Cellulase Induction in *Trichoderma reesei* by Cellulose Requires Its Own Basal Expression*

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The induction of cellulases by cellulose, an insoluble polymer, in the filamentous fungus *Trichoderma reesei* is puzzling. We previously proposed a mechanism that is based on the presence of low levels of cellulase in the uninduced fungus; this basal cellulase activity would digest cellulose-releasing oligosaccharides that could enter the cell and trigger expression of cellulases. We now present experiments that lend further support to this model. We show here that transcripts of two members of the cellulase system, *cbh1* and *egl1*, are present in uninduced *T. reesei* cells. These transcripts are induced at least 1100-fold in the presence of cellulose. We also show that a construct containing the hygromycin B resistance-encoding gene driven by the *cbh1* promoter confers hygromycin B resistance to *T. reesei* cells grown in the absence of cellulose. Moreover, cellulose-induced production of the *cbh1* transcript was suppressed when antisense RNA against three members of the cellulase system was expressed *in vivo*. Experiments are presented indicating that extracellular cellulase activity is the rate-limiting event in induction of synthesis of the cellulose transcripts by cellulose. The results reveal a critical requirement for basal expression of the cellulase system for induction of synthesis of its own transcripts by cellulose.

Cellulose, a β-(1,4)-linked glucose polymer, is a product of the utilization of solar energy and carbon dioxide by plants, through photosynthesis, a process which is estimated to produce $7.2 \times 10^{10}$ tons of cellulolitic biomass annually (1). The degradation and oxidation of cellulose to carbon dioxide, a process carried out mainly by microorganisms, is a key transformation step in the biological carbon cycle in nature (2). The filamentous fungus *Trichoderma reesei* is considered to be the most efficient producer of cellulases (3, 4). Its cellulases are classified into two broad classes: cellobiohydrolases (*CBH*), whose major activity involves the cleavage of cellobiose resi-

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1 The abbreviations used are: CBH, cellobiohydrolase; EG, endoglucanase; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s).

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EXPERIMENTAL PROCEDURES

**Materials**—[*γ*-32P]ATP, [*α*-32P]dATP, and [*α*]-32PdCTP (specific radioactivity: 3000 Ci/mmol) were purchased from Amersham Corp., GeNeAmp DNA Amplification kit was from Perkin-Elmer Co. Avicel (PH101, microcrystalline cellulose) was generously provided by Forlab-Kelrio S/A, Brazil.

**Construction of Plasmids and Fusion Genes**—The plasmids pCBH1-Hph-2.2 and pCBH1R-Hph-2.2 containing the 5′-flanking region of

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Basal Expression and Cellulase Induction

the cbh1 gene fused in the correct and the opposite orientation to the hygromycin B resistance gene (hph) were constructed as follows. A 2.2-kb EcoRI-BglII DNA fragment containing the 5′-flanking region of the cbh1 gene was treated with T4 DNA polymerase and ligated to a BamHI linker. The coding region for the hph gene was amplified using the plasmid pLG90 (22) in conjunction with PCR in which a BamHI site was embedded at the 5′ ends of the primers. The 5′-flanking region of the cbh1 gene and the amplified hph coding sequence were digested with BamHI, ligated and then inserted into the BamHI site of a Bluescript containing a polyadenylation signal. A construct containing the 5′-flanking region, the hph gene and the polyadenylation signal placed in the correct orientation was isolated and designated pCBH1-Hph-2.2. The pCBH1R-Hph-2.2 was constructed by placing the 5′-flanking region in opposite orientation using the SacII sites located 10 bp 5′ of the ATG.

The antisense plasmid, pJO51, was constructed as follows. Specific oligonucleotides, in which suitable restriction enzymes sites were placed at the 5′ end, were used in conjunction with PCR to amplify the required cbh2 and eg12 sequences. The amplified cbh2 DNA fragment contained 399 bp (from base 1477 to 1876) from the fourth exon of the gene (14). The amplified eg12 DNA fragment contained 399 bp (from base 992 to 1380) from the second exon of the gene (11). A 587-bp KpnI-EcoRI DNA fragment (from base 86 to 673) from the first exon of the eg1 gene (10) was isolated and used in the construction of the plasmid pJO51. After digestion with the suitable restriction enzymes, the fragments were ligated and inserted in the reverse orientation between the TrpC promoter and the 3′ terminator from Aspergillus nidulans (23, 24).

