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Structure, Organization, and Transcription of a Cellobiohydrolase Gene Cluster from *Phanerochaete chrysosporium*

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Restriction mapping and sequence analysis of cosmid clones revealed a cluster of three cellobiohydrolase genes in *Phanerochaete chrysosporium*. *P. chrysosporium cbh1-1* and *cbh1-2* are separated by only 750 bp and are located approximately 14 kb upstream from a cellulase gene previously cloned from *P. chrysosporium* (P. Sims, C. James, and P. Broda, Gene 74:411–422, 1988). Within a well-conserved region, the deduced amino acid sequences of *P. chrysosporium cbh1-1* and *cbh1-2* are, respectively, 80 and 69% homologous to that of the *Trichoderma reesei* cellobiohydrolase I gene. The conserved cellulose-binding domain typical of microbial cellulases is absent from *cbh1-1*. Transcript levels of the three *P. chrysosporium* genes varied substantially, depending on culture conditions. *cbh1-1* and *cbh1-2* were not induced in the presence of cellulose, nor did they appear to be subject to glucose repression. Therefore, aspects of the chromosomal organization, structure, and transcription of these genes are unlike those of any previously described cellulase genes.

The white rot fungus *Phanerochaete chrysosporium* has been widely studied as a model organism for lignocellulose degradation (see references 7 and 24 for reviews). In submerged culture, *P. chrysosporium* secretes a complex array of degradative enzymes, including three classes of hydrolytic cellulases: endoglucanases (EG) (E.C.3.2.1.4.), cellobiohydrolases (CBH) (E.C.3.2.1.91.), and β-glucosidases (E.C.3.2.1.21). Eriksson and Pettersson have identified five EG (9), one CBH (10), and two β-glucosidases (2) from *P. chrysosporium* cultures. More recently, multiple CBH-like enzymes have been characterized (19, 45, 46). Similar to other fungal cellulases, the EG and CBH are reported to act synergistically on crystalline cellulose (42). *P. chrysosporium* cellulases are induced by cellulose and certain cellulose derivatives, such as cellobiose, sophorose, and carboxymethyl cellulose; they are catabolite repressed by glucose (8).

Studies of bacterial and fungal cellulases have revealed a common structural design composed of discrete functional domains: a catalytic core, a conserved cellulose-binding terminus, and an intervening, highly glycosylated hinge region (see references 14 and 25 for reviews). *P. chrysosporium* cellulases also appear to be organized in accordance with this model. For example, as with other cellulases, papain cleavage of *P. chrysosporium* CBH separates the catalytic domain from the hinge and binding domains (20, 46). In addition, a CBH gene cloned from *P. chrysosporium* ME446 (39) is similar in sequence to other fungal CBH genes, especially *Trichoderma reesei cbh1* (38) and *Humicola grisea cbh-1* (1).

The most widely studied cellulolytic fungus, *T. reesei*, contains four distinct cellulase genes which encode CBHI and CBHII and EGI and EGIII. No evidence has been presented for linkage among the *T. reesei* genes, and multiple copies of closely related cellulase genes have not been reported to exist in *T. reesei* or in *P. chrysosporium*. We describe here a cluster of three structurally related CBH genes from *P. chrysosporium*, one of which lacks the cellulose-binding domain common to other microbial cellulases. We also show that the transcript levels from these genes vary considerably under different culture conditions.

MATERIALS AND METHODS

Isolation of clones. A *P. chrysosporium* BKM-1767 (ATCC 24725) genomic library was constructed in cosmid pKBY2 (48) and probed under conditions of low stringency (0.125 M Na_2HPO_4, 35% formamide, 7% sodium dodecyl sulfate, 1 mM EDTA at 37°C) with a 700-bp *Eco*RI fragment from *T. reesei cbh1*. The *T. reesei cbh1* clone was provided by Genencor (South San Francisco, Calif.). Several hybridizing cosmids were identified and characterized. One of these, pD76, was found to contain two *cbh1*-like genes, *P. chrysosporium cbh1-1* and *cbh1-2*. A second genomic library, constructed in cosmid pWE15 (Stratagene, LaJolla, Calif.), was probed as described above, with a 520-bp *Bam*HI fragment from *P. chrysosporium cbh1-1*. The probe was derived from a region of *P. chrysosporium cbh1-1* which is well conserved relative to other *cbh1*-like genes. Several *cbh1*-containing subclones of the cosmids were constructed, and the dideoxy-chain termination method (36) was used to sequence these M13 mp18/mp19 and double-stranded templates. Both strands were sequenced by using universal primers and synthetic oligonucleotides.

A restriction map of pWE15 cosmid pO7, which contains the three genes, was constructed by subcloning fragments and by Southern blot analyses. The veracity of this map and the genomic contiguity between genes were demonstrated by Southern blots in which genomic DNA was digested with *Sma*I, *Not*I, *Eco*RV, and *Pst*I, blotted, and probed with different fragments from pO7 (Fig. 1 and data not shown).

