig. 1). Four distinct *cenA*-specific hybrids were protected from S1 nuclease degradation (Fig. 3A, lane 6), three of which were closely spaced, with the fourth running a little higher in the gel. The two predominant hybrids (Fig. 3A, +1 and +2) were considered to map the transcriptional start sites for the *cenA*

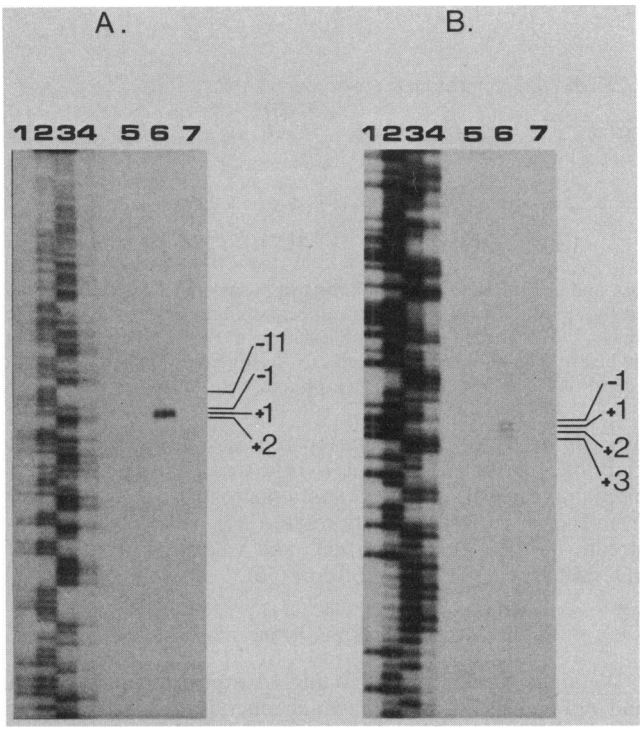

FIG. 3. Mapping 5′ end of *cenA* and *cex* mRNA. After hybridization with RNA and treatment with S1 nuclease, *cenA*- or *cex*-specific ^{32}P-labeled DNA probes were analyzed on 8% polyacrylamide–7 M urea sequencing gels alongside probe sequenced by the base-specific chemical cleavage method of Maxam and Gilbert (30). (A) S1 protection of *cenA* *Sma*I-*Sal*I (site of 5′ end labeled with ^{32}P) fragment (Fig. 1, A-2) by RNA from glucose-grown *C. fimi* (lane 5), RNA from CMC-grown *C. fimi* (lane 6), and yeast tRNA (lane 7). Lanes 1 through 4 contained chemical sequencing ladders G > A, G + A, T + C, and C > T, respectively. (B) S1 protection of *cex* *Pst*I-*Ban*I (site of 5′ end labeled with ^{32}P) fragment (Fig. 1, B-2). Lanes are as indicated in panel A. Numbers on the right identifying species of protected hybrids.

gene. These corresponded to two adjacent G residues 51 and 50 bases upstream of the translational initiation codon (ATG) for the unprocessed *cenA* gene product (see Fig. 5) (52). The other two protected hybrids mapped to a C and a G, 52 and 62 bases upstream, respectively, from the ATG.

Four adjacent *cex*-specific hybrids were resolved by S1 mapping (Fig. 3B, lane 6). These were not observed in the control lanes (lanes 5 and 7). The two predominant bands mapped to adjacent C residues, located 28 and 27 bases upstream of the translational initiation codon for the unprocessed *cex* gene product (36). These were considered start sites for *cex* gene transcription. The assignment of S1 bands to a position in a DNA sequence reflects a correction of 1.5 bases to compensate for the slower mobility of the S1-cleaved hybrids relative to the chemical degradation ladder (33, 49).

S1 mapping using capped RNA to confirm location of transcription initiation. Since the locations of the 5′ termini for the *cenA* and *cex* transcripts were detected by S1 nuclease mapping with DNA probes that were each labeled at the 5′ terminus, the transcript map obtained in this fashion could represent the 5′ ends of processed transcripts and not primary transcript initiation sites. Therefore, an S1 mapping experiment was performed with total RNA from CMC-grown *C. fimi* which had been labeled in vitro with the vaccinia virus capping enzyme. In this system, only RNA species with 5′ di- or triphosphates (i.e., the primary transcripts) are suitable substrates for guanylyltransferase (34). Hence, only RNA transcripts having intact, labeled 5′ ends can be resolved in the final analysis and only if they can form a hybrid with a DNA probe and be protected from S1 digestion.

