APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1994, p. 4387–4393
0099-2240/94/$04.00+0

Vol. 60, No. 12

Isolation, Characterization, and Analysis of the Expression of the *cbhII* Gene of *Phanerochaete chrysosporium*

CORINNE A. M. TEMPELAARS, PAUL R. J. BIRCH, PAUL F. G. SIMS,* AND PAUL BRODA

Department of Biochemistry and Applied Molecular Biology, University of Manchester Institute of Science and Technology, Manchester M60 1QD, United Kingdom

Received 14 July 1994/Accepted 30 August 1994

Two cDNA sequences representing putative allelic variants of the *Phanerochaete chrysosporium cbhII* gene were isolated by hybridization to the *Trichoderma reesei cbhII* gene. Both of the equivalent genomic sequences were subsequently isolated by the inverse PCR technique. DNA sequencing showed that the *cbhII* open reading frame of 1,380 bp codes for a putative polypeptide of 460 amino acids which is interrupted by six introns. The domain structure found in *T. reesei cbhII* is conserved in the equivalent *P. chrysosporium* protein. The overall similarity between the two gene products is 54%, with the region of highest conservation being found in the cellulose-binding domain (65%). Unlike the *cbhI* gene of *P. chrysosporium*, *cbhII* does not appear to be a member of a class of closely related genes. CBHII is a new member of family B of the β-1, 4-glucanases. Alignment of the *P. chrysosporium* and *T. reesei* CBHII protein sequences showed that all of the residues important for the formation of the extended loops of the catalytic domain and those residues that are involved in the catalytic action of the *T. reesei* enzyme are also present in the *P. chrysosporium* equivalent. The profiles of *cbh* gene expression in *P. chrysosporium* reveal that while cbhI.1 and cbhI.2 could be coregulated, *cbhII* can be independently controlled. The latter is so far the only cellulase gene found to be expressed when the fungus is grown on oat spelt arabinoxylan, suggesting that it may play an active role in the xylanolytic as well as the cellulolytic systems.

Cellulose is the world's most abundant biopolymer, and as such, its degradation is of considerable ecological, agricultural, and commercial importance. Biological degradation involves the action of cellulases, which are produced by a wide range of bacterial and fungal species. Typically, any such organism produces a number of different cellulases which frequently act synergistically to effect cleavage of the β-1, 4-chain. In the best-studied cellulolytic organism, *Trichoderma reesei*, four different activities have been identified. These are two exocellobiohydrolases (CBHI and CBHII) and two endoglucanases (EGI and EGIII). Each of these is coded for by a single gene which has been characterized at the molecular level (3, 17, 24, 26, 29, 30, 33).

In nature, cellulose is usually found in association with both lignin and hemicelluloses as a component of lignocellulose. Unlike *T. reesei*, the white rot fungus *Phanerochaete chrysosporium* is able to degrade all of these components of lignocellulose. This is achieved through the action of a large number of extracellular enzymes, including two distinct classes of peroxidases believed to degrade lignin (14), a number of xylanases (4), and several cellulases (8, 32). Genes coding for both types of peroxidases and one class of cellulase (exocellobiohydrolase I, or CBHI) have been described, and each of the three activities has been found to be coded for by a family of closely related genes (2, 5, 10, 18, 28).

To date, there is no information concerning the gene or genes coding for the exocellobiohydrolase II (CBHII) activity isolated by Uzcategui et al. (32). Here we show that the *cbhII* gene of *T. reesei* hybridizes to a single sequence within the *P. chrysosporium* genome and that this codes for a CBHII-like protein. This is thus the first instance of a class of extracellular enzyme activity produced by this organism which is not coded for by a gene family.

* Corresponding author. Mailing address: Department of Biochemistry and Applied Molecular Biology, University of Manchester Institute of Science and Technology, P.O. Box 88, Manchester M60 1QD, United Kingdom.

MATERIALS AND METHODS

Organism. *P. chrysosporium* ME446 (ATCC 34541) was maintained on slopes of 2% (wt/vol) malt extract.

Culture conditions for expression studies. Culture medium was modified Vogel's medium (15) containing 0.01 M dimethyl succinate buffer (pH 4.5), 0.23 g of $NH_4H_2PO_4$ per liter (low nitrogen), 0.068 g of veratryl alcohol per liter, and 0.2% (wt/vol) of a carbon source from glucose, Avicel (microcrystalline cellulose), ball-milled straw (BMS; lignocellulose), carboxymethyl cellulose, or oat spelt arabinoxylan.

General molecular biological techniques. DNA manipulations were performed according to methods described by Sambrook et al. (25) unless otherwise stated. Restriction enzyme digestions were carried out according to the manufacturer's recommendations.

Isolation and characterization of clones. A *P. chrysosporium* ME446 expression library constructed from cDNA derived from a BMS-grown culture and cloned into λ-NM1149 (28) was hybridized under nonstringent conditions (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 45% formamide, 5× Denhardt's solution, and 0.1% sodium dodecyl sulfate [SDS] at 37°C) to a 3.9-kb *Hin*dIII fragment containing the *T. reesei cbhII* gene (29). Three clones were isolated, and their inserts were cloned into pUC18 and characterized by restriction analysis. The complete primary sequence of one of the clones (pC1.2.1) and partial sequence of another clone (pC2.2.2) were determined by the dideoxy chain termination method. Sequenase version 2.0 (U.S. Biochemicals) was used for these reactions according to the manufacturer's recommendations. Universal primers, synthetic oligonucleotides, and subclones were used to obtain the complete sequence.

