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REGULATION OF *TRICHODERMA* CELLULASE FORMATION: LESSONS IN MOLECULAR BIOLOGY FROM AN INDUSTRIAL FUNGUS

A REVIEW*

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The present article reviews the current understanding of regulation of cellulase gene transcription in *Hypocrea jecorina (= Trichoderma reesei)*. Special emphasis is put on the mechanism of action of low molecular weight inducers of cellulase formation, the presence and role of recently identified transactivating proteins (Ace1, Ace2, Hap2/3/5), and the role of the carbon catabolite repressor Cre1. We also report on some recent genomic approaches towards understanding how cellulase inducers signal their presence to the transcriptional apparatus.

Keywords: cellulase formation, industrial fungus, *Trichoderma*

Introduction

Species of fungal genus *Trichoderma* are saprophytes, and their respective teleomorphs lignicolous and necrotrophic ascomycetes of the genus *Hypocrea*. It is believed that evolution drove these fungi from mere parasitism on wood-degrading basidiomycetes to adaptation to the partially destroyed wood [1]. Hence, in nature they encounter a wide variety of polysaccharides including cellulose – a β-1,4-glucosidically-linked homopolymer of about 8000–12000 glucose units, held together by hydrogen bonds thus forming an insoluble crystalline

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macromolecule – making up a significant part of the total amount. *Trichoderma* spp. also play a significant role in cellulose degradation in the soil.

Trichoderma reesei (the anamorph of the pantropical ascomycete *Hypocrea jecorina*), is probably the most prominent cellulose degrader within the genus *Trichoderma*. Originally isolated in the South Pacific (Solomon Islands) during the Second World War, where it was observed to degrade cotton fabric, tents and belts, it was first recognized as a potential industrial producer of cellulases in the late sixties. It uses at least three different types of enzymes for cellulose degradation, i.e. exoglucanases (i.e. cellobiohydrolases EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21), which occur in various isozymic forms (Table I). The industrial potential of this enzyme mixture led to a detailed investigation of the biochemical properties, three-dimensional protein structures and mechanism of action of several of these proteins [2–13].

Table I

Cellulase genes and their encoded proteins as identified in *H. jecorina*

Gene	Protein
cbh1	Cel7A
cbh2	Cel6A
egl1	Cel7B
egl2	Cel5A
egl3	Cel12A
egl4	Cel61A
egl5	Cel45A
bgl1	β-glucosidase 1
bgl2	β-glucosidase 2

Yet it should be noted here that Saloheimo et al. [14] recently added a new facette to the picture of fungal cellulose degradation, by cloning the gene encoding a protein with a sequence similarity to plant expansins. These are plant cell wall proteins which are thought to disrupt hydrogen bonding between cell wall polysaccharides without hydrolyzing them [15]. The protein, named swollenin, has an N-terminal fungal type cellulose binding domain connected by a linker region to the expansion-like domain, and also contains regions similar to mammalian fibronectin type III repeats. Overexpressed swollenin was found to disrupt the structure of cotton fibers without detectable formation of reducing sugars. Whether or not swollenin is necessary for the growth of *T. reesei* on cellulose is unknown, disruption of the swollenin gene had, however, no effect. The authors speculated that there may be multiple swollenin genes in *T. reesei*.

Apart from studies on the enzymology of cellulose degradation, there has also been a continuing interest in understanding how the synthesis of these enzymes is regulated. Such knowledge is required to understand the physiology of the organism, to provide strategies for improving enzyme production through nutritional or recombinant strategies, and for using cellulase promoters for the overproduction of heterologous proteins in *Trichoderma*.

The present review attempts to summarize the current state of knowledge on how an insoluble molecule like cellulose triggers its own degradation in *T. reesei*.

