Production of *Trichoderma reesei* Cellulases on Glucose-Containing Media

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The filamentous fungus *Trichoderma reesei* was shown to secrete active cellobiohydrolase I and the endoglucanase I catalytic core domain into the culture medium when the fungus was grown on glucose-containing medium. The expression of the proteins was driven by the promoters of the elongation factor 1 α , *tef1*, and the unidentified gene for cDNA1. The cDNA1 promoter gave the best yields. The highest amounts of cellobiohydrolase I and the endoglucanase I core, being 50 to 100 mg/liter, accounted for more than half of the total protein secreted by the fungus. The levels obtained with the *tef1* promoter were 20 to 50 times lower.

The filamentous fungus *Trichoderma reesei* has a long history in the production of hydrolytic enzymes widely used in the food and feed industries and recently also in the textile and pulp and paper industries. Among the hydrolases produced by *T. reesei* is the complete set of cellulolytic enzymes. Systematic improvement of production strains by mutagenesis and screening has resulted in strains producing reported levels of extracellular protein of over 40 g/liter, with approximately half of the proteins produced consisting of the main cellulase, cellobiohydrolase I (CBHI) (5). The relative proportions of the different hydrolases can be influenced only to some extent by varying the growth media and cultivation conditions (see reference 1 for an example). With genetic engineering techniques such as gene inactivation and overproduction under the control of the strong *cbh1* promoter, it has been possible to construct *T. reesei* strains with novel cellulase profiles (6) and enzyme mixtures highly enriched in certain activities such as that of xylanase (26).

It is crucial in many applications that the enzyme mixture produced by the fungus does not contain any harmful side activities. For instance, xylanases are used for enzymatic bleaching of pulp and the enzyme preparation should not contain activities destroying the quality of the cellulose fiber. It is to be expected that new specific applications for hydrolysis and controlled modification of plant polysaccharides will be developed. These applications may require highly specific enzyme preparations, even preparations with only one individual hydrolytic enzyme. Purification of the desired activities is costly for large scale applications, and it is desirable to use the whole enzyme mixture produced by the fungus. However, it is not conceivable that a production strain lacking all harmful side activities could be easily constructed by gene inactivation. Consequently, new production strategies are needed in order to obtain specific enzyme preparations for future applications.

Because of its excellent secretion capacity and cheap and easy cultivation, *T. reesei* is also a potent host for the large-scale production of heterologous proteins. The strong *cbh1* promoter has been used for the production of heterologous proteins on media containing cellulose or its derivatives (7, 12, 20, 27, 31). A synthetic glucose-containing medium would, however, be beneficial, because it is expected to induce lower

amounts of proteases, a condition important to production level (4), and it allows easier purification of the product. Because the *cbh1* promoter is repressed by glucose (23), other promoters are needed to produce heterologous proteins on glucose-containing media.

For the above-mentioned purposes, we have previously isolated *T. reesei* genes highly active in the presence of glucose (17). Because the expression of fungal hydrolytic genes is generally repressed by glucose, the promoters of these genes would allow production of a certain protein essentially free of most contaminating activities. In this article we describe the use of the promoters of the gene encoding translation elongation factor 1 α , *tef1*, and of a still-unidentified gene for cDNA1 in the construction of strains that secrete as model proteins active CBHI and the catalytic core domain of endoglucanase I (EGI) into the culture medium when *T. reesei* is grown on glucose-containing medium.