Southern blotting. Genomic DNA was isolated from cul-

tures of *P. chrysosporium* by methods described before (20). Separate aliquots of this DNA were digested with *Xba*I, *Sac*I, *Pvu*I, *Pst*I, *Hin*dIII, and *Eco*RI, all enzymes for which a site is not present within the *cbhII* gene. These digests were blotted and probed under stringent conditions (4× SSC, 50% formamide, 5× Denhardt's solution, and 0.1% SDS at 37°C) with the 1.0-kb *Bam*HI fragment from pC1.2.1.

PCR reactions. For inverse PCR reactions, 1 μg of genomic DNA was digested with *Pst*I. An aliquot (approximately 0.2 μg) of the digest was used in a ligation reaction. The ligated DNA was precipitated, briefly dried, and resuspended in 10 μl of distilled water. The reaction mixture for the PCR was as follows: 40 ng of digested and ligated genomic DNA, 200 ng of each primer, 200 μM deoxynucleoside triphosphates, 1× *Pfu* polymerase buffer, 2.5 U of *Pfu* polymerase (Stratagene), and distilled water to 100 μl. The reaction mixture was overlaid with paraffin oil, and the following thermocycling programs were run: 95°C for 5 min and 55°C for 1 min for 1 cycle; 92°C for 2 min, 55°C for 2 min, and 72°C for 2 min for 30 cycles; and 72°C for 10 min for 1 cycle. The primers used were 5′-GAC GTCTAGAGGCTGGTGATAACGG-3′ and 5′-ATCAAGC TTGCCGGCTTCGGTACCC-3′. For the conventional PCR step following the inverse PCR, the above conditions were used with 10 ng of genomic DNA and the following primers: 5′-G CGAATTCCACATTCCAGCATTTCATCCGTTG-3′ and 5′-CGCTGCAGAGATGCTAACCGAATTTCAACCGG-3′.

Preparation of cDNA for expression studies. RNA was prepared by the method of James et al. (13) and was precipitated with LiCl. Poly(A)$^+$ mRNA was prepared from this with Dynal's Dynabeads mRNA extraction kit according to the manufacturer's recommendations. cDNA was synthesized from poly(A)$^+$ mRNA according to the manufacturer's protocol with the Pharmacia first-strand cDNA synthesis kit.

Design of PCR primers for expression studies. Criteria for the design of gene-specific primer pairs for the *cbhI.1*, *cbhI.2* (28), and *cbhII* genes of *P. chrysosporium* were those of Brooks et al. (1). In each case, absolute specificity was confirmed by restriction analysis of amplification products. Under the conditions employed, only template DNA derived from clones containing the specific gene sequence yielded such products. The primer pairs used were 5′-ACAATGTTCCGCACTGCT ACTT-3′ (G36) and 5′-AGGGTGCCCGCGGAGGTGCC-3′ (G32) for *cbhI.1*, 5′-CACTCCTCGCATTCACTTGTCT-3′ (G34) and 5′-CTGCCGGTCTCGGTCCAGTTGC-3′ (G35) for *cbhI.2*, and 5′-CCTCAGCCCTTACTACGC-3′ (#29) and 5′-CCAATCTACCTCTACAGC-3′ (#32) for *cbhII*.

PCR amplification for expression studies. Approximately 50 ng of cDNA was subjected to 30 cycles of amplification by the PCR. In addition to template DNA, the amplification mixtures contained (in a final volume of 100 μl) 2.5 U of *Taq* polymerase (Promega), 1× *Taq* polymerase buffer, 100 μM each deoxynucleoside triphosphate, and 100 ng of each of the primers (see above). Annealing temperatures for the *cbhI.1*, *cbhI.2*, and *cbhII* primer pairs were, respectively, 61, 61, and 55°C.

RESULTS

Isolation and sequence analysis of a cDNA clone. Screening of the *P. chrysosporium* cDNA library with the *T. reesei cbhII* gene probe resulted in the isolation of two clones, pC1.2.1 and pC2.2.2, both containing a 1.5-kb insert. On the basis of restriction analysis, it appeared that pC1.2.1 and pC2.2.2 were very similar; partial sequencing confirmed this.

The nucleotide sequence of pC1.2.1 was determined and found to contain an open reading frame of 1,380 bp capable of coding for a protein of 460 amino acid residues (Fig. 1). The extreme N-terminal portion of this putative protein sequence is positively charged and is separated from the rest of the molecule by a stretch of predominantly hydrophobic residues. These are features typical of a signal peptide (34). The GC content of the nucleotide sequence was 61.1%, which is in close agreement with the calculated value of 59% for *P. chrysosporium* genomic DNA (19). The codon usage within the open reading frame is biased; only 5.4% of the codons used have A at their third position. This usage is similar to but not as marked as that seen with the *cbhI* genes from this organism (27, 28).

Cellulases typically consist of three domains, a cellulose-binding domain (CBD), a catalytic domain, and a hinge region linking these two (9). Alignment of the polypeptide sequence derived from the *P. chrysosporium cbhII* cDNA with the *T. reesei* CBHII protein sequence shows that the domain structure of the latter is conserved, with the N-terminal CBD separated from the C-terminal catalytic domain by a hinge region. The latter appears to be duplicated by comparison with that seen in the corresponding CBHI proteins (27–29) but is typically rich in serine and threonine residues. It is therefore likely that these residues are O-glycosylated, as in the case of the *T. reesei* protein (23).

The highest degree of similarity between the two CBHII proteins is seen within the CBD regions (65% identity), and although the overall relationship between the CBHI and CBHII proteins from the two fungi is not very close, the CBD regions from both classes of protein show a high level of similarity (Fig. 2A). This is in agreement with previous observations that such domains are conserved among the cellulases of both the same and different fungal species (31). The equivalent domains within bacterial cellulases have been shown to be related to each other but are quite different, both in terms of sequence and length, from those seen within the fungal enzymes (9).