RNA Isolation and Analyses—Total RNA was isolated as described (25). RNA (10 μg) was separated by electrophoresis on a 1.2% agarose gel, after denaturation with glyoxal and dimethyl sulfoxide (26), and transferred to a Zeta-Probe membrane. Membranes were hybridized with a random primer [α-32P]dCTP radiolabeled EcoRI fragment (720 bp) or KpnI-PstI fragment (1050 bp) containing part of the coding region of the cbh1 or eg1 respectively (10, 12).

Primers and Polymerase Chain Reaction—Each of the forward primers (cb1 for cellobiohydrolase I and eg 1 for endoglucanase, respectively) and a reverse oligonucleotide complementary to the mRNA; cb1 and eg1, primers correspond to DNA sequences 740–760 and 885–905 of the eg1 gene (10) was used and isolated in the construction of the plasmid pJO51. After digestion with the suitable restriction enzymes, the fragments were ligated and inserted in the reverse orientation between the TrpC promoter and the 3′ terminator from Aspergillus nidulans (23, 24).

RESULTS

Transmission of cbh1 and eg1 in Uninduced Cells—The transcripts of two members of the cellulase system, cbh1 and eg1, could not be detected by Northern analysis in total RNA isolated from uninduced T. reesei cells (Fig. 1). Moreover, the physical heterogeneity of substrates and the complexity of the cellulase system’s multiple enzymes, synergism, and glucanases with overlapping specificity (6), present obstacles to assaying the expression of a single member of the cellulase system, especially at low activity levels. To detect low levels of transcription of cellulases in uninduced T. reesei cells, we assayed for the presence of cbh1 and eg1 transcripts using reverse transcription-PCR. The strategy of the amplification process is presented in Fig. 2A. The position of the primer and the expected amplified product using DNA or RNA as a template is also shown. cDNAs were synthesized from total RNA extracted from Avicel-induced and glycerol-grown (uninduced) T. reesei cells and then amplified by PCR. This procedure produced 82- and 125-bp segments of the cbh1 and eg1 transcripts, respectively (Fig. 2B; refer to details in Fig. 2A). However, if T. reesei genomic DNA was used as a template instead of mRNA, the presence of introns in cbh1 and eg1 genes, resulted in fragments of 149 and 196 bp, respectively (Fig. 2C). In addition, digestion of amplified products by appropriate restriction enzymes produced fragments of the expected sizes (Fig. 2C; see also Fig. 2A). This excluded any possible artifactual amplification of contaminating DNA and confirmed that the amplified DNA fragments corresponded to spliced cbh1 and eg1 transcripts. Quantitation of both transcripts from uninduced relative to induced T. reesei cells was performed using 5′-32P-labeled reverse primers (cb2 for cbh1 and eg2 for eg1) and comparing the extent of amplification achieved after different numbers of PCR cycles as described by Chelly et al. (29). The levels of cbh1 and eg1 transcripts were, respectively, 1156- and 1148-fold lower in uninduced cells relative to induced cells. It is worth mentioning that similar results were obtained when total RNA was isolated from uninduced T. reesei cells grown in a culture medium in which peptone was omitted.

The Promoter of the cbh1 Gene Drives Basal Expression of a Heterologous Gene Placed Under Its Control—We used a heterologous gene fusion to demonstrate that the promoter of the major member of the cellulase system, cbh1, is functionally active in the absence of cellulose, and that this low basal promoter activity is sufficient to endow a new phenotype on transformed T. reesei cells. Series of plasmids were constructed in which the Escherichia coli gene encoding hygromycin B phosphotransferase (22) was placed downstream from the 5′-flanking DNA sequence of the cbh1 gene. The enzyme, hygro-
mycin B phosphotransferase, catalyzes the phosphorylation and subsequent inactivation of the antibiotic hygromycin B, a potent inhibitor of protein synthesis in both pro- and eu-karyotes (30). The resistance to hygromycin B of transformed T. reesei cells is shown in Fig. 3. The results demonstrate that the cbh1 promoter fused to the hygromycin B phosphotransferase gene in the correct orientation (plasmid pCBH1-Hph-2.2) conferred resistance to the antibiotic in noninduced cells. No transformants were obtained in control experiments in which the cbh1 promoter was fused in the opposite orientation (plasmid pCBH1R-Hph-2.2) or with promoterless construct (pHph). In six independent transformations, an average of 500 transformants/mg of the plasmid pCBH1-Hph-2.2 was usually obtained (Fig. 3). It is important to note that in T. reesei transformants harboring the plasmid pCBH1-Hph-2.2, the transcript of hygromycin B phosphotransferase was found to be induced with cellulose or sophorose in a manner resembling that of the cbh1 gene (data not shown). These results indicate that the cbh1 promoter has basal transcription activity in the absence of an inducer and that low levels of mRNA do indeed give rise to hygromycin B phosphotransferase activity and subsequent resistance of transformed T. reesei cells to the antibiotic.