After putative *cbh1* genes were identified by sequence

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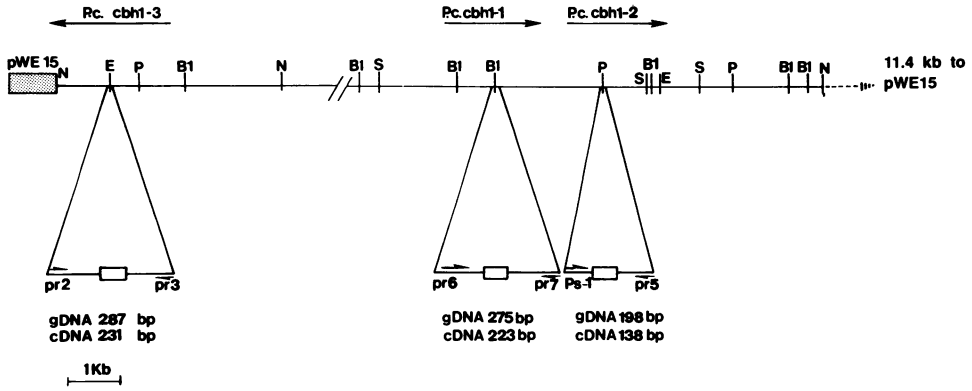

FIG. 1. Partial restriction map of pWE15 cosmid pO7 and PCR amplification strategy. The positions and transcriptional orientations of genes are shown by arrows. Approximately 9 kb of the map is not shown between the diagonal hatch marks. Thus, the distance between the translational start codons of *P. chrysosporium cbh1-3* and *cbh1-1* is approximately 14 kb. To illustrate the PCR strategy, amplified regions surrounding a conserved intron (open box) are shown below, with half arrows identifying primers and their relative positions. The predicted sizes of target cDNAs and the competitive templates (genomic DNAs [gDNAs]) are indicated. Known restriction sites for *Bam*HI (B1), *Pst*I (P), *Eco*RV (E), and *Not*I (N) are shown.

analysis of cosmids, full-length cDNAs that encode *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3* were amplified by the polymerase chain reaction (PCR) and subcloned. Modifications of standard procedures were used (e.g., see reference 23). Primers were located 3′ and 5′ of translational stop and start codons, respectively. These downstream and upstream primers were as follows:

5′-TGAATTCCACCAATATTCACGCAGG-3′ and
5′-GAGAATTCTGAAACCGCTACACATT-3′
for *P. chrysosporium cbh1-1*
5′-CCTTGTGACCCCAGCATTAGATACA-3′ and
5′-GAGAATTCGAGGTTGAGACGCGATA-3′
for *P. chrysosporium cbh1-2*
5′-GAGAATTCCTTAGTAGCACTGGGA-3′ and
5′-GAGAATTCTACCGTCTGCTCACACT-3′
for *P. chrysosporium cbh1-3*

First-strand synthesis of *P. chrysosporium cbh1-3* and *cbh1-1* involved reverse transcription of total RNA derived from cellulolytic cultures by using the downstream primers, whereas *cbh1-2* was amplified by PCR from double-stranded cDNA synthesized with an oligo(dT) primer (Amersham Inc.). Amplified *P. chrysosporium cbh1-2* cDNA was subcloned directly into pPCR1000 (28). Following digestion with *Eco*RI, *P. chrysosporium cbh1-1* and *cbh1-3* cDNAs were ligated into pT7T3 18U (Pharmacia Inc.) and pKSII (Stratagene Inc.), respectively.

Northern (RNA) analysis. *P. chrysosporium* was grown at 37°C and 120 rpm in 330 ml of modified Norkran's medium (8) amended with one of three carbon sources: 0.5% glucose, 1.0% sucrose, or 0.1% cellobiose plus 0.4% Whatman CF11 powdered cellulose. Earlier work demonstrated that when used alone, insoluble powdered cellulose serves as a poor carbon source for *P. chrysosporium*. The cellobiose, therefore, was added as an easily utilized, soluble carbon source that is also a powerful inducer of endoglucanases in *P. chrysosporium* (8). Glucose-grown cultures were harvested after 3 and 7 days. Sucrose-grown cultures were harvested on day 5. The cellobiose-cellulose cultures were harvested when cellulose azure (Calbiochem, LaJolla, Calif.) indicated cellulase activity in the medium (approximately 4 days). Total RNA was isolated from cultures as previously described (43). Four micrograms of total RNA per well was size fractionated on a 1% agarose–formaldehyde gel, blotted onto Hybond-N (Amersham, Arlington Heights, Ill.), and cross-linked with a UV Stratalinker 1800 (Stratagene). The blot was probed as previously described (30), with prehybridization at 50°C for 2.5 h, hybridization at 40°C overnight, two washes at room temperature for 15 min each, and one wash at 48°C for 5 min. Oligonucleotide probes corresponding to poorly conserved regions of the *P. chrysosporium cbh1*-like genes were chosen to avoid hybridization to related transcripts. Oligonucleotides 5′-CGGATTGAGAATC CCCACATCCGC-3′, 5′-ATGCTGGCAGAGTCCTGAGGA CAG-3′, and 5′-AGGAGCTCGTGGACGTGGAGGAGT-3′ were used to detect transcripts of *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3*, respectively.