The catalytic domains of the *P. chrysosporium* and *T. reesei* CBHII proteins are also clearly related. Moreover, in this case, a significant relationship can also be seen if the comparison is extended to include some bacterial cellulases (Fig. 2B). These particular bacterial proteins as well as the *T. reesei* CBHII are members of the β-1, 4-glucanase family (9, 12), and we therefore suggest that the *P. chrysosporium* protein should also be considered a member of this class of cellulase.

Isolation and analysis of the *cbhII* genomic sequence by inverse PCR. Southern blot analysis revealed that a *Pst*I restriction fragment within the genome was likely to contain the entire *cbhII* gene (Fig. 3, lane A). The genomic *cbhII* sequence was therefore isolated by the inverse PCR method of Ochman et al. (16). Two reverse PCR primers were designed so that after they were hybridized to the coding region of the *cbhII* sequence, they pointed out from the center of the gene. These primers included restriction sites for *Xba*I and *Hin*dIII which were introduced at their 5′ ends to facilitate cloning of the fragments produced.

The inverse PCR resulted in the amplification of a 1.1-kb fragment. This was digested with *Pst*I, and the two resulting fragments were cloned into pUC18. The nucleotide sequences of the inserts immediately adjacent to the *Pst*I sites were determined so that the design of primers that would themselves promote amplification of a region containing the complete *cbhII* genomic sequence via a further round of conventional PCR would be possible. In this way, a 2.3-kb PCR product was isolated and cloned into pUC18. This was sequenced by the use of synthetic oligonucleotides which were designed for the sequencing of the cDNA clone and which

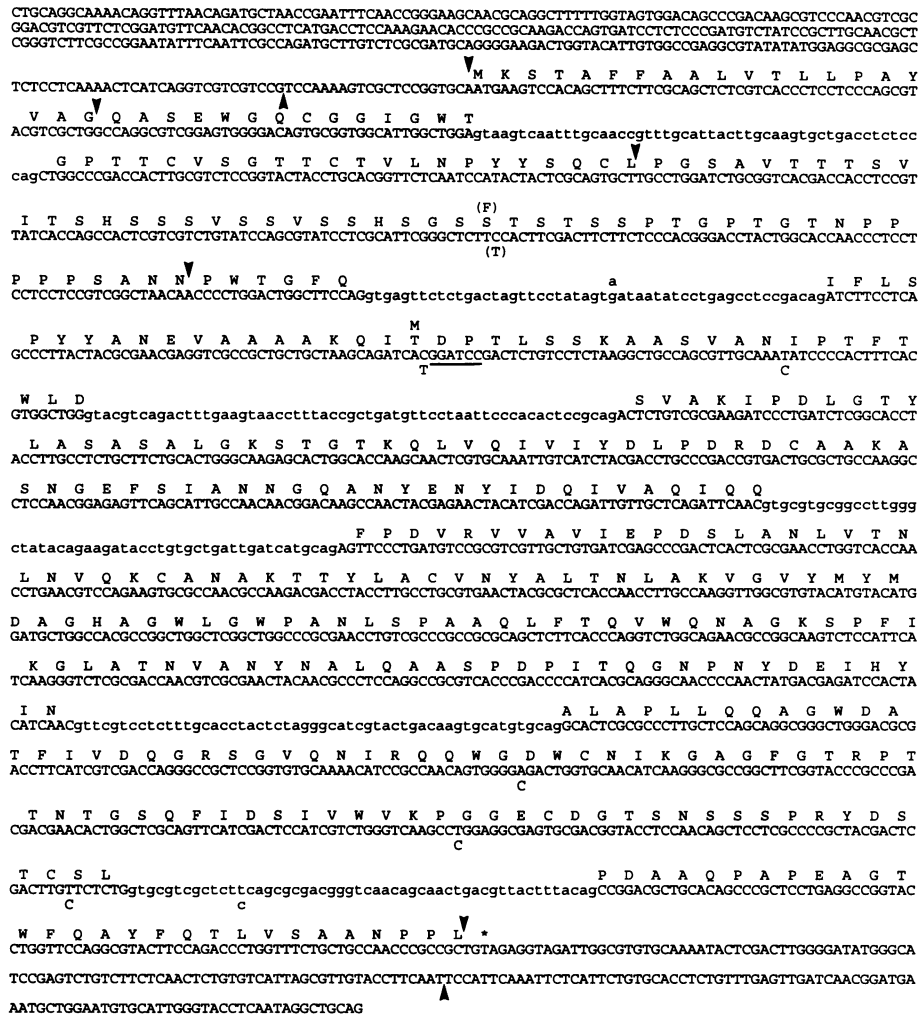

FIG. 1. The complete nucleotide sequence of the *cbhII* gene of *P. chrysosporium* ME446. The sequences determined from the different genomic clones fell into two classes designated type 1 and type 2. These show a total of seven base pair differences between them, five in the coding region and two in the introns. The sequence presented is that of a type 2 variant and contains data derived from the analysis of both genomic (pES5, pES6, pEB1, pEB4, pESA1, and pGCBH2H) and cDNA (pC2.2.2) clones. The 5′ and 3′ extents of the data derived from the cDNA clone are indicated by the upward-pointing arrowheads below the nucleotide sequence. Base pair differences between this sequence and the type 1 sequence (typified by clones pES1 and pC1.2.1 [genomic and cDNA clones, respectively]) are indicated by the alternative bases at the appropriate positions beneath the type 2 nucleotide sequence. In the single instance in which such a change introduces an amino acid alteration, this is indicated by the residue located above the amino acid sequence at position 131. The single alteration of a nucleotide and the consequent amino acid change that results from the conflict between the type 1 cDNA and genomic sequences are similarly indicated, but in this instance, the variant base and residue are shown in parentheses. Intron sequences are indicated by lower-case letters. The approximate limits of the domains recognized within the protein sequence (signal peptide, CBD, hinge region, and catalytic region from the N terminus to the C terminus) are indicated by the downward-pointing arrowheads above the amino acid sequence. The *Bam*HI site at position 496 which was used in the production of the *cbhII*-specific probe is underlined.

were used in addition to the universal primer. Three full-length clones and five subclones were sequenced to allow for the possibility of misincorporation errors during the PCRs (7).