To map the *cenA* mRNA 5′ end in this fashion, the A-2 probe was tested for its ability to protect an RNA species of 162 bases after S1 digestion. This size was predicted based on the calculated distance between the +1 site identified in Fig. 3A and the *Sal*I end of the probe. A protected hybrid of about this size was indeed observed after S1 mapping (Fig. 4A, lane 2), which was conspicuously absent in the control lane (lane 1, RNA without probe). That the protected capped transcript migrated as a species which was a few nucleotides longer than expected may be explained by the presence of the 5′ cap ($G^{5'}ppp^{5'}Np. . .$), which is also known to sterically hinder S1 digestion (34, 51). In this experiment we were unable to detect a larger protected species which would correspond to the weak −11 band observed in Fig. 3A (see Discussion).

To map the *cex* mRNA 5′ end in a similar fashion, the B-2 probe was used to determine whether it could protect an RNA species about 69 bases long. However, the analysis could not resolve the specific signal from a background of nuclease-treated, unhybridized RNA in this size range (results not shown). Therefore the B-2a probe was used to protect an RNA species which would be slightly longer and resolve higher in the gel. This B-2a probe was tested for its ability to protect an RNA species of 246 bases after S1 digestion. This size was based on the calculated distance between the +1 site identified in Fig. 3B and the *Sau*3A1 site of the probe within *cex*. A protected hybrid of this size was observed (Fig. 4B, lane 2) which was not present in the control lane (lane 1, RNA without probe). These results confirmed the previous mapping of start sites for *cenA* and *cex* transcription.

The DNA sequences immediately upstream of the mapped mRNA start sites of *cenA* and *cex* are compared in Fig. 5.

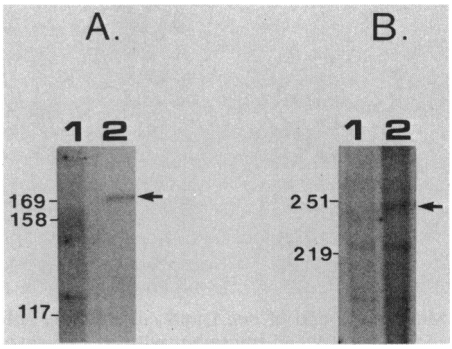

FIG. 4. S1 mapping with *C. fimi* RNA labeled with guanylyltransferase and [α-^{32}P]GTP. After hybridization between DNA probes and *C. fimi* RNA and S1 and RNase A treatment, *cenA*- and *cex*-specific hybrids were analyzed on 5% polyacrylamide–7 M urea gels. The numbers on the left indicate size and migration of M13mp11 *Hae*III fragments. The arrows on the right indicate the specific probe-protected RNA species (lane 2). Results of the parallel negative control experiments without probe added to the labeled RNA are also shown (lane 1). (A) S1 protection of labeled RNA by *cenA* *Sma*I-*Sal*I fragment (Fig. 1, A-2). (B) S1 protection of labeled RNA by *cex* *Sau*3A1 fragment (Fig. 1, B-2a).

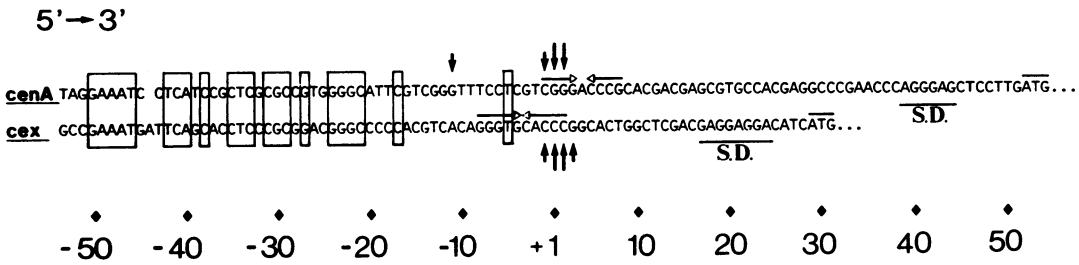

FIG. 5. DNA sequences corresponding to the 5'-terminal regions of *cenA* and *cex* mRNA. The numbering of bases starts with the mRNA start site (+1). The 3' nucleotides of the protected fragments are denoted by arrows whose lengths are approximately proportional to the intensities of the bands in the gels shown in Fig. 3. The ATG initiation codons are overlined. Putative Shine-Dalgarno-type ribosomal binding sites (S.D.) are underlined. Inverted repeats are overlined, with open arrowheads. The sites of conserved DNA sequences located upstream of the mapped mRNA start sites are boxed. The transcriptional maps of *cenA* and *cex* are shown at the top and bottom, respectively.