PCR quantitation of transcripts. Relative transcript levels of *P. chrysosporium cbh1* genes were established by competitive PCR. First-strand cDNAs were prepared by reverse transcription with downstream primers. Double-stranded cDNAs were then amplified by PCR by using upstream primers. Included in the PCRs was a competitive template in the form of genomic DNA. The competitor was added in a series of dilutions with known concentrations. Introns within the competitive template allowed the target cDNA and genomic product to be size fractionated on agarose gels.

Primers were synthesized by the β-cyanoethyl phosphoramidite method and used without further purification. Downstream primers for *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3* were 5′-CAAAAGCCCGTGTTGGAGGT-3′ (pr7), 5′-CGTGGTACCAAGCCAGCCTT-3′ (pr5), and 5′-GCAGT CGCTTCCGGAGCAGC-3′ (pr2), respectively. Upstream primers were 5′-CACAGTGTGTCCAAGGGATGTC-3′ (pr6), 5′-GCCGATGGTGGACTTGC-3′ (Ps1), and 5′-GCTAAGT ACGGTACCGGCTA-3′ (pr3). Primer positions flanked an intron common to all three genes (Fig. 1).

First-strand syntheses and subsequent PCR amplifications were done as previously described (16), with minor modifications. Reverse transcription reactions took 45 min at 42°C with 50 U of Moloney murine leukemia virus enzyme (GIBCO BRL, Gaithersburg, Md.). Each 20-μl reaction also included 40 U of RNasin (Promega Biotech Inc., Madison, Wis.), 18 pmol of a downstream primer, and 20 μg of total RNA. PCRs were done in 100-μl total volumes by using 5 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk,