Inducing conditions and inducer paradigms

Mechanism of induction by cellulose

In 1960, Mary Mandels and Elwyn T. Reese [16] already raised the central question of regulation of cellulase formation: "Cellulases are adaptive enzymes, but the natural substrate – cellulose – is insoluble. So how does induction occur?" It is generally believed that oligosaccharides, which are released from cellulose, serve as the actual compounds triggering the high level of induction of cellulase expression (Figure 1). This hypothesis is supported by the high cellulase-inducing

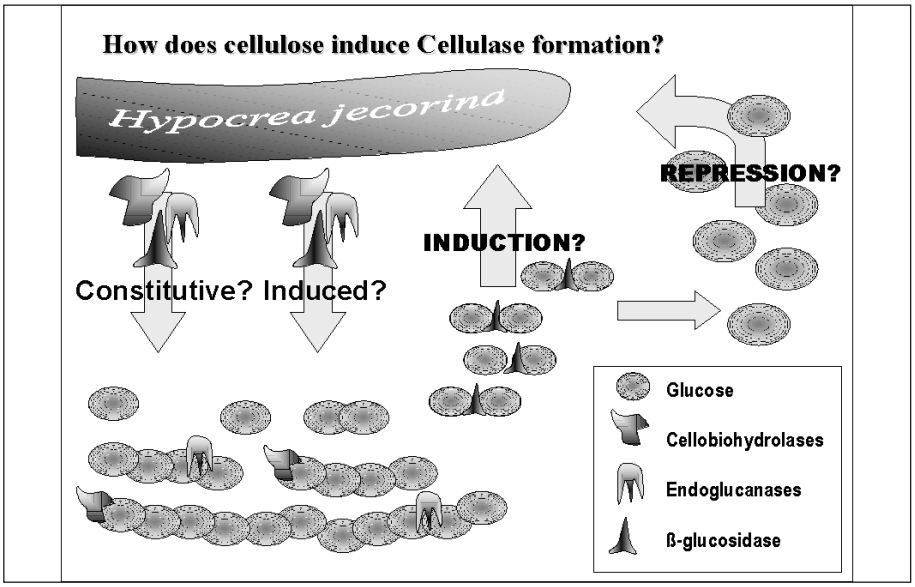

Figure 1. Current hypothesis of how cellulases are formed by *H. jecorina*

power of several disacharides such as cellobiose (a β-1,4-linked diglucoside), δ-cellobiono-1,5-lactone, lactose and sophorose (a β-1,2 linked diglucoside).

If these low molecular weight compounds are indeed the physiological inducers of cellulases, then how do they arise in the presence of the fungus? Three different models have been offered. One of them – already proposed in the seventies – suggests that the fungus produces low, constitutive cellulase levels, which would enable an initial attack on cellulose and release of oligosaccharides which may act as inducers for more abundant cellulase biosynthesis [17–20]. While the evidence presented in favour of this explanation has not unequivocally been accepted, the occurrence of similar induction mechanisms in several other microbial systems strengthens this hypothesis. Generally speaking, binding of a transcriptional regulator to its target sequence in the promoter is an equilibrium, and thus even strongly repressed genes or genes strongly relying on induction can always give rise to very low levels of "constitutive" expression which, however, may escape detection by the methods available.

An alternative explanation has been presented by Kubicek and coworkers, which was originally based on the observation that only conidia but not mycelia are able to start growing on cellulose in fermenter cultures: conidia of *Trichoderma* spp. contain a whole set of different enzymes capable of hydrolysing a wide range of polysaccharides and related compounds [21, 22], and may therefore be an option for initial degradation of cellulose under conditions of nutritional stress. They are located on the conidial surface, are formed irrespectively of the nutrient conditions used to induce sporulation, and are able to hydrolyse crystalline cellulose [21]. The essential role of the conidial-bound cellulases in the formation of the cellulase inducer has been demonstrated by depriving the conidia from their cellulases by washing with a soft detergent, which impaired the ability of *T. reesei* to grow on cellulose [21]. Seiboth et al. [23, 24], using isogenic strains in which the major cellulase genes (*cbh1, cbh2, egl1* and/or *egl2*) had been deleted, showed that different cellulases exhibit different abilities for inducer formation: strains in which *cbh2* and *egl2* displayed only marginal cellulase induction, whereas a strain containing an *egl1* deletion was almost indistinguishable from the wild-type strain. Conidia from a strain in which both *cbh1* and *cbh2* had been replaced were completely unable to induce *egl1* or *egl2* gene expression by cellulose. Addition of 2 mM sophorose to cultures of the strain in which both *cbh1* and *cbh2* had been deleted restored this ability. These results are in accordance with a mechanism by which several conidial-bound cellulases synergistically carry out an initial degradation of cellulose molecules, thereby generating the inducers of cellulase biosynthesis, and hence lead to cellulase gene expression and growth on cellulose.