The sequences are arranged so that the 5' termini as identified by the S1 protection experiments are aligned. The DNA sequence between positions −21 and −51 from the mRNA start sites of both genes is 66% conserved. Only one gap was introduced, 44 bases upstream from the *cenA* +1 site.

Mapping *cenA* and *cex* 3' termini with S1 nuclease. To identify the 3' ends of the *cenA* and *cex* mRNA, transcripts synthesized in vivo were analyzed by S1 nuclease mapping with the 3'-end-labeled probes A-3 (labeled at the *Bgl*II site) and B-3 (labeled at the *Sau*3A1 site) as shown in Fig. 1.

Three distinct *cenA*-specific hybrids were protected from S1 nuclease degradation (Fig. 6A). These three termination sites mapped to positions 1,438, 1,449, and 1,464 bases from the initiation site (+1) of *cenA* mRNA. In Fig. 7 (top), it can be seen that these termination sites fell 41, 52, and 67 bases, respectively, downstream of the *cenA* stop codon in a region of *C. fimi* DNA where inverted repeats can be found (52). Only one strong *cex*-specific hybrid was identified by 3' S1 mapping (Fig. 6B). This termination site mapped 1,564 bases from the *cex* mRNA initiation site (+1), is 83 bases downstream of the *cex* stop codon, and is also in a region of inverted repeats (Fig. 7, bottom) (36).

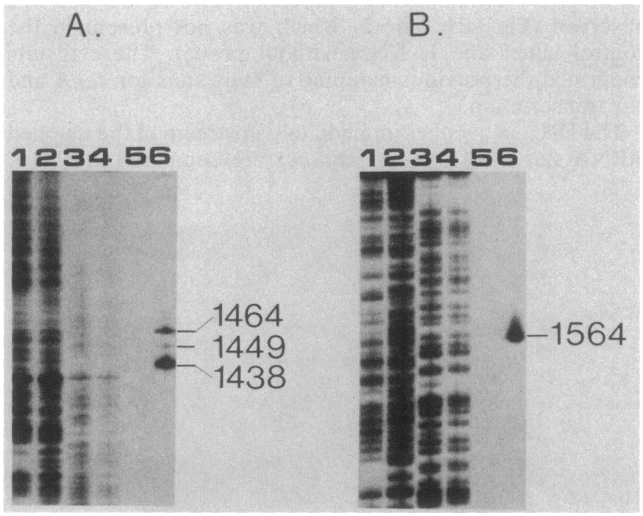

FIG. 6. Mapping 3' end of *cenA* and *cex* mRNA. After hybridization with *C. fimi* RNA and treatment with S1 nuclease, *cenA*- or *cex*-specific ^{32}P-labeled DNA probes were analyzed on 5% polyacrylamide–8.3 M urea sequencing gels alongside probe sequenced by the method of Maxam and Gilbert (30). (A). S1 protection of *cenA Bgl*II (site of 3' end labeled with ^{32}P)-*Sma*I fragment (Fig. 1, A-3) by RNA from glucose-grown cells (lane 5) and from CMC-grown cells (lane 6). Lanes 1 through 4 contained chemical sequencing ladders G > A, G + A, T + C, and C > T, respectively. (B) S1 protection of *cex Sau*3A1 (site of 3' end labeled with ^{32}P)-*Sal*I fragment (Fig. 1, B-3). Lanes are as indicated in panel A. Numbers on the right denote distance (in nucleotides) from the +1 site (see Fig. 5).

DISCUSSION

We investigated the in vivo mRNA transcripts of the *cenA* and *cex* genes of *C. fimi*, which encode the extracellular enzymes Eng and Exg respectively.