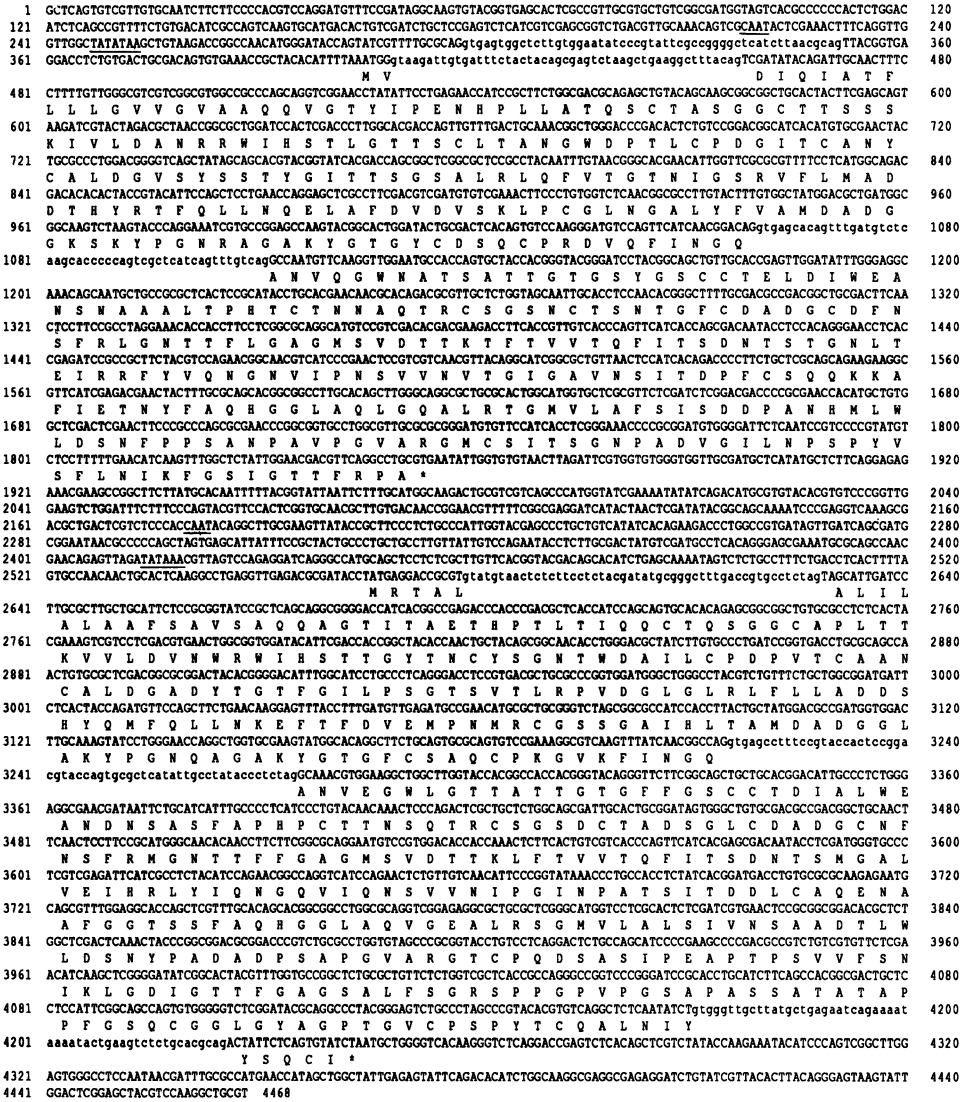

FIG. 2. Nucleotide sequence of the 4.5-kb region containing *P. chrysosporium cbh1-1* and *cbh1-2* and their deduced amino acid sequences. Asterisks denote stop codons. Introns are indicated by lowercase letters. Putative CAAT and TATA promoter elements are underlined. Introns were identified by PCR amplification of cDNAs (data not shown).

Conn.) and 18 pmol of each primer. A competitive template in the form of cosmid pO7 was present at concentrations ranging from 10^{-7} to 10^{-20} g/100 μl of the reaction mixture. This broad range of competitive template concentrations was adequate for the level of precision sought, but finer titrations of templates would allow precision to approach twofold differences in cDNA concentrations (16). Reactions were subjected to an initial cycle of denaturation (6 min, 94°C), annealing (2 min, 54°C), and prolonged extension (40 min, 72°C), followed by 35 cycles of denaturation (1 min, 94°C), annealing (2 min, 54°C), and extension (5 min, 72°C). A final 15-min extension at 72°C was also included. Following amplification, 20-μl quantities of the PCRs were size fractionated in 2% SeaKem–1% NuSieve agarose gels (FMC Inc., Rockland, Maine), ethidium bromide stained, and photographed.

Nucleotide sequence accession number. The nucleotide sequence of the DNA strand containing *P. chrysosporium cbh1-1* and *cbh1-2* has been deposited in the GenBank library under accession no. X54411.

RESULTS

Cloning and sequence analysis. Cosmid pD76 was isolated from a *P. chrysosporium* library in pKBY2 by probing at low stringency with a 700-bp *Eco*RI fragment from *T. reesei cbh1*. The nucleotide sequence of a 4.5-kb region was determined (Fig. 2), and two open reading frames (*P. chrysosporium cbh1-1* and *cbh1-2*) with homology to *T. reesei cbh1* were identified. *P. chrysosporium cbh1-1* and *cbh1-2* are encoded on the same DNA strand and are separated by approximately 750 bp.

A second cosmid library in pWE15 was probed with a 520-bp *Bam*HI fragment from *P. chrysosporium cbh1-1*. Two of the 23 clones that hybridized to the *P. chrysosporium cbh1-1* fragment closely resembled pD76, the cosmid from