Yet a third explanation for the initial attack on cellulose has been offered by Ilmen et al. [25], who observed significant expression of all cellulases after depletion of glucose from the culture. The mechanism of this triggering of cellulase formation is not understood at the moment, as it seems to be independent of carbon starvation alone or relieve from carbon catabolite repression. Release of inducing compounds from fungal cell walls or inducer formation through transglycosylation of the glucose previously present in culture are yet unverified suggestions to explain these results.

Soluble inducers

Whichever of these models is in fact responsible for the initial hydrolytic attack on the cellulose molecule, the final product must be a low-molecular weight inducer which is taken up by the fungal cells. Its identity is unknown, but several oligosaccharides can function as cellulase inducers in laboratory experiments:

Cellobiose. As the major soluble end-product formed from cellulose is cellobiose, its appearance in the cell could signal the presence of extracellular cellulose and would therefore be a logical candidate for the natural inducer of cellulase biosynthesis. Growth on cellobiose or addition of cellobiose to *T. reesei* cultures growing on cellulose, however, inhibits cellulase formation [26]. This is probably due to rapid hydrolysis of cellobiose to glucose, which represses cellulase formation [25, 27]. These results must not be interpreted, however, as evidence against cellobiose as an inducer, as one must bear in mind that transport of cellobiose, sophorose, gentiobiose and laminaribiose into *T. reesei* mycelia occurs by a constitutively formed β-linked disaccharide permease [28], which exhibits a much higher affinity for this substrate than the extracellular β-glucosidase. Therefore, at a low disaccharide concentration, as it would be expected to arise from cellulose degradation, uptake of cellobiose (a prerequisite for induction) is favoured over hydrolysis. Induction by cellobiose therefore depends on the ratio of the *in vivo* activity of the cellobiose permease to the activity of β-glucosidase and only occurs when the ratio is high. Consistent with this assumption, slow feeding of cellobiose, addition of β-glucosidase inhibitors, or addition of cellobiose analoga which are poor substrates of β-glucosidase (viz. δ-cellobiono-1,5-lactone) [29, 30] all lead to cellulase formation in amounts comparable to those observed on cellulose [26].

Sophorose. Sophorose, which is also a poor substrate for β-glucosidase, induces cellulase gene expression at a high level and has been considered as the natural inducer of cellulase formation for a long time, particularly as its presence in culture fluids of *T. reesei* has been demonstrated [16, 18, 31]. Sophorose seems to be formed by transglycosylation activity of β-glucosidase [32], but one should bear in mind that some endoglucanases are also capable of performing this reaction. Genetic support for sophorose formation by β-glucosidase was obtained with a recombinant strain, however, in which the β-glucosidase *(bgl1)* gene had been disrupted and which consequently showed less efficient growth and cellulase production on cellulose [33]. Results consistent with these findings have also been reported using β-glucosidase inhibitors [34] or β-glucosidase-mutant strains [35, 36]. Addition of sophorose to the medium restored cellulase induction and growth on cellulose in all of these cases, indicating that β-glucosidase was important for cellulase inducer formation from cellulose.

These results do not prove, however, that the natural inducer is indeed sophorose. Indeed, *T. reesei* strains carrying multiple copies of *bgl1* and displaying enhanced β-glucosidase activity showed an increased efficacy of cellulase induction not only by cellulose but also by sophorose and sophorose induction of cellulase formation in a *bgl1* disruptant strain was still inhibited by the β-glucosidase inhibitor nojirimycin [37]. One possible explanation for this finding would be that formation of the final inducer involves the action of at least one more β-glucosidase. A candidate for this enzyme would be the constitutive, plasma-membrane bound, and methyl-β-D-glucoside-inducible β-glucosidase described by Umile and Kubicek [38] and Chen et al. [39]. In addition, Saloheimo et al. [40] recently characterized an intracellular β-glucosidase enzyme (BGLII, = Cel1a) and its gene *(bgl2)*, and demonstrated that the respective protein exhibited transglycosylation activity. So the nature of the β-glucosidase involved in cellulase induction is not known yet.