MATERIALS AND METHODS

Strains, media, and growth conditions. *T. reesei* QM9414 (ATCC 26921) (15) was used as a production host. *T. reesei* QM9414 and transformants were maintained on potato dextrose agar (PD) slants (Difco) or preserved as spore suspensions in 20% glycerol at -70°C . *T. reesei* strains were cultivated at 28°C in 96-well microtiter plates or in shake flasks for 3 to 4 days in 50 ml or for 5 to 6 days in 250 ml of *Trichoderma* minimal medium (22) buffered to pH 6 and supplemented with 3% glucose. Microtiter plates were inoculated with spore suspension or directly from PD slants with a toothpick. Other cultivations were inoculated with 10^6 spores per ml of culture. The glucose concentration in the culture medium was monitored daily with a GOD-Perid method-based kit (Boehringer Mannheim) and was kept above 1% by the addition of aliquots of 20% glucose when necessary. The Solka Floc cultivation was carried out for 5 days in shake flasks with 50 or 250 ml of *Trichoderma* minimal medium (22) supplemented with 1% Solka Floc cellulose (James River Corporation, Berlin, N.H.) and 0.2% Bacto Peptone (Difco). Fermentor cultivation was carried out on minimal medium (22) containing 2% glucose, 0.4% Bacto Peptone, and 0.1% yeast extract (Difco) in a 15-liter fermentor. The glucose concentration was monitored throughout the cultivation as described above and was kept above 1% by glucose feeding. Growth media and mycelia were separated by filtration through GF/B glass microfiber filters (Whatman).

Cloning and sequencing of the *tef1* and cDNA1 genes. Isolation of the *tef1* and cDNA1 cDNA clones has been described by Nakari et al. (17). The genomic copies of the *tef1* and cDNA1 genes were isolated with the corresponding cDNA clones pcDNA33 and pcDNA1, respectively, as probes as described by Nakari et al. (17). A 2.9-kb *Xho*I fragment containing 1.7 kb of the 5' coding region and 1.2 kb of the promoter region of the *tef1* gene was subcloned into pSP73 (Promega), resulting in the plasmid pTHN1. Similarly, a 3.2-kb *Eco*RI fragment containing the coding region and 1.2 kb of the 5' and 1.6 kb of the 3' flanking regions of the cDNA1 gene was subcloned into pSP73 (Promega), resulting in the plasmid pTHN3. The cDNA and genomic copies of the *tef1* and cDNA1 genes were sequenced by the dideoxynucleotide method with a Sequenase sequencing kit (U.S. Biochemical).

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Cloning of *tefl* and cDNA1 promoters. A 1.2-kb *tefl* promoter extending from the 5' end to the initiator ATG was amplified by PCR with Vent polymerase (New England Biolabs) according to the manufacturer's instructions. The primer pair used was 5'-ACC GGA ATT CAT ATC TAG AGG AGC CCG CGA GTT TGG ATA CGC C (sense) and 5'-ACC GCC GCG GTT TGA CGG TTT GTG TGA TGT AGC G (antisense).

The exact location of the promoter of the cDNA1 gene with an unknown function could not be determined on the basis of homology comparisons. The translation initiation site of the gene was thus predicted by sequence analyses and by Northern (RNA) hybridization experiments. The longest cDNA clone started 190 bp upstream of the putative coding region (longest open reading frame), which was preceded by no open reading frames in the cDNA and chromosomal sequences. In Northern analysis, only those probes which were specific for the region contained in the longest cDNA clone gave signals, but no signal was obtained with probes specific for only regions upstream of the cDNA (data not shown). The analysis by the PC/GENE 6.85 COD_FICK program, which predicts coding and noncoding sequences, was consistent with the Northern hybridization and sequence analyses. Furthermore, no putative intron consensus signals were present in the sequence preceding the actual intron verified by comparison of the cDNA and chromosomal copies, thus excluding the possibility of an extra intron in the 5' end of the gene.

Consequently, 1.2 kb of the cDNA1 promoter preceding the putative translational initiator ATG or a 1.8-kb fragment containing at its 5' end an additional 600 bp of bacterial vector sequences derived from the plasmid pSP73 was amplified. The antisense primer 5'-GAT GCA TCG ATC GTC CGC GGG TTG AGA GAA GTT GTT GGA TTG ATC AAA AAG was used in combination with the sense primers 5'-TAG CAG GTC GAC GAA TTC GGT CTG AAG GAC and 5'-CGT ACC ATA TGA CAG AAT TCG GCG GCG ATA AGT CGT GTC TTC CGG GTT G for the 1.2- and 1.8-kb fragments, respectively. The oligonucleotide primers used included a cleavage site for *KspI* at the 5' end of the antisense primer and a site for *EcoRI* at the 5' end of the sense primer to facilitate subsequent cloning of the promoter fragments. The amplification protocol consisted of an initial cycle of denaturation at 96°C for 5 min, annealing at 55°C for 2 min, and polymerization at 72°C for 5 min followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and polymerization at 72°C for 2 min.