Comparison of the genomic and cDNA sequences shows that the coding region of the gene is interrupted by six intervening sequences (Fig. 1). These are located at different positions from those seen within the equivalent *T. reesei* gene. In all cases, the *P. chrysosporium* introns contain sequences conforming to consensus 5′ and 3′ splice and lariat sites and are of the characteristically small size found in filamentous fungal genes (11).

Comparison of the sequences from the various genomic clones revealed that there were two classes of sequence (designated type 1 and type 2) showing five base pair differences between them in the coding regions of the genes and an additional two changes in two of the introns (Fig. 1). Only one of the changes in the coding region of the sequence results in an amino acid substitution (threonine replaced with methionine at position 131); the other four are silent changes. Comparison of the cDNA sequence of pC1.2.1 and that of the relevant regions in pC2.2.2 revealed that these clones were also divided between the same two classes. In addition, clone pC1.2.1 shows one base pair difference from its likely genomic equivalent which would result in an amino acid substitution

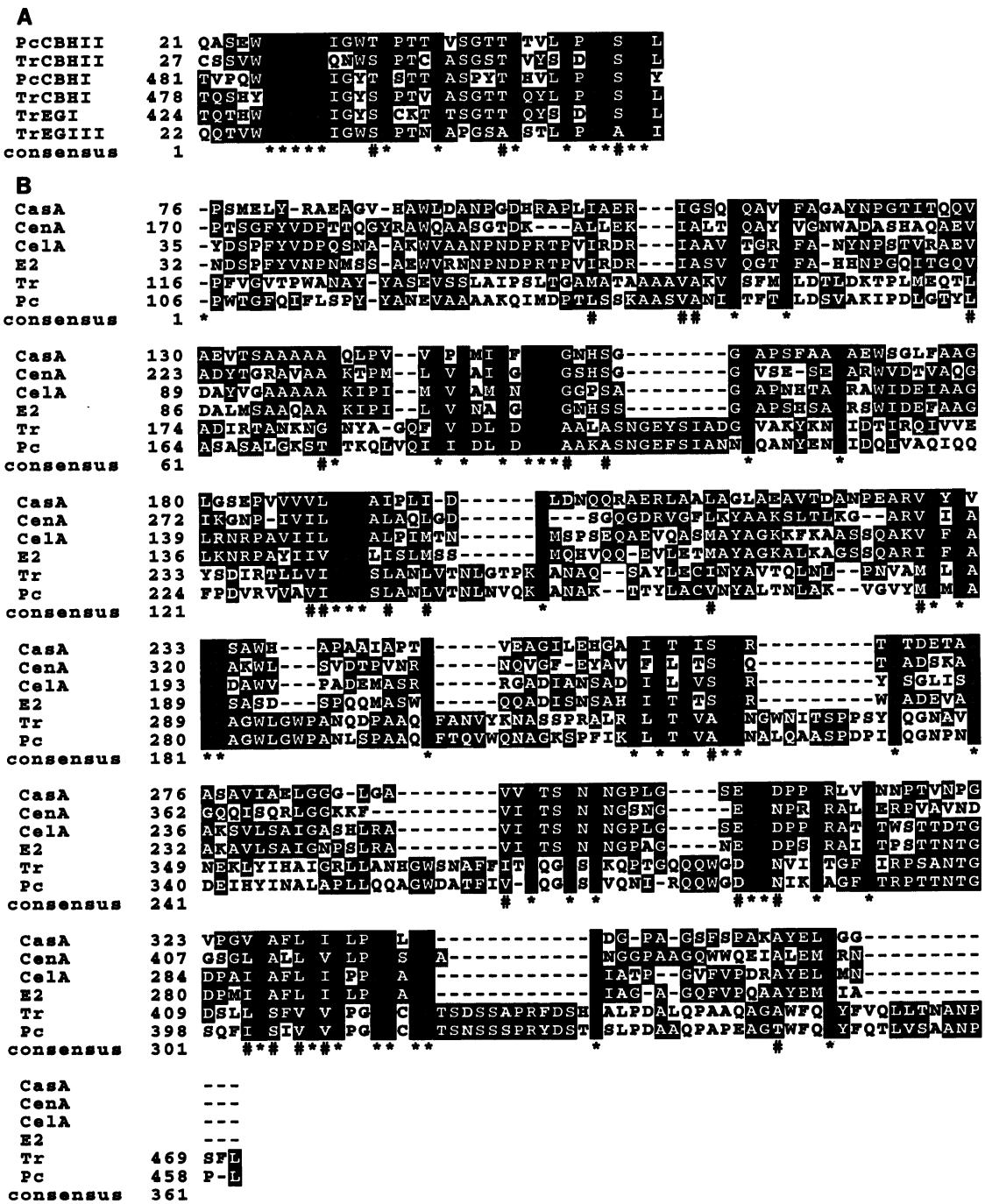

FIG. 2. Comparison of cellulase amino acid sequences. The alignments presented were generated with the Clustal V program, and the output was enhanced with Boxshade. Positions at which an identical residue is present in all sequences are indicated by * in the consensus, whereas # indicates those positions at which similar but not identical residues are present. The numbers indicate the positions at which the first residue presented here occurs in the corresponding protein sequence. (A) Comparison of fungal cellulose-binding domains. Enzyme designations are given at the left of the figure. They are preceded by either Pc or Tr to define the source organism as either *P. chrysosporium* or *T. reesei*, respectively. (B) Comparison of the *P. chrysosporium* CBHII catalytic domain with those from members of family B of the β-1, 4-glucanases. Enzyme names are as follows: CasA, *Streptomyces* strain KSM-9 endoglucanase; CenA, *Cellulomonas fimi* endoglucanase; CelA, *Microbispora bispora* endoglucanase; E2, *Thermomonospora fusca* endoglucanase; Tr, *T. reesei* exocellobiohydrolase II; Pc, *P. chrysosporium* exocellobiohydrolase II.

(serine replaced by phenylalanine at position 85). This may have been the result of a nucleotide misincorporation during PCR. However, only one genomic clone of this class was analyzed, so it was not possible to confirm that this was the case. *P. chrysosporium* is a dikaryon in which two genome equivalents show restriction fragment length polymorphism differences (21, 22). The two classes of clone described above are thus likely to be allelic variants. However, none of the base

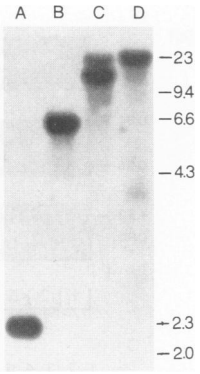