Both *cenA*- and *cex*-specific transcripts were detected by Northern blot analysis of *C. fimi* RNA prepared from cells grown on CMC, a soluble cellulosic substrate known to induce both Eng and Exg production in *C. fimi* (Fig. 2A and B, lanes 3) (28). These transcripts were found to be about 1,400 and 1,500 nucleotides long, respectively. Interestingly, a 1,400-nucleotide mRNA species was detected by the *cenA* probe in significant amounts with RNA prepared from cells grown on glycerol (Fig. 2A, lane 1), a carbon source not known to induce cellulase synthesis in *Cellulomonas* spp. (1). No *cex* mRNA was detected in these preparations (Fig. 2B, lane 1). Although not quantitative, these results do indicate that under growth conditions which are noninducing, the level of *cenA* mRNA in the cell appears to be higher than the level of *cex* mRNA, assuming the turnover rates of these mRNAs are the same.

A low level of constitutive expression of extracellular cellulases, most notably endoglucanases, has been observed in many cellulolytic organisms (11, 18, 22, 23). The function of the constitutively expressed enzymes is presumably to generate low-molecular-weight cellulose-specific degradation products which can act as inducers once a suitable substrate is encountered (39). This could explain why *cenA* mRNA was detected in RNA prepared from glycerol-grown cultures of *C. fimi*.

With the Northern blot technique, neither *cenA* nor *cex* mRNAs could be detected in RNA prepared from glucose-grown cultures of *C. fimi*, even after prolonged exposure of the film (results not shown). This strongly suggests that both *cenA* and *cex* are subject to repression at the transcriptional level by the readily assimilated glucose carbon source.

Catabolite repression has been recognized as one form of regulatory control of cellulase biosynthesis in *C. fimi* (1) and other procaryotic and eucaryotic cellulolytic organisms (10, 11, 14, 18, 35, 37, 38, 43). However, the molecular mechanism of this repression in cellulolytic bacteria has remained relatively uncharacterized apart from attempts to isolate strains insensitive to catabolite repression (14, 43) or to

FIG. 7. DNA sequences corresponding to the 3'-terminal regions of *cenA* and *cex* mRNA. Shown are the DNA sequences downstream of the translational stop codons of *cenA* (top) and *cex* (bottom). Only the noncoding strands are shown (5'→3', left to right). The numbering corresponds to the number of bases from the +1 sites (Fig. 5). The stop codons are overlined. The arrows, whose lengths are approximately proportional to the intensities of the bands on the S1 gels (Fig. 6), denote the corresponding 5' nucleotides of the protected fragments. The inverted repeats are overlined, with open arrowheads.

reconstruct cellulolytic systems in more suitable hosts by molecular cloning (2, 11, 16, 27). While the effect of catabolite repression on gene expression has been well studied in *E. coli* (24, 38), we believe that this is the first report of repression of cellulase biosynthesis at the level of transcription in *C. fimi* as a consequence of growth on glucose.

To map the *C. fimi* promoters for *cenA* and *cex*, the 5' ends of *cenA* and *cex* mRNAs were mapped by nuclease S1 protection studies with DNA probes that had been 5' labeled to high specific activities. With the 5'-end-labeled probe A-2, four *cenA* mRNA 5' termini were found between 62 and 50 bases upstream from the ATG codon, three closely spaced and one about 11 bases further upstream. This suggested that *cenA* transcription was being directed from two promoters: promoter $cenAp_1$ directed transcription from position +1, which appeared to represent the strongest signal on the S1 gels, and the promoter $cenAp_2$ directed transcription from position −11, which was a weak signal relative to the +1 signal.

With the 5'-end-labeled probe B-2, four *cex* mRNA 5' termini were found between 26 and 29 bases from the ATG codon. We could not detect any bands higher in the S1 gels, even after prolonged exposure (data not shown). Transcription of the *cex* gene appears to be directed from only one promoter in this region.

Since the transcript maps obtained for *cenA* and *cex* with high-specific-activity 5'-labeled DNA probes could represent the location of 5' termini of processed message, we took advantage of the vaccinia virus capping enzyme to label, in vitro, the *C. fimi* RNA from CMC-grown cells and map it by nuclease S1 studies with unlabeled DNA restriction fragments. Guanylyltransferase can only cap the 5' di- and triphosphate ends of RNA primary transcripts with GMP (34). Using an [α-^{32}P]GTP donor, we were able to label the RNA to a specific activity sufficient to detect mRNAs present in the 0.003 to 0.01% range. The rationale was that since only primary transcripts could be labeled, only labeled primary transcripts would be protected by *cenA*- or *cex*-specific, unlabeled, DNA probes in an S1 mapping study.