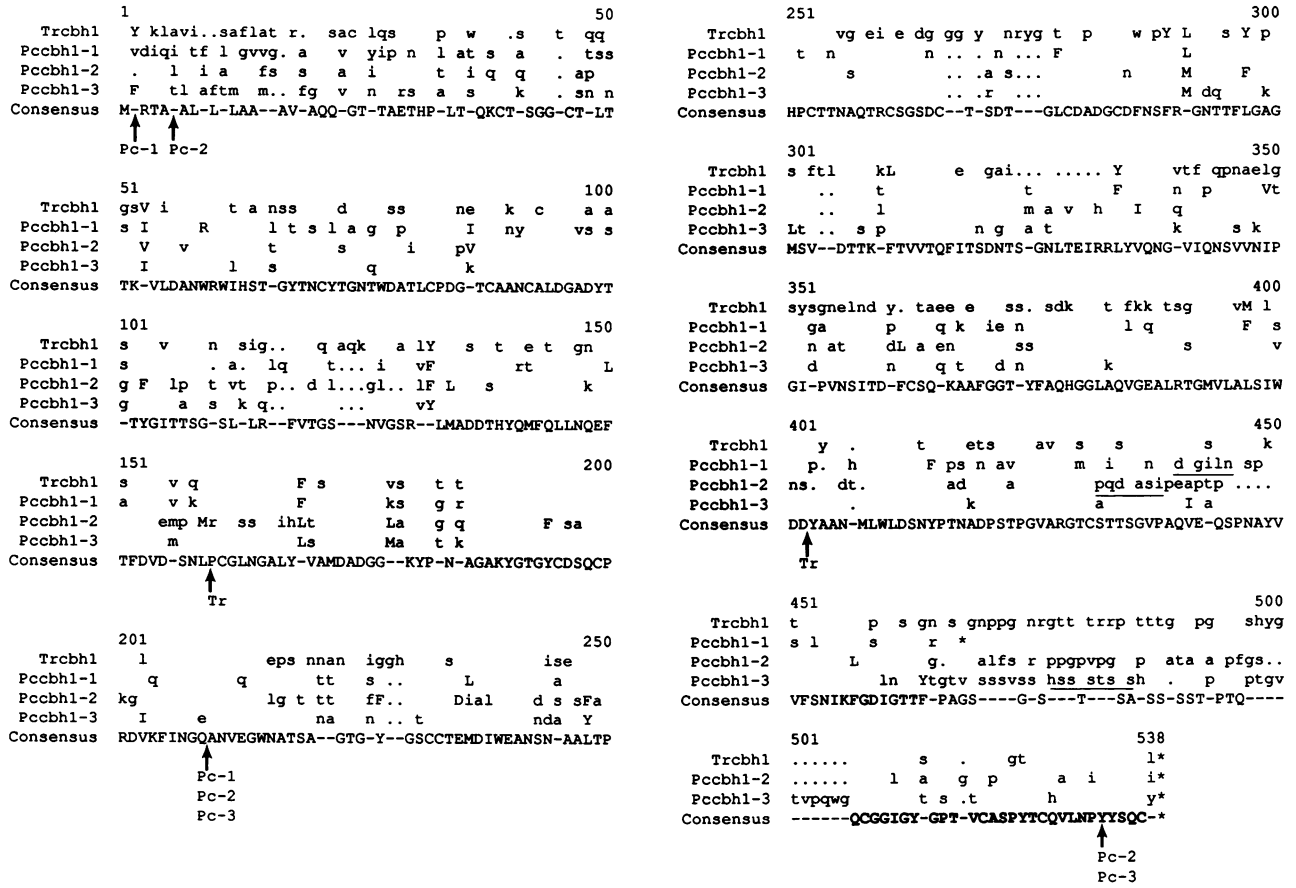

FIG. 3. Deduced amino acid sequence alignment of *T. reesei cbh1* (38) and *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3* (39). Amino acid residues are printed only when they differ from the consensus sequence. Asterisks denote stop codons. Periods indicate spaces introduced to improve alignment. Deduced amino acid homologies (threshold = 1.0) and the consensus sequence (threshold = 1.5) were calculated with the Dayhoff table (37), in conjunction with the University of Wisconsin Genetics Computer Group software (3). A hyphen indicates an absence of consensus. Amino acids highly similar to the consensus or to each other at a given position are shown in uppercase. Underlining indicates the locations of the corresponding oligonucleotides used to probe total RNA. The crystalline cellulose-binding domain is shown in boldface in the consensus. This region is immediately preceded by the hinge domain, which is rich in serine, proline, and threonine residues. Intron positions are indicated by arrows beneath the consensus sequence; Pc-1, Pc-2, Pc-3, and Tr refer to *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3* and *T. reesei cbh1*, respectively.

which *P. chrysosporium cbh1-1* and *cbh1-2* were isolated. Southern analyses of these two cosmids indicated the presence of a third *cbh1*-like open reading frame (*P. chrysosporium cbh1-3*) approximately 14 kb from the previously cloned pair of genes (Fig. 1). *P. chrysosporium cbh1-3* was subcloned and partially sequenced, and particular attention was paid to two areas: the 5′ end of the gene, including 409 bp of the 5′ untranslated region, and a highly conserved region within the core of the gene that is split by an intron. The sequence of this gene, including its 5′ untranslated region and intron, is 99.8% identical to the gene sequence reported previously by Sims et al. (39). There is a single base pair difference within the introns of the two clones; the C at position 1046 in *P. chrysosporium cbh1-3* is a T in our clone. We therefore conclude that the two clones represent allelic variants of the same gene. The clone of Sims et al. (39) was derived from a different *P. chrysosporium* strain, ME446.

Intron positions were determined by sequence analysis of full-length cDNA clones. The location of *P. chrysosporium cbh1-3* introns, previously assigned by alignment with *T. reesei cbh1* and the presence of conserved splice sites (39), was confirmed. Unlike *P. chrysosporium cbh1-3*, *cbh1-1* and *cbh1-2* have introns within the first 15 bp of their coding regions. The third intron of *cbh1-1* and the second intron of *cbh1-2* correspond to the first intron of *cbh1-3*, and the amino acid sequence in this region is highly conserved in all three *P. chrysosporium* genes and in *T. reesei cbh1*. Both *P. chrysosporium cbh1-2* and *cbh1-3* have introns within the last 15 bp of their coding regions. All of the introns in *P. chrysosporium cbh1-1* and *cbh1-2* contain sequences similar to the fungal consensus sequences (17) at their 5′ and 3′ splice sites. Relative to the *cbh1*-like genes in *P. chrysosporium*, intron position in *T. reesei cbh1* is not conserved (Fig. 3). The presence of additional introns within the 5′ and 3′ untranslated regions cannot be ruled out, as the entire transcripts were not amplified.