Lactose. Lactose (a 1,4-O-β-D-galactopyranosyl-D-glucose) is not a normal degradation product of cellulase or of other plant polymers. Its demonstration as a potent cellulase inducer is therefore puzzling, and was interpreted to be due to its structural resemblance to cellobiose. It is at present the only soluble carbon source which can be used economically for cellulase production or production of heterologous proteins under cellulase promoters by *T. reesei* [41].

The major pathway of lactose metabolism by *T. reesei* occurs by extracellular hydrolysis into glucose and galactose, and subsequent metabolism of the

galactose moiety via the Leloir pathway (B. Seiboth and C. P. Kubicek, unpublished data). In order to identify the mechanism of lactose induction, mutants in the various steps were prepared. Thereby, knock-out mutants in the galactokinase-encoding gene *gal1* resulted in a strong impairment of cellulase induction by lactose [42], whereas knock-out of *gal7* (encoding the subsequent uridyltransferase step) did not affect cellulase induction but lead to a prolonged abundance of the cellulase gene transcript [43]. These two findings therefore suggest that the cellular concentration of galactose-1-phosphate may signal the presence of lactose. This induction seems, however, to be influenced by other as yet unknown environmental conditions, as growth of *T. reesei* on galactose (which obviously also leads to formation of galactose-1-phosphate) does not induce cellulase formation, even in a carbon catabolite repression-negative background [42]. We have some evidence that a slow growth rate may be essential for induction by lactose.

At the moment, nothing is known about the mechanism by which galactose-1-phosphate activates transcription. The fact that mutants of *T. reesei*, in which cellulase induction by cellulose or sophorose is impaired, are still induced by lactose (M. Mandels, S. Zeilinger and C. P. Kubicek, unpublished data) suggests that lactose induction works by a different signalling pathway or uses different transacting factors.

Other soluble compounds. A limited number of other low-molecular weight compounds have been reported to promote cellulase gene expression, but their relationship to *in vivo* induction by cellulase has not been tested. An interesting case is the finding by Margolles-Clark et al. [44] that transfer of pregrown mycelia of *T. reesei* to L-arabitol results in expression of *cbh1* with a transcript abundance comparable to lactose cultivations. This finding has not been pursued further but may constitute a link between hemicellulose and cellulose degradation.

Also L-sorbose was found to induce coordinately cellulase genes at the transcriptional level [41], but also the physiological meaning of this is unclear. We have noted (C. P. Kubicek, unpublished data) that D-sorbose is rapidly converted into sorbitol. It will be interesting to learn whether arabitol and sorbitol induce cellulases by the same mechanism.

Regulation of cellulase gene expression

Whatever the nature of the *in vivo* inducer of cellulase is, it is commonly accepted that their formation is regulated at transcriptional level, and the expression

of the different cellulase genes has been reported to be coordinate [10, 19, 24, 25, 27, 33, 45, 47–50]. *cbh1* is the most highly expressed gene, followed by *cbh2, egl5* and *egl1*.

Analysis of cellulase gene promoters (mainly *cbh1* and *cbh2*) has shown that expression of these genes is subject to both positive and negative regulation. These are treated in detail below.

Transcriptional activators of cellulase gene expression

To clone genes encoding positive-acting transcriptional regulators of *T. reesei* cellulase expression, Saloheimo et al. [51] employed the one-hybrid system using a full-length *T. reesei cbh1* promoter coupled to the *S. cerevisiae HIS3* gene as a bait. This strategy led to the identification of two putative transcriptional activators, viz. the *ace1* (activator of cellulase gene expression) gene, encoding a novel DNA-binding protein (Ace1) that contains three zinc finger motifs of Cys(2)-His(2) type; and *Ace2* encoding a DNA-binding protein (Ace2) belonging to the class of zinc binuclear cluster proteins found exclusively in fungi. Possible Ace1 homologues were found among expressed sequence tags of *Aspergillus* and *Neurospora*, indicating that this gene also occurs in other filamentous fungi.

According to the *in vitro* binding data, there are at least eight binding sites for Ace1 in the *cbh1* promoter. Ace1 recognized all AGGCAAA sites and some AGGCA sites preceded by a relatively A-T rich region. The available evidence does not exclude that other variants of the binding sequence exist. Identical or very closely related sequences occur, e.g. in the promoters of *T. reesei* cellulase genes *egl1, egl5,* and *cbh2* and the xylanase gene *xyn1*. Disruption of the *ace1* gene in *T. reesei* resulted in retarded growth of the fungus on a cellulose-containing medium, on which cellulases are normally highly expressed.