As a result of this procedure, we were able to confirm that we had mapped transcriptional start sites for both *cenA* and *cex*. However, we could not confirm that there are two promoters for the *cenA* gene, although this had been indicated in the previous experiments. One possibility is that there are indeed two tandem promoters for *cenA*. The *cenA*-specific 5'-labeled DNA probe displayed a weaker signal at −11 than at +1 (Fig. 3A). In the RNA population labeled in vitro with guanylyltransferase, the specific activity of the RNA labeled in this fashion was only about 1/10 that of the 5' labeled probe. Therefore, the signal from the mRNAs originating at −11 would probably go undetected in our system. Of course, further biochemical studies would be needed to confirm this observation.

The finding that only G and C corresponded to the start sites mapped by nuclease S1 protection studies was not unexpected considering the unusually high G+C content of *C. fimi* DNA. However, this observation is of interest since most procaryotic gene transcripts initiate with G or A in A+T rich stretches of DNA (20, 33, 40), although exceptions are known (6, 7, 20, 21, 33, 40).

When the regions of DNA upstream of the sites where both genes initiate transcription were compared they displayed considerable sequence homology (Fig. 5). The highly conserved sequences 20 bases upstream of the start sites of both genes do not resemble known consensus promoters as recognized by *E. coli*, *Bacillus subtilis*, or *Streptomyces* RNA polymerase holoenzymes (6, 7, 19, 20). However, as shown in Fig. 8, there are a number of similarities between positions only 4 bases upstream of the identified start sites of both *cenA* and *cex* and −10 regions of other characterized promoters. Of particular interest are the −10 region homologies between the putative $cenAp_1$ and *cex* promoters and the *tet* promoter of pBR322. In addition, the putative $cenAp_2$ promoter, which probably does not direct efficient transcription in *E. coli* (52), shows considerable −10 region homology with the $tsrp_1$ promoter of *Streptomyces azureus*, which is

PROMOTER	-35 REGION		-10 REGION		mRNA START
E. coli / B. subtilis	TTGACa	-17bp-	TAtAaT		
pBR322 tet	GTTTGACA	-17bp-	TTTAAT	-6bp-	G
cenA P1	TCGCGCCG	-16bp-	TTTCCT	-4bp-	G
cex	TCCCGCGG	-16bp-	ATTCGT	-4bp-	C
cenA P2	TCCTCATC	-16bp-	CAGGGT	-4bp-	G
tsr P1	TCAGGGCA	-19bp-	TAGGGT	-6bp-	A
Streptomyces	TTGaca	-17bp-	tAGgaT		

FIG. 8. Homology of putative *C. fimi* promoters for "−10" and "−35" regions with other procaryotic consensus promoter sequences. Shown are comparisons of the putative $cenAp_1$, $cenAp_2$, and *cex* promoters with the *E. coli*/*B. subtilis* consensus promoter (20), pBR322 *tet* promoter (20), *Streptomyces* consensus promoter (21), and $tsrp_1$ promoter (21). Matches are denoted by vertical lines.

not known to direct efficient transcription in *E. coli* or *B. subtilis* (21).

The high degree of sequence conservation in the region 20 to 50 bases upstream from the +1 sites of *cenA* and *cex*, which overlaps their putative −35 regions, suggests that these sequences have a common regulatory function. Possibly these sequences are binding sites for regulatory elements and, together with the promoter sequences, could play a role in the regulation of transcription of *cenA* and *cex*.

The inverted repeats immediately downstream of the translational stop codons resemble rho-independent termination signals as found in *E. coli* (40, 54). The mapping of transcript 3′ ends in these regions suggested that these sequences function as termination signals for transcription in *C. fimi*.

The complete nucleotide sequences of *cenA* (52) and *cex* (36) have been determined. The minimum length of genetic material needed to encode their characterized Eng and Exg proteins, from the first base of the initiation codon to the last base of the stop codon, is 1,350 bases for *cenA* and 1,455 bases for *cex*. We mapped the 5′ and 3′ termini of the *cenA* and *cex* transcripts and found them to be about 1,464 and 1,564 bases long, respectively. ENG and EXG, then, appear to be translated from monocistronic mRNAs.