DNA constructions. To construct the expression vector pTHN100B for EGI core expression under control of the *tefl* promoter, the *cbh1* promoter in the previously constructed vector pEM-3A (16) was replaced with the *tefl* promoter. The PCR-amplified *tefl* promoter (see above) was digested with *KspI* and partially digested with *EcoRI* and cloned into pEM-3A (16) digested with *EcoRI* and partially digested with *KspI* to release the *cbh1* promoter. pEM-3A (16) contains the *eglI* cDNA (21) in between a 2.3-kb *cbh1* promoter fragment and a 0.7-kb *cbh1* terminator fragment. A stop codon is constructed after the catalytic core domain of *eglI* cDNA at Asn-394. Additionally, pEM-3A (16) contains the acetamidase gene from the plasmid p3SR2 (9), which is linked 3' of the *cbh1* terminator and serves as the selection marker in fungal transformation.

For the construction of the cDNA1 promoter-EGI core expression cassette (THN300), 8 µg of the *KspI*-digested amplified 1.8-kb cDNA1 promoter fragment was ligated with 6 µg of the 2.2-kb EGI core-*cbh1* terminator fragment released from pEM-3 with *KspI* and *SphI*. pEM-3 is same as pEM-3A (16) mentioned above, except that it is missing the acetamidase gene. The cDNA1 promoter-CBHI expression cassette (THN302) was constructed by ligating 1 to 2 µg of the *KspI*-digested 1.2-kb promoter fragment with 2.5 to 5 µg of the 3.2-kb *KspI*-*SphI*-digested fragment containing the *cbh1* gene and the 0.7-kb *cbh1* terminator released from pMLO17, which is a descendant of pMLO16 (23) in which *lacZ* has been replaced with a *cbh1* protein-coding region. The total volume of each ligation mixture was 10 µl.

Fungal transformations. In the fungal transformation, 5 µg of the plasmid pTHN100B was used. In cotransformations, 2 to 12 µg of the plasmid p3SR2 (9) was used with a 10-µl volume of each ligation mixture (see above) containing either the cDNA1 promoter-EGI core expression cassette (THN300) or the cDNA1 promoter-CBHI expression cassette (THN302). Transformation and selection of *Amd*⁺ transformants were carried out as described by Penttilä et al. (22), and transformants were purified through conidia.

Initial screening for production of CBHI and EGI core. Transformants producing CBHI or EGI core in microtiter or shake flask cultures were screened by analyzing 200 µl of culture medium by slot blotting. CBHI was detected by monoclonal anti-CBHI antibody 89 and EGI core was detected by monoclonal anti-EGI antibody A2 (kindly provided by Rolf Bühler, Alko Research Laboratories), with alkaline phosphatase-labelled polyvalent anti-mouse immunoglobulins (Sigma) as secondary antibodies. The alkaline phosphatase conjugate was detected with the ProtoBlot Western blot (immunoblot) AP (Promega).

Analysis of proteins in culture medium. To remove small peptides, culture supernatants were gel filtered with Econopac DG10 columns (BioRad) prior to protein concentration and activity measurements. Total protein concentrations were determined according to the method of Lowry et al. (14). CBHI (33) and EGI (2, 10) activities were measured by using soluble 4-methylumbelliferyl-β-D-cellobiopyranoside (Koch-Light) and hydroxyethyl cellulose (Fluka 54290), respectively, as substrates. CBHI and EGI core were detected by Western analysis with the anti-CBHI antibody 89 and the anti-EGI antibody A2 as described above for the screening procedure.