FIG. 3. Southern hybridization analysis of *P. chrysosporium* genomic DNA. Aliquots (2 μg) of *P. chrysosporium* genomic DNA were digested with each of the following restriction enzymes: *PstI*, *Hin*dIII, *Eco*RI, and *Bam*HI (lanes A to D, respectively). The resulting fragments were fractionated by agarose gel electrophoresis and then transferred to nitrocellulose for hybridization. The probe used was a 1.0-kb *Bam*HI DNA fragment from clone pC1.2.1. This was generated by digestion at the site located at position 496 (Fig. 1) and a site downstream of the coding region which is derived from the vector's polylinker. The fragment thus produced codes for the catalytic domain and hinge region of the *cbhII* gene. The numbers to the right of the figure indicate the positions and sizes in kilobases of lambda DNA digested with *Eco*RI-*Hin*dIII that was used as a molecular weight marker.

pair changes altered or introduced a restriction site, so it was not possible to visualize restriction fragment length polymorphisms via Southern blotting. The identification of two cDNA clones apparently equivalent to the two genomic homologs shows that both alleles of the *cbhII* gene are probably expressed.

Is the *P. chrysosporium cbhII* gene part of a gene family? Studies of the *cbhI* genes in *P. chrysosporium* have shown that these form a family of closely related genes (5, 6, 28). To test if this was also the case for *cbhII*, an experiment was carried out under hybridization conditions identical to those that reveal the presence of the *cbhI* family when a *cbhI*-specific probe is used. Thus, genomic DNA was digested with six restriction enzymes for which no sites were present within the *cbhII* gene sequence, and under conditions identical to those used for *cbhI*, this was hybridized to the 1.0-kb fragment produced by *Bam*HI digestion of the pC1.2.1 insert (Fig. 1). This fragment contains the entire coding region of *cbhII* except for the N-terminal portion encoding the CBD and hence does not cross-hybridize to other non-*cbhII* cellulase genes. The autoradiograph obtained (Fig. 3) reveals that hybridization of the *cbhII* gene occurred to only one fragment in each of the restriction digests, with the exception of the *Eco*RI digest. The extra band that is visible in this track (Fig. 3, lane C) is due to incomplete digestion of the genomic DNA. Additional genomic DNA digests (*Pvu*I, *Sac*I, and *Xba*I) probed with the same *Bam*HI DNA fragment also revealed only a single hybridizing fragment (result not shown). We therefore have no evidence for the existence of multiple copies of *cbhII*-like genes in the genome of *P. chrysosporium* ME446.

PCR analysis of *cbh* gene expression. Previous reports have described the existence of a *cbhI*-like family in *P. chrysosporium*. In contrast, the data presented above provide evidence for the occurrence of only a single *cbhII* gene (Fig. 3). The reasons for the complexity of the cellulolytic system in this organism are unclear and beg the question of whether all of the genes involved are coordinately regulated or whether expression is dependent on the substrate used.

PCR was used in an earlier study to analyze the expression of closely related lignin peroxidase genes (1). Low levels of gene expression could be detected because of the greater sensitivity of this technique when compared with that of Northern (RNA) analysis. In order to carry out a similar study on the cellulase system, primer pairs specific for the *cbhI.1*, *cbhI.2*, and *cbhII* genes were designed to generate an amplification product that spanned at least one intron. This allows products derived from both cDNA and contaminating genomic DNA (gDNA) to be differentiated on the basis of size (Fig. 4A).