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LITERATURE CITED

1. **Béguin, P., H. Eisen, and A. Roupas.** 1977. Free and cellulase-bound cellulases in a *Cellulomonas* species. J. Gen. Microbiol. **101**:191–196.
2. **Béguin, P., M. Rocancourt, M.-C. Chebrou, and J.-P. Aubert.** 1986. Mapping of mRNA encoding endoglucanase A from *Clostridium thermocellum*. Mol. Gen. Genet. **202**:251–254.
3. **Bergey, D. H., R. S. Breed, R. W. Hammer, F.-C. Harrison, and F. M. Huntoon.** 1923. Bergey's manual of determinative bacteriology, 1st ed. p. 165. The Williams & Wilkins Co., Baltimore.
4. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNA's by gel electrophoresis of S1 endonuclease digested hybrids. Cell **12**:721–732.
5. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNA's. Cell **14**:695–711.
6. **Bibb, M. J., M. J. Bibb, J. M. Ward, and S. N. Cohen.** 1985. Nucleotide sequences encoding and promoting expression of three antibiotic resistance genes indigenous to *Streptomyces*. Mol. Gen. Genet. **199**:26–36.
7. **Bibb, M. J., G. R. Janssen, and J. M. Ward.** 1985. Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene **38**:215–226.
8. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
9. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, A. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
10. **Canevascini, G., M. R. Coudray, J. P. Rey, J. G. Southgate, and H. Meier.** 1979. Induction and catabolite repression of cellulase synthesis in the thermophilic fungus *Sporotrichium thermophile*. J. Gen. Microbiol. **110**:291–303.
11. **Coughlan, M. P.** 1985. The properties of fungal and bacterial cellulases with comment on their production and application. Biotechnol. Genet. Eng. Rev. **3**:39–109.
12. **Ehrenberg, L., I. Fedorcsak, and F. Solymosy.** 1976. Diethylpyrocarbonate in nucleic acid research. Prog. Nucleic Acid Res. Mol. Biol. **16**:189–262.
13. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
14. **Fennington, G., D. Neubauer, and F. Stutzenberger.** 1984. Cellulase biosynthesis in a catabolite repression-resistant mutant of *Thermomonospora curvata*. Appl. Environ. Microbiol. **47**:201–204.
15. **Gilkes, N. R., D. G. Kilburn, M. L. Langsford, R. C. Miller, Jr., W. W. Wakarchuk, R. A. J. Warren, D. J. Whittle, and W. K. R. Wong.** 1984. Isolation and characterization of *Escherichia coli* clones expressing cellulase genes from *Cellulomonas fimi*. J. Gen. Microbiol. **130**:1377–1384.
16. **Gilkes, N. R., D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1984. A mutant of *Escherichia coli* that leaks cellulase activity encoded by cloned cellulase genes from *Cellulomonas fimi*. Bio/Technology **2**:259–263.
17. **Gilkes, N. R., M. L. Langsford, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1984. Mode of action and substrate specificities of cellulases from cloned bacterial genes. J. Biol. Chem. **259**:10455–10459.
18. **Gong, C. S., and G. T. Tsao.** 1979. Cellulase and biosynthesis regulation. Annu. Rep. Ferment. Processes **3**:111–140.
19. **Grossman, A. D., and R. Losick.** 1986. RNA polymerase heterogeneity in bacteria. Symp. Soc. Gen. Microbiol. **39**:127–138.
20. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11**:2237–2255.
21. **Hopwood, D. A., M. J. Bibb, K. F. Chater, G. R. Janssen, F. Malpartida, and C. P. Smith.** 1986. Regulation of gene expression in antibiotic producing *Streptomyces*. Symp. Gen. Microbiol. **39**:251–276.
22. **Hulme, M. A., and D. W. Stranks.** 1970. Induction and the regulation of production of cellulases by fungi. Nature (London) **226**:469–470.
23. **Hulme, M. A., and D. W. Stranks.** 1971. Regulation of cellulase production by *Myrothecium verrucaria* grown on non-cellulosic substrates. J. Gen. Microbiol. **69**:145–155.
24. **Jacob, F., and J. Monod.** 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**:318–356.
25. **Keddie, R. M.** 1974. Genus III. *Cellulomonas* Bergey et al. 1923, 154, emend. mut. char. Clark 1952, 50, p. 629–631. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
26. **Kennell, D., and I. Bicknell.** 1973. Decay of messenger ribonucleic acid from the lactose operon of *Escherichia coli* as a function of growth temperature. J. Mol. Biol. **74**:21–31.
27. **Koide, Y., A. Nakamura, T. Uozumi, and T. Beppu.** 1986. Molecular cloning of a cellulase gene from *Bacillus subtilis* and its expression in *Escherichia coli*. Agric. Biol. Chem. **50**: 233–237.
28. **Langsford, M. L., N. R. Gilkes, W. W. Wakarchuk, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1984. The cellulase system of *Cellulomonas fimi*. J. Gen. Microbiol. **130**:1367–1376.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
31. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–79.
32. **Miller, R. C., Jr., E. T. Young II, R. H. Epstein, H. M. Krisch, T. Mattson, and A. Bolle.** 1981. Regulation of the synthesis of the T4 DNA polymerase (gene 43). Virology **110**:98–112.
33. **Moran, C, P., N. Lang, S. F. C. LeGrice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and transla-