Fungal DNA and RNA isolation and Southern and Northern hybridizations. Chromosomal DNA was isolated according to the method of Raeder and Broda (25), and 2 µg of DNA was cleaved with *SmaI* or *XhoI* in the case of EGI core-producing transformants and with *NcoI*, *BglII*, or *XmaI* in the case of CBHI-producing transformants. These restriction enzymes had known recognition sequences within the cDNA1 promoters, in the *cbh1* and *eglI* genes, and in the *cbh1* terminator fragment. Southern hybridizations with *cbh1*- and *eglI*-specific probes were carried out under stringent conditions (28). Total cellular RNA was isolated as described by Chirgwin et al. (3) from the shake flask cultivations on the last day. Total RNAs (2 or 0.2 µg) were glyoxylated (28), electrophoresed on 1% agarose, and transferred onto Hybond N nylon filters (Amersham). Filters were hybridized with *cbh1*- and *eglI*-specific probes at 42°C and were washed twice with 5× SSPE (1× SSPE is 0.15 M NaCl plus 0.01 M sodium phosphate plus 1 mM EDTA) for 15 min at 42°C, twice with 1× SSPE-0.1% sodium dodecyl sulfate (SDS) for 15 min at 42°C, and twice with 0.1× SSPE-0.1% SDS for 15 min at 42°C.

RESULTS

DNA constructions and transformations. *T. reesei* strains producing EGI core under the *tefl* promoter were constructed by placing 1.2 kb of the PCR-amplified *tefl* promoter in front of the EGI core coding region in the previously constructed plasmid pEM-3A (16). The resulting expression vector, pTHN100B, contains the *eglI* cDNA in between the *tefl* promoter and the *cbh1* 3' flanking region containing the transcription terminator. Within the *eglI* gene, Asn-394, a potential N-glycosylation site, is mutated to a stop codon, resulting in premature translation termination and consequently giving rise to the ~43-kDa EGI core protein, which lacks the cellulose binding domain and the flexible linker region which connects the catalytic core to the cellulose binding domain. pTHN100B also contains the *Aspergillus nidulans amdS* (9) gene allowing the transformants to be selected by their abilities to use acetamide as their sole nitrogen source (22, 29).

The 1.8-kb promoter region preceding the presumed translation initiation site of the cDNA1 gene was amplified by PCR. Problems were encountered in subcloning of the cDNA1 promoter, and for this reason the PCR-amplified fragment was ligated to a fragment containing the EGI core expression cassette and the ligation mixture (THN300) was directly transformed into the fungus. The same strategy was also used in constructing CBHI-producing strains. In this case, the 1.2-kb cDNA1 promoter fragment was amplified by PCR and ligated to the expression cassette containing the CBHI protein coding region and the *cbh1* terminator (ligation mixture THN302).

The *T. reesei* strain QM9414 was transformed with the plasmid pTHN100B, and transformants were selected on the basis of acetamide utilization. The ligation mixtures THN300 and THN302 were cotransformed into strain QM9414 together with the separate plasmid p3SR2 (9) carrying the acetamidase marker. The *Amd*⁺ transformants obtained were cultivated for 3 to 4 days on glucose-containing medium on microtiter plates or in shake flasks, whereafter EGI core and CBHI production was screened with monoclonal antibodies. The transformation with pTHN100B resulted in only one clone with an EGI core expression level clearly detectable by the screening method used. On the other hand, out of the 94 THN300 transformants and the 107 THN302 transformants obtained, 14 and 22% released EGI core and CBHI, respectively, into the culture medium. The transformants were purified through single spores and were once more tested for EGI core and CBHI production as previously to identify the best producers.

Shake flask and fermentor cultivations. To analyze production of EGI core, the *tefl* transformant pTHN100B-16C and the three best EGI core-producing cDNA1 transformants (THN300-3B, THN300-60A, and THN300-182A) were grown for 6 days in shake flasks on glucose-containing medium to-

TABLE 1. Shake flask cultivations of EGI core- and CBHI-producing cDNA1 transformants on glucose-containing medium

<i>T. reesei</i> strain	Total protein (mg/liter)	Amt (mg/liter [%]) of product determined by ^a :			
		EGI core		CBHI	
		HEC activity	Western analysis	umbG ₂ activity	Western analysis
THN300-3B	50	15 (30)	13 (26)	ND ^b	
THN300-60A	60	20 (30)	27 (45)	ND	
THN300-182A	70	21 (30)	53 (76)	ND	
THN302-47C	80			43 (54)	53 (66)
THN302-137E	40			9 (22)	7 (18)
THN302-158E	50			12 (24)	8 (16)
QM9414	50–60				

^a Values in parentheses are percentages of the total protein. HEC, hydroxyethyl cellulose; umbG₂, 4-methylumbelliferyl- β -D-cellobiopyranoside.