In contrast to *ace1*, homologues of *Ace2* were not found. Ace2 binds *in vitro* to the 5'-GGCTAATAA site present in the *cbh1* promoter at –779 relative to ATG. Mutation of the triplet GGC completely abolished Ace2 binding, and changing the next two TAA triplets reduced it. The binding site is not a repeated sequence as has been shown for most other transcription factors with zinc binuclear cluster DNA-binding domains reviewed by Todd and Andrianopoulos [52]. In this respect Ace2 appears to be similar to the *A. niger* XlnR. It is important to note in this respect that Ace2 does not bind to the XlnR consensus (5'-GGCTAAA-3'). The *A. niger* XlnR was first identified as a xylanase regulator but was later shown to regulate also the transcription of other hemicellulase and cellulase genes. The suggested putative binding sites for XlnR and ACEII contain an identical core. How-

ever, Ace2 and XlnR are different in size and show no amino acid sequence similarity except in the DNA-binding domain. Even here the identity is only 20%, much less than when Ace2 is compared with other proteins with zinc binuclear cluster domains, and it is thus highly unlikely that Ace2 is the *Trichoderma* homologue of XlnR. An XlnR homologue has recently been cloned and characterized (R. Rauscher, E. Würnleithner and R. L. Mach, unpublished results; [54]), but no *ace2* homologue is present in the genome of *A. nidulans* or *A. fumigatus* (C. P. Kubicek, unpublished data). It is therefore likely that *Trichoderma* developed a specific transactivator for cellulase regulation which is not present in other fungi.

Deletion of the *Ace2* gene led to lowered induction kinetics of *cbh1, cbh2, egl1* and *egl2* mRNAs and to 30–70% reduced cellulase activity when the fungus was grown on medium containing Solka floc cellulose. Cellulase induction by sophorose was not affected by *Ace2* deletion, however, suggesting that sophorose and cellulose induction may involve different DNA-binding proteins.

Other transacting factors involved in cellulase gene expression were detected by studying the regulation of the *cbh2* promoter. Using cell-free extracts from sophorose-induced and noninduced mycelia and various fragments of the *cbh2* promoter of *H. jecorina* in electrophoretic mobility shift assay (EMSA) analysis and performing *in vitro* and *in vivo* footprinting analysis, Zeilinger et al. [54] detected the nucleotide sequence 5'-ATTGGGTAATA-3' (consequently named *cbh2*-activating element, CAE) to bind a protein complex. *In vivo* footprinting showed that binding to CAE occured both under induced as well as non-induced conditions. EMSA analysis, however, revealed that the CAE-protein complex formed by cell-free extracts from induced mycelia migrated faster than that from non-induced mycelia, suggesting that the former either lacks a protein component present in the latter, or becomes enzymatically modified (e.g. by phosphorylation or proteolysis). EMSA analysis with specifically mutated versions of CAE revealed that protein binding requires the presence of an intact copy of either one of two adjacent motifs: a CCAAT (= ATTGG) box on the template strand and a GTAATA box on the coding strand, whereas a simultaneous mutation in both completely abolished binding. *H. jecorina* transformants, containing correspondingly mutated versions of the *cbh2* promoter fused to the *Escherichia coli hph* gene as a reporter, expressed *hph* in a manner paralleling the efficacy of CAE-protein complex formation in EMSA, suggesting that the presence of either of both motifs is required for induction of *cbh2* gene transcription.

EMSA supershift experiments using an antibody against *Aspergillus nidulans* HapC suggested that the complex which binds to the *H. jecorina* CCAAT box contains a HapC homologue. To obtain direct evidence for this, Zeilinger et al.

[55] cloned the *hap2, hap3* and *hap5* genes from *H. jecorina*. They encode proteins whose core regions display great similarity to *Aspergillus* HapB, HapC and HapE and to known Hap homologues from other organisms. All three proteins (Hap2, Hap3 and Hap5) were needed to bind to the CAE in the *H. jecorina cbh2* gene promoter in vitro. Thus the CCAAT box on the template strand in CAE is bound by the *H. jecorina* equivalent of the Hap protein complex.