Cellulases are composed of three structural domains: a core which contains the hydrolytic site, a hinge which protrudes from the core and tends to be glycosylated, and attached to the hinge a highly conserved tail which binds crystalline cellulose (25). The overall structure of *P. chrysosporium cbh1-2* is similar to that of *T. reesei cbh1* in that it contains both the hinge region and the highly conserved tail at its 3′ end (Fig. 3). In contrast, *P. chrysosporium cbh1-1* is

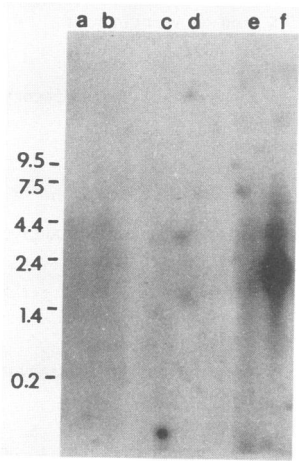

FIG. 4. Northern analysis of total RNA from *P. chrysosporium* grown in glucose or cellobiose-cellulose. Lanes a, c, and e contained RNAs from 3-day-old glucose-grown cultures. Lanes b, d, and f contained RNAs from cellobiose-cellulose-grown cultures. Lanes a and b, c and d, and e and f were probed with *P. chrysosporium cbh1-1*-, *cbh1-2*-, and *cbh1-3*-specific oligonucleotide probes, respectively, as described in Materials and Methods. The sizes of markers are shown on left in kilobases.

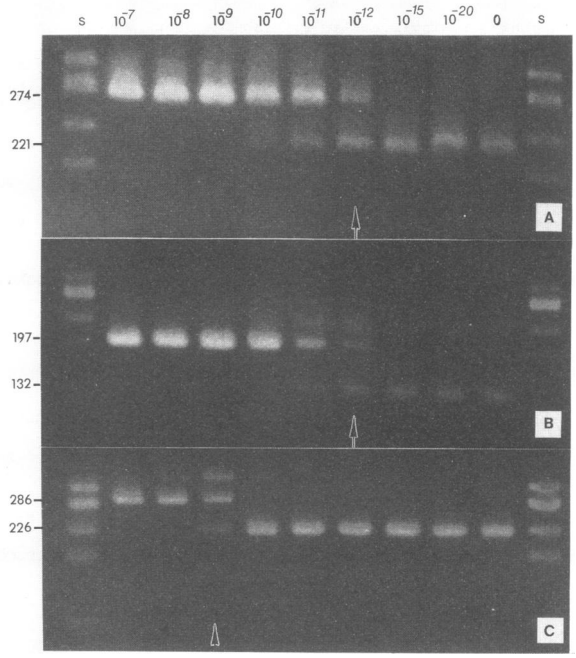

FIG. 5. Competitive PCR quantitation of mRNAs of *P. chrysosporium cbh1-1* (A), *cbh1-2* (B), and *cbh1-3* (C) in cellulose-grown cultures. One-hundred-microliter PCR reactions received from 10^{-7} to 10^{-20} g of a competitive template in the form of cosmid pO7. No cosmid was added to reactions labeled zero. Molecular size standard ϕX174 digested with *Hae*III is shown in lanes S. Predicted sizes of target cDNAs and the larger competitive products are shown on left margins in base pairs. Vertical arrows indicate reactions in which the product target and competitive target were approximately equivalent.

unlike previously described fungal cellulase genes in that it does not contain the hinge or the tail at either terminus. The nucleotide sequence downstream of its stop codon does not encode an amino acid sequence that bears any resemblance to the cellulose-binding tail in any reading frame. However, within a particularly well-conserved region corresponding to residues 124 to 209 of *T. reesei cbh1* (38) (owing to alignment, these appear as residues 130 to 215 in Fig. 3), *P. chrysosporium cbh1-1* is more homologous to *T. reesei cbh1* than is *P. chrysosporium cbh1-2*. Within this region, *P. chrysosporium cbh1-1* is 80% homologous and *P. chrysosporium cbh1-2* is 69% homologous to *T. reesei cbh1*. Since this region is highly conserved among all three sequences, it appears likely to contain residues important in catalysis.

The nucleotide sequences of *P. chrysosporium cbh1-1* and *cbh1-2* are distinct from that of *cbh1-3* (39). *P. chrysosporium cbh1-3* (at 80%) is as homologous as *cbh1-1* to *T. reesei cbh1* within the highly conserved region described above. However, the amino acid sequences of the *P. chrysosporium* genes within this region are not identical (Fig. 3). In addition, both *P. chrysosporium cbh1-1* and *cbh1-2* exhibit substantially reduced codon bias relative to *cbh1-3*. For example, ACA (Thr) is not utilized in *cbh1-3* but represents 16 and 14% of the Thr codons in *cbh1-1* and *cbh1-2*, respectively; CAU (His) is not utilized in *cbh1-3* but represents 33 and 43% of the His codons in *cbh1-1* and *cbh1-2*; and UGU (Cys) represents only 5% of the Cys codons in *cbh1-3* while it is 44 and 36% of the Cys codons in *cbh1-1* and *cbh1-2*. *cbh1-1* and *cbh1-2* encode proteins with predicted molecular masses and isoelectric points of 45 kDa and 5.5 and 50 kDa and 4.3, respectively.