^b ND, not determined.

gether with *T. reesei* QM9414, which was used as a control. To analyze production of CBHI, a similar 5-day glucose cultivation of the three best CBHI-producing cDNA1 transformants (THN302-47C, THN302-137E, and THN302-158E) and the best EGI core-producing cDNA1 transformant (THN300-182A) was carried out. Strain THN300-182A was also cultivated for 4 days on glucose-containing medium in a 15-liter fermentor. In all experiments, extra glucose was added during the cultivations in order to keep the level higher than 1%. Aliquots of the culture supernatants were collected on the last day of the shake flask cultivations, were collected daily from the fermentor, and were analyzed for CBHI and EGI core expression.

The amounts of CBHI and EGI core produced by the transformants were estimated on the basis of enzyme activity measurements and Western analysis, with purified CBHI and EGI proteins as standards. Because of the low level of EGI core produced with the *tef1* promoter (roughly 2 mg/liter), further analysis was carried out with the cDNA1 transformants only. Table 1 summarizes the results obtained with the cDNA1 transformants.

The production levels of EGI core and CBHI under the cDNA1 promoter in the shake flask cultivations were 13 to 53 and 7 to 53 mg/liter, respectively, depending on the transformant and the method of analysis (Table 1). These values correspond to 16 to 76% of total extracellular protein in these cultivations. In the Coomassie blue-stained SDS-polyacrylamide gel of the shake flask culture samples (Fig. 1A), EGI core and CBHI produced by the cDNA1 transformants are seen among the most prominent bands. Western analysis (Fig. 1B) shows that no production of endogenous EGI or CBHI protein on glucose medium could be detected in untransformed strain QM9414. Because of the size difference between EGI core and the full-length EGI, the lack of leakage of EGI is also evident in the transformants. This is in agreement with our earlier results showing tight glucose repression of the cellulase genes on glucose-containing media (23).

In the fermentor cultivation on glucose, the EGI core-producing cDNA1 transformant THN300-182A yielded 100 mg of the product per liter after 96 h of cultivation, which corresponds to 40% of total secreted protein (Fig. 2 and Table 2). Accumulation of EGI core during the fermentor cultivation can be clearly detected by SDS-polyacrylamide gel electrophoresis (Fig. 3).

FIG. 1. (A) Coomassie blue-stained SDS-polyacrylamide gel of culture media (200 μ l each) from 5-day shake flask cultivations of EGI core- and CBHI-producing cDNA1 transformants on glucose-containing media. Lane 1, untransformed host strain QM9414; lanes 2 to 4, CBHI-producing transformants THN302-47C, THN302-137E, and THN302-158E, respectively; lane 5, EGI core-producing transformant THN300-182A; lane 6, untransformed host strain QM9414 cultivated on Solka Floc cellulose medium. Arrows show the positions of CBHI and EGI core. The positions of the molecular mass markers are shown on the right. (B) Western analysis of similar shake flask cultivations with anti-CBHI or anti-EGI antibody. Purified CBHI (500 ng) and EGI (500 ng) proteins were used as controls. Lanes 1 to 3, transformants THN302-158E, THN302-137E, and THN302-47C, respectively; lanes 4 to 6, transformants THN300-182A, THN300-60A, and THN300-3B, respectively; lane 7, transformant pTHN100B-16C producing EGI core under the control of the *tef1* promoter; lane 8, untransformed host strain QM9414. Aliquots (30 μ l each) of culture media of strains THN100B-16C and QM9414 and 10- μ l aliquots from all other cultivations were loaded. The molecular masses of CBHI, EGI, and EGI core are shown on the left.