PCR with these primers was performed on cDNA populations prepared from mycelium grown on a number of carbon sources (Fig. 4B). In agreement with previous observations (27, 28), the *cbhI* genes were not expressed in the presence of glucose, which served as the sole carbon source. The same repressive effect of glucose was also seen in the case of the *cbhII* gene. However, all three genes were expressed on Avicel (microcrystalline cellulose), BMS (lignocellulose), and cellobiose and, interestingly, in the presence of carboxymethyl cellulose. The last is typically thought of as an endoglucanase substrate but clearly induces expression of all three of the exoglucanase-like genes examined here. Oat spelt arabinoxylan was the only substrate that elicited a differential pattern of expression, with *cbhII* being the only gene examined that was expressed on this carbon source. When such expression was followed over time (from days 3 to 6 after inoculation; Fig. 4C), *cbhII* was found to be strongly expressed throughout the period studied. No such expression was detected for the *cbhI* class of genes.

DISCUSSION

The data described above suggest that *P. chrysosporium* contains a single gene which codes for CBHII and which is present in two allelic forms within the heterokaryotic strain ME446. This is in strong contrast to the situation found for genes coding for CBHI activity, which in this fungus is coded for by a family of at least six closely related, nonallelic genes (5, 6, 28) producing at least two discrete proteins (32).

The ME446 *cbhII* gene codes for a protein of 460 amino acid residues with a calculated molecular weight of 48,479. The equivalent *T. reesei* enzyme consists of 471 residues with a calculated molecular weight of 49,653 (29). Uzcategui et al. (32) have shown by protein sequencing that the CBHII of *P. chrysosporium* K3 contains a region of amino acid sequence that closely resembles a part of the *T. reesei* enzyme. However, in terms of amino acid sequence, the equivalent region derived from the ME446 gene is markedly different from that found for the protein isolated from strain K3, although the overall molecular masses and amino acid compositions of the enzymes from the two strains are very similar. Since we have been unable to find any evidence for the existence of multiple *cbhII* genes within the genome of strain ME446, it seems that at least some regions of the CBHII molecule may thus show significant strain-to-strain variation.

Rouvinen et al. (23) determined the three-dimensional structure of the catalytic domain of *T. reesei* CBHII by X-ray crystallography. The active site was shown to be tunnel-shaped and to contain four separate binding sites for glycosyl units. Two aspartate residues, Asp-199 and Asp-245 (preprotein numbering), were proposed to be the catalytic residues; we note that the equivalent residues (191 and 237) are conserved in the *P. chrysosporium* sequence. Moreover, four cysteine

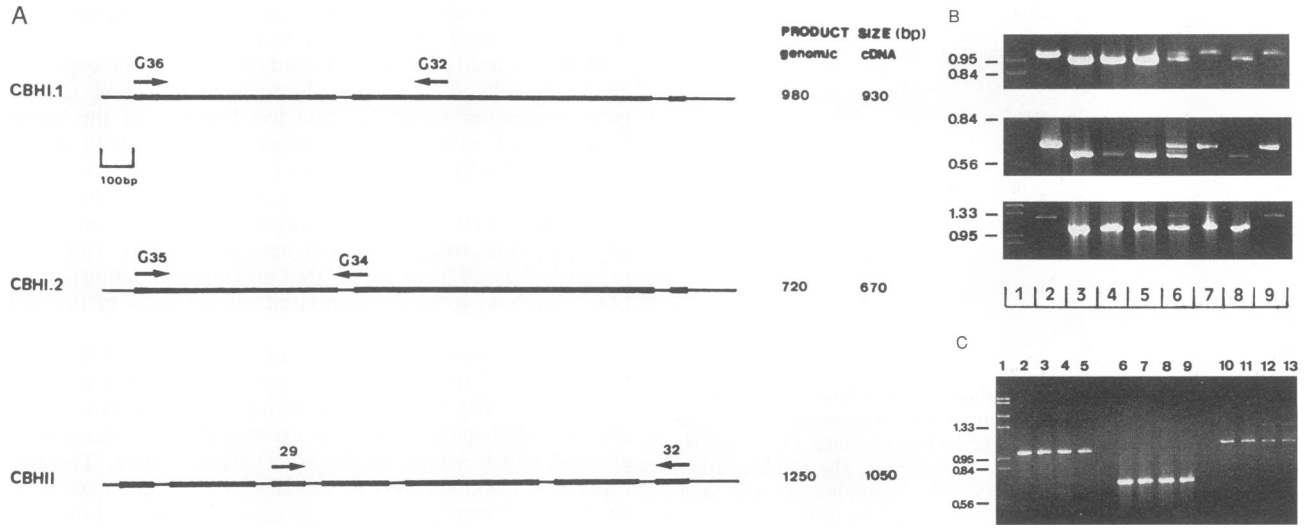

FIG. 4. PCR analysis of *P. chrysosporium* cellulase gene expression. (A) Diagrammatic representation of each of the *cbhI.1*, *cbhI.2*, and *cbhII* genomic sequences. Exons are represented by the thick lines, and introns and flanking sequences are represented by the thin lines. The arrows indicate the positions at which the PCR primers used in these analyses anneal to their target genes. The identity of each oligonucleotide is indicated above the corresponding arrow. The sizes of the amplification products expected from genomic- and cDNA-derived templates are given to the right of each diagram. (B) PCR amplification products generated with primer pairs specific for *cbhI.1* (upper panel), *cbhI.2* (middle panel), and *cbhII* (lower panel). Templates used were *P. chrysosporium* gDNA (lane 2), the corresponding cloned cDNA sequence (lane 3) and cDNA populations prepared from mycelium grown on BMS (lane 4), Avicel (lane 5), cellobiose (lane 6), xylan (lane 7), carboxymethyl cellulose (lane 8), and glucose (lane 9). Lambda DNA digested with *Eco*RI-*Hin*dIII was used as a size marker (lane 1), and the sizes (in kilobases) of the marker fragments closest in size to the amplification products are given to the left of each panel. (C) PCR amplification products generated with primer pairs specific for *cbhI.1* (lanes 2 to 5), *cbhI.2* (lanes 6 to 9), and *cbhII* (lanes 10 to 13). Templates used were cDNA populations prepared from mycelium grown on xylan for 3 days (lanes 2, 6, and 10), 4 days (lanes 3, 7, and 11), 5 days (lanes 4, 8, and 12), and 6 days (lanes 5, 9, and 13) after inoculation. The PCR products generated with the primer pairs specific for *cbhI.1* (lanes 2 to 5) and *cbhI.2* (lanes 6 to 9) have sizes expected for those derived from gDNA templates (980 and 720 bp, respectively). In contrast, the products generated from the *cbhII*-specific primers (lanes 10 to 13) have sizes expected for products from cDNA templates. *cbhII* gDNA is also present in these cDNA preparations but is excluded from the amplification reactions by competition from the significantly larger amount of the equivalent cDNA. This results in a much reduced but sometimes visible gDNA product (see lanes 12 and 13). Size markers (lane 1) were as for panel B.