Transcription of *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3*. By using gene-specific oligonucleotide probes (Fig. 3), transcripts of *P. chrysosporium cbh1-3*, but not of *P. chrysosporium cbh1-1* and *cbh1-2*, were detected in total RNAs derived from cellulolytic cultures (Fig. 4). With prolonged exposure of Northern blots, faint hybridization bands were seen (Fig. 4, lanes a, b, and e), but the specificity of these signals was uncertain. Competitive PCR techniques were employed to assess the relative transcript levels of the three genes more precisely. As described by Gilliland et al. (16), the strategy involved reverse transcription, followed by PCR amplification of cDNAs. Several dilutions of a competitive genomic template were added to these PCR amplifications. The initial concentrations of specific cDNAs were determined by estimating dilution points at which the target cDNAs and competitive templates were equivalent.

With this approach, cDNA levels in cultures grown on cellulose-cellobiose were approximately 1 pg, 1 pg, and 1 ng for *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3*, respectively (Fig. 5). Thus, under these conditions, *cbh1-3* transcripts were approximately 1,000-fold more abundant than those of *cbh1-1* and *cbh1-2*.

These analyses were extended to glucose- and sucrose-grown cultures (Table 1). Estimated *P. chrysosporium cbh1-3* cDNA levels were lowest in glucose cultures harvested after 3 days (<1 fg) and approximately equal in sucrose-grown and 7-day-old glucose-grown cultures (10 to 100 pg). Relative to those in cellulose-grown cultures, the *P. chrysosporium cbh1-2* transcript levels increased approximately 10-fold in sucrose-grown cultures and *cbh1-1* showed slight increases in sucrose-grown and 7-day glucose-grown cultures.

DISCUSSION

Cellulase clones appear to encode CBH. The cellulase genes described here bear strong nucleotide similarity to *T. reesei cbh1* (38). Conservation within a region believed likely to

TABLE 1. Transcription of *P. chrysosporium* CBH genes in different carbon sources[a]

Gene	Amt of cDNA produced in the following carbon source[b] (culture age):			
	Cellulose	Sucrose	Glucose (3 days)	Glucose (7 days)
cbh1-1	1 pg	1–10 pg	1 pg–1 fg	10 pg
cbh1-2	1 pg	10 pg	1 pg	1 pg–1 fg
cbh1-3	1 ng	10–100 pg	<1 fg	10–100 pg

[a] Estimated cDNA in competitive PCRs as described in Materials and Methods and illustrated in Fig. 5 for cellulose-derived RNA samples.

[b] Norkran's medium (8) amended with 0.4% cellulose plus 0.1% cellobiose, 1.0% sucrose, or 0.5% glucose. Glucose-grown cultures were harvested on day 3 or during the late stationary phase on day 7.

contain a catalytic site(s) is particularly high. Although the *T. reesei* EG-encoding gene (*egl*) shares significant homology with *T. reesei cbh1* and, by consequence, with *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3*, it is unlikely that the *P. chrysosporium* genes encode EG because they show greater homology to *T. reesei cbh1* than to *T. reesei egl*. In addition, they appear to fit the active-site model proposed to be typical for CBH (35). The deduced amino acid sequences of *P. chrysosporium cbh1-1* and *cbh1-2* contain four regions that are absent from EGI when it is compared with CBHI (33). Rouvinen et al. (35) suggest that the lack of these regions results in an open active site common to EG, whereas their presence creates a tunnel-shaped active site like that found in CBHII.

Although the multiplicity of *cbh1*-like genes is unusual, it is consistent with recent results of Uzcategui et al. (46) and Ishihara et al. (19, 45), who have purified and characterized multiple isozymes from *P. chrysosporium* cultures. Specifically, six exo-β-glucanases were purified by fast protein liquid chromatography by Ishihara and coworkers (19, 45). Interestingly, comparisons of deduced and experimentally determined amino acid sequences have shown that *P. chrysosporium cbh1-3* probably encodes CBH62, not the dominant isozyme (46). The major CBH was fourfold more abundant than CBH62, and its amino acid sequence does not correspond to that of any of the three genes reported here. Southern blots of genomic DNA and other cosmids suggest that there are as many as six *cbh1*-like genes in *P. chrysosporium* (1a). In contrast, *T. reesei* has been reported to contain only one *cbh1* gene.