To compare the production of EGI core under the control of the cDNA1 promoter on glucose with that on cellulose-containing medium and with the expression levels of the cellulases naturally obtained, cultivations were carried out with the EGI core-producing strain THN300-182A on media containing Solka Floc cellulose. It can be seen that the cDNA1 promoter was also functional when the fungus was growing on cellulose (Fig. 4). However, because of variations in growth state and rate on cellulose- and glucose-containing media, it is impossible to fully compare the capacities of the strain for extracellular protein production on the two media. The difference in size of EGI core and the wild-type EGI could be used to estimate the production specific to the cDNA1 promoter on cellulose medium. Western analysis (Fig. 4) clearly demonstrates the much higher production level of endogenous EGI compared with that of EGI core.

Southern and Northern analyses of transformants. Southern analysis was carried out to verify the presence of correctly ligated and integrated CBHI and EGI expression cassettes in the genomes of the transformants THN300-3B, THN300-60A, THN300-182A, THN302-47C, THN302-137E, and THN302-

FIG. 2. The cDNA1 transformant THN300-182A on glucose-containing medium in a fermentor. Production of EGI core is shown in Table 2.

158E. The presence of such cassettes is especially important because ligation mixtures, which might lead to incorrectly ligated and integrated promoters and expression cassettes, were used in transformation. In all the transformants characterized in the present study, at least one copy of the gene correctly linked to the promoter in question could be detected (data not shown). Besides containing these expression cassettes, some of the transformants also seemed to contain other copies in which the joint between the promoter and the gene was correct but in which some deletions within 400 bp of the 5' upstream area of the promoter or in the terminator region had occurred. Because only one correctly sized mRNA could be detected in the best EGI core-producing transformant and in the three best CBHI-producing cDNA1 transformants, these other copies either are not expressed or result in correct transcription (see below). In all cases the expression cassettes were integrated into unknown locations in the genome, except for one EGI core-producing transformant (THN300-60A) in which integration into the cDNA1 locus had occurred (data not shown).

Transcription efficiencies driven by the cDNA1 promoter were determined by Northern analysis with probes similar in length and specific activity (Fig. 5). Glucose cultivations of the CBHI- and EGI core-producing transformants (THN302-47C and THN300-182A, respectively) were compared with two different Solka Floc cultivations. The mRNA levels of *cbh1* and *egl1* produced from the cDNA1 promoter on glucose medium seem to be roughly five to ten times lower than those of the endogenous transcripts on cellulose. Because of similarities in size, the amounts of *cbh1* and *egl1* mRNA derived from the cDNA1 promoter cannot be distinguished from the endogenous ones on cellulose cultivations. However, no notable increase in the total amount of *cbh1* or *egl1* mRNA can be seen in the transformants on the cellulose cultivations compared with those of the host strain. This indicates that the mRNA levels produced from the endogenous promoters clearly exceed

TABLE 2. EGI core production by the cDNA1 transformant THN300-182A in a fermentor

Time in fermentor (h)	Amt (mg/liter) of EGI core determined by:		Total protein (mg/liter)	% of EGI core of total protein
	Western analysis	HEC ^a activity		
20	1	0.8	110	0.7–1
45	8	1.0	130	0.5–6
72	80	36	180	20–44
96	100	41	230	18–43

^a HEC, hydroxyethyl cellulose.

FIG. 3. Coomassie blue-stained SDS-polyacrylamide gel of fermentor culture medium of the EGI core-producing cDNA1 transformant pTHN300-182A cultivated on glucose medium. Lane 1, untransformed host strain QM9414 cultivated for 6 days in 50 ml of glucose medium (185 μ l of culture supernatant); lanes 2 to 5, transformant at 20, 45, 72, and 96 h of cultivation (with 230, 85, 85, and 85 μ l of culture supernatant), respectively. The positions of the molecular mass markers are shown on the left.

those of the expression cassettes, which is in agreement with the protein data presented above (Fig. 4). The difference seen in the endogenous cellulase mRNA levels in the two cellulose cultivations is probably due to the different growth stages of the fungus.