Expression of Antisense RNA against cbh2, egl1, and egl2 mRNA Leads to Suppression of Cellulose-induced Expression of the cbh1 Transcript—We designed an antisense strategy to establish convincingly that the inductive mechanism of the cellulase mRNA by cellulose in T. reesei requires basal expression of the cellulase transcripts. The cellulase system of T. reesei is made up of hydrolases catalyzing the cleavage of β-(1,4)-glycosidic bonds in the cellulase chain. The members of this system include at least two cellobiohydrolases, CBHI and CBHII, and two major endoglucanases, EGI and EGII, that act synergistically (7, 8) in the hydrolysis of cellulose to oligosaccharides. Therefore, we decided to examine the effect that the expression of antisense RNA against CBHII, EGI, and EGII mRNAs would have on the induction of cbh1. We reasoned that if the basal activity of cbh2, egl1, and egl2 was necessary for induction by cellulose, the expression of antisense transcripts against those three cellulases should inhibit the induction of cbh1 by cellulose but not by sophorose. A 399-bp DNA sequence from the fourth exon of the cbh2 gene, a 587-bp DNA sequence from the first exon of the egl1 gene and a 398-bp DNA sequence from the second exon of the egl2 gene were ligated together and then inserted in reverse orientation between the TrpC promoter and the 3’ terminator (23, 24) from A. nidulans (Fig. 4, 10171).
The TrpC promoter was found to be functional in T. reesei (16). This antisense construct, pJO51, was transformed into QM9414 T. reesei cells, and five stable transformants were isolated. Analyses of DNA from those transformants showed that the plasmid pJO51 was integrated in the fungal genome (data not shown). The effect of cellulose and sophorose on the expression of the cbh1 transcript was analyzed and the results of one of those transformants, QMJO51, and the original QM9414 cells are presented in Fig. 4. The cbh1 transcript was examined by Northern blot analysis of total RNA from transformed cells. The nucleotide and amino acid sequences presented below the pCBH1-Hph-2.2 construct represent the fusion region between the cbh1 and the hph as indicated. The arrowhead represents the orientation of the cbh1 promoter.

**Fig. 3. Basal expression of a heterologous gene construct controlled by the 5'-flanking cbh1 DNA region.** The plasmid pCBH1-Hph-2.2 contains a 2.2-kb DNA fragment from the 5'-flanking cbh1 region, including the nucleotide sequence coding for the first 9 amino acids of the cbh1 gene, fused in frame to the gene coding for hygromycin B phosphotransferase. The plasmid pCBH1R-Hph-2.2 contains the 5'-flanking cbh1 DNA region, including the TATA sequence, but not the ATG codon, fused in the opposite orientation. T. reesei was transformed with Bluescript (as a control plasmid), pCBH1-Hph-2.2, pCBH1R-Hph-2.2, and promoterless constructs and cells were plated on minimal medium (see “Experimental Procedures”). After 48 h, two comparable 2.5-cm circles, containing at least 20 transformants, were removed from each plate, placed on top of minimal medium containing no inducer, and grown for 4–5 days in the absence or the presence of 200 μg/ml hygromycin B as indicated. To calculate the number of resistant transformants, aliquots containing comparable number of transformed protoplasts were plated on minimal medium containing 200 μg/ml hygromycin B and resistant colonies were counted after 48 h. Results are the average of six independent transformations. The nucleotide and amino acid sequences presented below the pCBH1-Hph-2.2 construct represent the fusion region between the cbh1 and the hph as indicated. The arrowhead represents the orientation of the cbh1 promoter.