tion in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.
34. **Moss, B.** 1981. 5′ end labelling of RNA with capping and methylating enzymes, p. 253–266. *In* J. G. Chirikjian and T. S. Papas (ed.), Gene amplification and analysis, vol. 2. Elsevier/North-Holland Publishing Co., Amsterdam.
35. **Nisizawa, T., H. Suzuki, and K. Nisizawa.** 1972. Catabolite repression of cellulase formation in *Trichoderma viride*. J. Biochem. **71**:999–1007.
36. **O'Neill, G., S. H. Goh, R. A. J. Warren, D. G. Kilburn, and R. C. Miller, Jr.** 1986. Structure of the gene encoding the exoglucanase of *Cellulomonas fimi*. Gene **44**:325–330.
37. **Paigen, K., and B. Williams.** 1970. Catabolite repression and other control mechanisms in carbohydrate utilization, p. 251–324. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, vol. 4. Academic Press, Inc. (London), Ltd., London.
38. **Postma, P. W.** 1986. Catabolite repression and related processes. Symp. Soc. Gen. Microbiol. **39**:21–49.
39. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41**:711–753.
40. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
41. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
42. **Stackebrandt, E., and O. Kandler.** 1979. Taxonomy of the genus *Cellulomonas*, based on phenotypic characters and deoxyribonucleic acid-deoxyribonucleic acid homology, and proposal of seven neotype strains. Int. J. Syst. Bacteriol. **29**:273–282.
43. **Stewart, B. J., and J. M. Leatherwood.** 1976. Derepressed synthesis of cellulase by *Cellulomonas*. J. Bacteriol. **128**: 609–615.
44. **Thayer, D. W., S. V. Lowther, and J. G. Phillips.** 1984. Cellulolytic activities of strains of the genus *Cellulomonas*. Int. J. Syst. Bacteriol. **34**:432–438.
45. **van Wezenbeek, P. M. G. F., T. J. M. Hulsebos, and J. G. G. Schoenmakers.** 1980. Nucleotide sequence of the filamentous bacteriophage M13 DNA genome: comparison with phage fd. Gene **11**:129–148.
46. **Vieira, J., and J. Messing.** 1982. The pUC plasmids and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
47. **von Gabain, A., J. G. Belasco, J. L. Schottel, C. Y. Chang, and S. N. Cohen.** 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA **80**:653–657.
48. **Wakarchuk, W. W., D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1984. The preliminary characterization of the β-glucosidases of *Cellulomonas fimi*. J. Gen. Microbiol. **130**:1385–1389.
49. **Weaver, R. F., and C. Weissman.** 1979. Mapping of RNA by a modification of the Berk and Sharp procedure: the 5′ termini of 15S β-globin mRNA precurssor and mature 10S β-globin mRNA have identical map units. Nucleic Acids Res. **7**:1175–1193.
50. **Whittle, D. J., D. G. Kilburn, R. A. J. Warren, and R. C. Miller, Jr.** 1982. Molecular cloning of a *Cellulomonas fimi* cellulase gene in *Escherichia coli*. Gene **17**:139–145.
51. **Wich, G., H. Hummel, M. Jarsch, U. Bar, and A. Bock.** 1986. Transcription signals for stable RNA genes in *Methanococcus*. Nucleic Acids Res. **14**:2459–2479.
52. **Wong, W. K. R., B. Gerhard, Z. M. Guo, D. G. Kilburn, R. A. J. Warren, and R. C. Miller, Jr.** 1986. Characterization and structure of an endoglucanase gene of *Cellulomonas fimi*. Gene **44**:315–324.
53. **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.
54. **Yanofsky, C.** 1981. Attenuation in the control of expression of bacterial operons. Nature (London) **289**:751–758.