residues that form two disulfide bridges (from residues 200 to 259 and 392 to 439) within the *T. reesei* molecule are also conserved in *P. chrysosporium* (positions 192, 251, 382, and 429). Therefore, the structures and thus the catalytic mechanisms of the two proteins are likely to be very similar. In addition, we have already noted that comparison of amino acid compositions of the catalytic domains of known cellulases identifies the *P. chrysosporium* CBHII as a new member of the B family of β-1, 4-glycanases (9, 12).

In the case of the *P. chrysosporium cbhI.2* gene, comparison of genomic and cDNA copies revealed that an intron at the 3′ end of the gene was not always excised (28). Because this intron does not contain an in-frame stop codon, translation of such an unspliced transcript would result in a protein product with a significantly altered C terminus. The equivalent intron in the *cbhI.1* gene also lacks a stop codon, raising the possibility that differential splicing may occur in this gene as well (27). The second intron in the *cbhII* gene is located near the border of the hinge region and the catalytic domain and does not contain a stop codon, and moreover, translation through this sequence would maintain the correct reading frame of the subsequent exons. Differential splicing of this intron resulting in the addition of a 17-amino-acid insert that is serine rich would therefore also be possible and could therefore extend the hinge region of the enzyme, increasing the distance between the CBD and the catalytic domain. In the two cDNA clones (pC1.2.1 and pC2.2.2) that were isolated, this intron was excised; more cDNA clones would have to be sequenced to determine whether nonexcision of this intron does in fact occur.

PCR analysis of the expression of *cbhI.1*, *cbhI.2*, and *cbhII* revealed that, for all three genes, transcription is repressed when glucose is the sole carbon source. The substrates Avicel, BMS, cellobiose, and carboxymethyl cellulose, however, elicited expression of these genes. Expression of the *cbhII* gene on the last substrate is perhaps surprising, since activity against carboxymethyl cellulose is generally thought to be diagnostic of endoglucanases. Enzymes with such activity have previously been shown to be among the cellulase repertoire of *P. chrysosporium* (8). However, we have been consistently unable to detect DNA sequences within the *P. chrysosporium* genome that are related to the endoglucanase genes of *T. reesei*. It has been noted before (32) that the product of the *cbhI.1* gene in *P. chrysosporium* K3 does have significant carboxymethyl cellulase activity, and we have previously suggested that the multiple *cbhI*-like sequences in this organism might code for endoglucanase as well as exoglucanase activities (28).

The *cbhII* gene of *P. chrysosporium* was the only *cbh* gene among those investigated to be expressed when mycelium was grown on xylan. It is possible that this gene is coordinately regulated with the xylanase genes. This is plausible on two counts. On one hand, the two substrates xylan and cellulose occur in intimate association in nature, and thus it might well be efficient for genes coding for enzymes effecting their degradation to be expressed together. On the other hand, it has been clearly demonstrated that both xylanase and cellulase

genes have evolved from common ancestors by domain shuffling (9). It is therefore possible that elements responsible for the regulation of these genes might also have been derived from a common source.

Further work will be needed to characterize the mechanism of action of the *cbhII* gene product via heterologous expression and to investigate whether its unique feature among the *cbh* genes of expression on xylan is indeed a reflection of an active role within the xylanolytic system of *P. chrysosporium*.

ACKNOWLEDGMENTS

We thank Venture Research International and the Agriculture and Food Research Council for support.