**Fig. 4. Effect of expression of antisense RNA against cbh2, egl1, and egl2 mRNAs on cellulose- and sophorose-induced expression of the cbh1 transcript.** A, restriction maps of the genes coding for CBHII, EGL, and EGII. Boxes represent exon and lines introns; shaded ports within the fourth exon of the cbh2 gene and the second exon of egl2 gene were amplified using the primer presented on the top and the bottom of each of the restriction map. A restriction site was added at the 5' end of each primer (bold letters), as indicated, to facilitate the construction of the plasmid pJO51. The shaded part within the first exon of the egl1 gene was isolated using the indicated restriction sites. These fragments were ligated and inserted in the opposite orientation between the TrpC promoter and terminator as presented in B and named pJO51. In T. reesei cells, QM9414 was transformed with pJO51, and a stable transformant, QMJO51, was isolated in which the plasmid pJO51 was integrated within the fungal genome. QM9414 and QMJO51 were induced with 1% cellulose (C) or 3 M sophorose (D). Aliquots were removed at the indicated time, and total RNA was isolated and analyzed by Northern blot with labeled cbh1 probes as indicated. The transcript of actin (act) of T. reesei (33) was analyzed, and is included as a control.
RNA isolated from QMJO51 and QM9414 cells exposed to cellulose for 17, 20, and 24 h, or sophorose for 4 and 6 h. Using cellulose as an inducer, the results revealed reduction of the expression of the cbh1 transcript by at least 85% in cells carrying the antisense construct QMJO51, compared with the original QM9414 (Fig. 4C). However, expression of the antisense RNA did not repress the synthesis of the cbh1 transcript if the soluble disaccharide sophorose was used as an inducer (Fig. 4D).

Effect of the Addition of Exogenous Cellulase on the Induction of cbh1 and egl1 Transcripts—Studies on the nature of the physiological inducer of the cellulases by growing T. reesei on cellobiose have indicated that sophorose might be one of the naturally occurring inducers of the cellulase system (18). Therefore we examined the kinetics of induction of the cbh1 and egl1 transcript by the soluble disaccharide sophorose was used as an inducer (Fig. 4D).

DISCUSSION

In T. reesei, cellulase is an inducible enzyme system that has drawn considerable interest regarding the mechanism by which the insoluble inducer cellulose triggers synthesis of the cellulase transcripts. The proposed mechanism of cellulase induction is that the fungus produces basal levels of cellulase and that the activity of these extracellular enzymes on cellulose produces a soluble inducer, which can enter the cell and effect induction (18, 31). In support of this mechanism, we have previously shown that antibodies against CBHI, CBHII, EGI, and EGII blocked the expression of cbh1 gene in the presence of cellulose but not the soluble inducer sophorose (21). However, these constitutive levels of cellulases and their direct role in...
cellulase induction have not been conclusively demonstrated.

The results presented here show that the mRNAs of the major members of the cellulase system, cbh1 and eg1, are transcribed under uninduced conditions, and that induction with cellulose results in at least 1100-fold increase of both transcripts. To examine further the basal activity of the promoter of the cbh1 gene, we constructed a chimeric vector in which the gene encoding hygromycin B phosphotransferase (22) was placed under the control of the 5'-flanking DNA sequence of the cbh1 gene. Indeed, under uninduced conditions, resistance to the antibiotic hygromycin B was observed with T. reesei cells transformed with this construct and grown on medium lacking cellulose. These sets of experiments indicate that the promoters of cbh1 and eg1 are transcriptionally active under uninduced conditions, and that the basal activity of the cbh1 promoter is sufficient to drive expression of a heterologous gene such as the hygromycin B resistance-encoding gene.

We also used an antisense RNA strategy to present direct and in vivo evidence for the requirement of the basal expression of the cellulase in induction of the cellulase transcripts by cellulose. The major cellulases from T. reesei, CBHII, CBHIII, EGI, and EGII, have glucanase activities with overlapping specificity (6). Therefore, we decided to express an antisense RNA composed of parts of the coding sequences against specificity (6). Therefore, we decided to express an antisense RNA composed of parts of the coding sequences against the inductive process is protein dependent (data not shown). In addition, it has been shown that the transcription of the cbh1 transcript by cellulose but not sophorose; and (iv) the kinetic of expression of the cbh1 and eg1 transcripts by cellulose is influenced by the addition of cellulose to the culture medium.

Taken together, these results support a model that requires the presence of basal activity of cellulases for the induction of their own transcripts by cellulose. The identification of genes involved in this process and their mode of action will be critical to completely understand how the induction occurs at the molecular level.

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