DISCUSSION

The production of both homologous and heterologous proteins by filamentous fungi is generally achieved with strong hydrolase promoters, whose disadvantages usually are their at least partial carbon catabolite repression by glucose. The number of promoters having regulatory properties that allow protein production on glucose-containing media is at the moment limited. Data on the use of the glyceraldehyde phosphate dehydrogenase promoter (*gpdA*) of *A. nidulans* (24) and other promoters such as *A. nidulans alcC* (alcohol dehydrogenase), *tpiA* (triose phosphate isomerase), *oliC* (subunit 9 of mitochondrial ATPase synthase), and *A. niger adhA* (alcohol dehydro-

FIG. 4. Comparison by Western analysis of 5-day cultivations on glucose- and Solka Floc cellulose-containing media of the EGI core-producing transformant THN300-182A. Supernatant samples (5 μ l) were analyzed with anti-EGI antibody. Purified EGI was used as control. Cultivations were carried out in 50 and 250 ml of Solka Floc cellulose medium (lanes 1 and 2, respectively) and 250 ml of glucose medium (lane 3). The molecular masses of the wild-type EGI and EGI core proteins are shown on the left.

FIG. 5. Northern analysis of *cbh1* and *egl1* expression of CBHI-producing transformant THN302-47C, EGI core-producing transformant THN300-182A, and untransformed host strain QM9414. Analysis was performed in the same cultivations as described in the legend to Fig. 3. Lane 1, 2 μ g of RNA from a 250-ml glucose cultivation; lane 2, 0.2 μ g of RNA from a 250-ml Solka Floc cellulose cultivation; lane 3, 0.2 μ g of RNA from a 50-ml Solka Floc cellulose cultivation.

genase) have been published (30, 35). We have tested various promoters—e.g., the *tefl* and cDNA1 promoters described here and previously, the *pgk1* promoter (32), and a modified *cbh1* (16, 23) promoter—for production of EGI core and CBHI on glucose-containing medium. The modified glucose-derepressed *cbh1* promoter (16, 23) was obtained by mutating in the promoter three hexamer sequences similar to the binding sites of the glucose repressor proteins MIG1 of *Saccharomyces cerevisiae* and CreA of *A. nidulans* (13, 18). A similar approach used to construct a derepressed expression system with a modified *alcA* promoter for protein production on glucose has recently been reported for *A. nidulans* (8), but no production levels have yet been reported. In addition, the *gpdA* promoter of *A. nidulans* has been used to produce single-chain antibodies in *T. reesei* on glucose-containing medium (19).

So far, the cDNA1 promoter has proven to be the most effective of the *T. reesei* promoters tested on glucose-containing media. Even though the strong *PGK* promoter is generally used for protein production in the yeast *S. cerevisiae*, the *pgk1* promoter of *T. reesei* proved to be insufficient in protein production in *T. reesei* (32). The production levels reported in this

study for shake flask cultures of both CBHI and EGI core under the control of the cDNA1 promoter clearly also exceed the amounts of cellulases obtained with the *tefl* and the derepressed *cbh1* promoters in the same *T. reesei* strain, being 10- to 50-fold higher. The efficiency of the cDNA1 promoter is further confirmed by the favorable product-to-total protein ratio, showing that roughly half of the secreted protein is either CBHI or EGI core. The *tefl* gene was isolated as one yielding abundant amounts of mRNA on glucose medium, but the promoter seems not to be suitable for protein production. It is possible that the high amounts of *tefl* mRNA previously observed in glucose cultivations reflect the high level of stability of the mRNA rather than the high level of transcription efficiency of the promoter. In mammalian cells, the regulation of the gene is also rather complex, including mechanisms such as translational control (11) and involvement of enhancers of transcription in the first intron (34), which might also operate in fungi.

Although the expression levels on glucose of both *cbh1* and *egl1* directed by the cDNA1 promoter were rather high, these and the levels of protein product obtained, 50 to 100 mg/liter, are not comparable to the endogenous cellulase mRNAs or the protein levels of this strain on inducing cellulose medium. Also, the amount of total secreted protein remains low, 0.05 to 0.23 g/liter, compared with that obtained with this strain on cellulose medium, 1.1 to 1.8 g/liter, and is far from the reported yields of about 40 g/liter obtained with the hypercellulolytic production strains (5). Production conditions for *T. reesei* have so far been optimized mainly for media containing complex plant material, and the optimization of glucose cultivations is urgently needed in order to demonstrate the secretion and production capacities of *T. reesei*. Furthermore, it would be instructive to know whether production could be increased by using any of the industrial production strains as hosts.

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