REFERENCES

1. **Brooks, P., P. F. G. Sims, and P. Broda.** 1993. Isozyme specific polymerase chain reaction analysis of differential gene expression: a general method applied to lignin peroxidase genes of *Phanerochaete chrysosporium*. Bio/Technology **11**:830–834.
2. **Brown, A., P. F. G. Sims, U. Raeder, and P. Broda.** 1988. Multiple ligninase genes from *Phanerochaete chrysosporium*. Gene **72**:77–85.
3. **Chen, C. M., M. Gritzali, and D. W. Stafford.** 1987. Nucleotide sequence and deduced primary structure of cellobiohydrolase II of *Trichoderma reesei*. Bio/Technology **5**:274–278.
4. **Copa-Patiño, J. L., Y. G. Kim, and P. Broda.** 1993. Production and initial characterisation of the xylan-degrading system of *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **40**:69–76.
5. **Covert, S. F., J. Bolduc, and D. Cullen.** 1992. Genomic organisation of a cellulase gene family in *Phanerochaete chrysosporium*. Curr. Genet. **22**:407–413.
6. **Covert, S. F., A. Vanden Wymelenberg, and D. Cullen.** 1992. Structure, organization, and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58**:2168–2175.
7. **Eckert, K. I., and T. A. Kunkel.** 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. Nucleic Acids Res. **18**:3739–3744.
8. **Eriksson, K.-E., and B. Pettersson.** 1975. Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. Eur. J. Biochem. **51**:193–206.
9. **Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller, Jr., and R. A. J. Warren.** 1991. Domains in microbial β-1, 4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. **55**:303–315.
10. **Godfrey, B. J., M. B. Mayfield, J. A. Brown, and M. H. Gold.** 1990. Characterisation of a gene encoding a manganese peroxidase from *Phanerochaete chrysosporium*. Gene **93**:119–124.
11. **Gurr, S. J., S. E. Unkles, and J. R. Kinghorn.** 1987. The structure and organisation of nuclear genes of filamentous fungi, p. 93–139. *In* J. R. Kinghorn (ed.), Gene structure in eukaryotic microbes. IRL Press, Oxford.
12. **Henrissat, B., M. Claeyssens, P. Tomme, L. Lemesle, and J.-P. Mornon.** 1989. Cellulase families revealed by hydrophobic cluster analysis. Gene **81**:83–95.
13. **James, C. M., M. S. Soares-Felipe, P. F. G. Sims, and P. Broda.** 1992. Expression of a single lignin peroxidase-encoding gene in *Phanerochaete chrysosporium* strain ME446. Gene **114**:217–222.
14. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41**:465–505.
15. **Kirk, T. K., E. Schultz, W. J. Conners, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117**:277–285.
16. **Ochman, H., M. M. Medhora, D. Garza, and D. L. Hartl.** 1990. Amplification of flanking sequences by inverse PCR, p. 219–227. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, London.
17. **Penttila, M., P. Lehtovaara, H. Nevalainen, R. Bhikhabhai, and J. K. C. Knowles.** 1986. Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. Gene **45**:253–263.
18. **Pribnow, D., M. B. Mayfield, V. J. Nipper, J. A. Brown, and M. H. Gold.** 1989. Characterisation of a cDNA encoding a manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J. Biol. Chem. **264**:5036–5040.
19. **Raeder, U., and P. Broda.** 1984. Comparison of the lignin degrading white-rot fungi *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum* at the DNA level. Curr. Genet. **8**:499–506.
20. **Raeder, U., and P. Broda.** 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. **1**:17–20.
21. **Raeder, U., and P. Broda.** 1986. Meiotic segregation analysis of restriction site polymorphisms allows rapid genetic mapping. EMBO J. **5**:1125–1127.
22. **Raeder, U., W. Thompson, and P. Broda.** 1989. RFLP-based genetic map of *Phanerochaete chrysosporium* ME446; lignin degrading genes occur in clusters. Mol. Microbiol. **3**:911–918.
23. **Rouvinen, J., T. Bergfors, T. Teeri, J. K. C. Knowles, and T. A. Jones.** 1990. Three dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science **249**:380–386.
24. **Saloheimo, M., P. Lehtovaara, M. Penttila, T. Teeri, J. Stahlberg, G. Johansson, G. Pettersson, M. Claeyssens, P. Tomme, and J. K. C. Knowles.** 1988. EGIII, a new endoglucanase from *Trichoderma reesei*: the characterisation of both gene and enzyme. Gene **63**:11–21.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Shoemaker, S., V. Schweickart, M. Ladner, D. Gelfand, S. Kwok, K. Myambo, and M. Innis.** 1983. Molecular cloning of exocellobiohydrolase I derived from *Trichoderma reesei* strain L27. Bio/Technology **1**:691–696.
27. **Sims, P., C. James, and P. Broda.** 1988. The identification, molecular cloning and characterisation of a gene from *Phanerochaete chrysosporium* that shows strong homology to the exocellobiohydrolase I gene from *Trichoderma reesei*. Gene **74**:411–422.
28. **Sims, P. F. G., M. S. Soares-Felipe, Q. Wang. M. E. Gent, C. Tempelaars, and P. Broda.** 1994. Differential expression of multiple exo-cellobiohydrolase I-like genes in the lignin-degrading fungus *Phanerochaete chrysosporium*. Mol. Microbiol. **12**:209–216.
29. **Teeri, T. T., P. Lehtovaara, S. Kauppinen, I. Salovuori, and J. K. C. Knowles.** 1987. Homologous domains in *Trichoderma reesei* cellulolytic enzymes: gene sequence and expression of cellobiohydrolase II. Gene **51**:43–52.
30. **Teeri, T. T., I. Salovuori, and J. K. C. Knowles.** 1983. The molecular cloning of the major cellulase gene from *Trichoderma reesei*. Bio/Technology **1**:696–699.
31. **Tomme, P., H. van Tilbeurgh, G. Pettersson, J. Vanderkerkhove, J. Knowles, T. Teeri, and M. Claeyssens.** 1988. Studies of the cellulolytic system from *Trichoderma reesei*: analysis of domain function in two cellobiohydrolases by limited proteolysis. Eur. J. Biochem. **170**:575–581.
32. **Uzcategui, E., A. Ruiz, R. Montesino, G. Johansson, and G. Pettersson.** 1991. The 1, 4-β-D-glucan cellobiohydrolases from *Phanerochaete chrysosporium*. I. A system of synergistically acting enzymes homologous to *Trichoderma reesei*. J. Biotechnol. **19**:271–286.
33. **Van Ardsell, L. N., S. Kwok, V. L. Schweickart, M. B. Ladner, D. H. Gelfand, and M. Innis.** 1987. Cloning, characterisation and expression in *Saccharomyces cerevisiae* of endoglucanase I from *Trichoderma reesei*. Bio/Technology **5**:60–64.
34. **Von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184**:99–105.