Novel structural aspects of *P. chrysosporium cbh1-1*. Among fungal cellulases, only one other gene besides *P. chrysosporium cbh1-1* has been reported to lack the highly conserved cellulose-binding domain: an EG-encoding gene from *Aspergillus aculeatus* (31). However, the *A. aculeatus* gene product is reported to demonstrate no appreciable amino acid homology to other cellulases. It remains possible that it contains a functionally similar but structurally novel domain that has not been identified. Limited proteolytic cleavage of *T. reesei* CBHI frees the core of the protein from the hinge and tail regions and produces a protein that is active on small, soluble substrates but inactive on insoluble substrates (44, 47). Because *P. chrysosporium cbh1-1* encodes only the core region, it is possible that its gene product has a similar substrate specificity. To investigate the substrate specificity of *P. chrysosporium cbh1-1* directly, it is necessary to either identify culture conditions under which it is expressed at higher levels or to express the cloned gene in a heterologous host.

Cellulase proteins without the cellulose-binding domain have been reported. An EG that lacks hinge and tail regions was isolated from a *T. reesei* culture filtrate, but it appeared to result from posttranslational processing of EGIII rather than from a truncated gene (41). In addition, Mischak et al. (29) demonstrated that if *T. reesei* was grown in unbuffered, high-nitrogen medium, CBHI and CBHII were partially degraded, with proteolysis occurring between the hinge and core domains. Similar proteolytic cleavage was demonstrated in vitro for the nonglycosylated forms of glucanases from *Cellulomonas fimi* (15). Although proteases are secreted by *P. chrysosporium* (5, 11), their specificity for cleavage of cellulase domains has not been established. Knowles et al. (25) suggested that cellulases are cleaved specifically as cultures age to diversify the substrate specificities of the available cellulolytic enzymes. With the cloning of *P. chrysosporium cbh1-1*, it appears that *P. chrysosporium* utilizes a different strategy to achieve the same goal.

Linkage among members of a complex gene family. Clustering of genes involved in a degradative or biosynthetic pathway is well known in filamentous fungi. Examples include the *qa* and *qut* clusters that control quinate-shikimate metabolism in *Neurospora crassa* (13) and *A. nidulans* (26), the *alcA* and *alcR* genes involved in ethanol metabolism in *A. nidulans* (32), the *crnA-niiA-niaD* gene cluster for nitrate assimilation in *A. nidulans* (21), and the penicillin-biosynthetic genes in *Penicillium pinophilum* (4, 40) and *A. nidulans* (27). None of these examples involve multiple copies of very similar genes like the *P. chrysosporium cbh1-1*, *cbh1-2*, and *cbh1-3* cluster.

Clustering of structurally related genes is unusual in fungi. The lignin peroxidase (LiP)-encoding gene family of *P. chrysosporium* is the only other similar example. There are at least six very closely related lignin peroxidase genes in *P. chrysosporium*, and close linkage between some of them has recently been reported (12, 18, 34). One particular LiP-encoding gene cluster is structurally similar to the *P. chrysosporium cbh* genes; the LiPA- and LiPB-encoding genes are 750 bp apart and reside approximately 15 kb downstream of a third LiP-encoding gene, *GLG5* (12). The significance of this form of genomic organization and its possible role in regulation remain to be established.

Regulation. Transcription of the three genes, especially *P. chrysosporium cbh1-3*, is altered by culture conditions. As expected, *cbh1-3* transcripts were abundant in cellulose-grown cultures. Transcription of *cbh1-3* increased on sucrose-grown and 7-day-old glucose-grown cultures relative to that in 3-day-old glucose-grown cultures. This pattern is consistent with recent studies (22) that show derepression of *T. reesei* cellulases during glucose starvation. Further, Eriksson and Hamp (8) have identified sucrose as a readily available carbon source not associated with cellulase repression in *P. chrysosporium*. Transcription levels of *P. chrysosporium cbh1-1* and *cbh1-2* were constitutively low in all four cultures, but in a 3-day-old glucose-grown culture they exceeded *P. chrysosporium cbh1-3* transcript levels. Thus, *cbh1-1* and *cbh1-2* do not appear to be subject to glucose repression. The detection of low transcript levels in uninduced cultures is consistent with a model of CBH regulation in *T. reesei* (6). This model holds that low levels of constitutive *cbh1* expression occur and are necessary for subsequent induction.

Conditions in lignocellulose, the natural substrate of *P. chrysosporium*, are certainly more complex than those in the media used here. It is possible, therefore, that under such conditions, in the presence of inducers that have not been

identified, *P. chrysosporium cbh1-1* and *cbh1-2* play a significant role in cellulose degradation. Alternatively, they may never be expressed at high levels relative to *cbh1-3*; the codon bias of the three genes is consistent with this hypothesis (17). A comprehensive understanding of the role of these genes in cellulose degradation requires elucidation of the number and structures of all of the genes within the family and then assessment of their expression in complex substrates. The latter may be facilitated by the competitive PCR techniques described here.

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