Interestingly, Abrahao-Neto et al. [45] reported that mitochondrial activity is required for maximal cellulase gene expression. Carraro et al. [56] showed that heterologous transcription of a reporter gene controlled by the *cbh1* promoter in *S. cerevisiae* was also dependent on active mitochondria, suggesting the presence of respective *cis*-acting sequences in cellulase promoters. It shall be mentioned that the Hap2/3/5 complex in yeast is involved in respiratory regulation [57], and it would be interesting to learn, whether such a regulation would also occur in *T. reesei*.

One protein binding to the GTAATA box in the *cbh2* promoter is Ace2, as shown by EMSA analysis with its overexpressed DNA-binding domain (Figure 2), thereby extending its consensus binding motif to GGSTAATA. It is intriguing to note that – in contrast to findings of Aro et al. [58] – deletion of its binding motif in the *cbh2* promoter also impairs *cbh2* induction by sophorose and not only by cellulose [54].

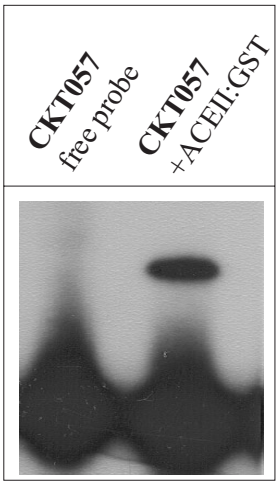

Figure 2. Binding of Ace2 to the *H. jecorina cbh2* promoter. Binding of ACEII:GST (aa 1–58, DNA binding domain; [57]) to the labeled oligonucleotide CKT057 (Zeilinger et al. 1998) containing CAE. Free probe is shown in the first lane. The amount of purified recombinant ACEII:GST fusion protein used in the binding reaction was 2 µg

A cellulase-negative mutant strain, originally isolated during the strain selection program of Reese and Mandels [59], was shown by Zeilinger et al. [60] to be defective in the functional formation of the CAE-protein complexes but not in their binding to the target sequences themselves. Cell-free extracts from a cellulase-negative mutant form CAE-protein complexes with higher mobility and lower binding-strength compared to the wild type, suggesting that the CAE-binding complexes either lack one of the protein components of the complex or that one of the proteins is truncated. EMSA analysis demonstrated an increased mobility of the Ace2-containing DNA complex and, supported by *in vivo* footprinting, a lowered binding strength of the Hap2/3/5 proteins. However, the *hap2/hap3/hap5* genes of the mutant are unaltered and transcribed normally, and the *Ace2* transcript has a similar size in the mutants and in the wild-type (M. Schmoll and C. P. Kubicek, unpublished data), thus rendering a mutation in one of these two binding partners unlikely.

As all the components identified as transcriptional regulators of cellulase gene expression bind their target sites constitutively, the question arose as to their way of action. Since the Hap2/3/5 complex has been shown in other fungi to enable transcription by chromatin remodelling, Zeilinger et al. [61] investigated the alterations in chromatin structure accompanying induction of cellulase gene expression on the *cbh2* promoter. The chromatin structure in the wild type strain showed a nucleosome free region (nfr) around CAE flanked by strictly positioned nucleosomes under repressing conditions. Induction results in a loss of positioning of nucleosomes –1 and –2 downstream of CAE, thus making the TATA box accessible. Simultaneous mutation of both motifs of CAE or of the CCAAT-box alone also led to a loss of positioning of nucleosome –1 covering the TATA-box, whereas mutation of the Ace2-binding-consensus sequence resulted in a narrowing of the nfr, indicating that the proteins binding to both motifs within CAE interact with chromatin, although in different ways. These data therefore suggest that both the Hap2/3/5 complex and Ace2 are all involved in nucleosome assembly on the *cbh2* promoter, and that they respond to inducing conditions by repositioning nucleosome –1.

Interestingly, the cellulase-negative mutant strain described above still displayed the induction-specific changes in nucleosome structure whereas it failed to trigger gene expression, thereby confirming that it is not impaired in the proteins directly interacting with CAE and that nucleosome rearrangement and induction of *cbh2* expression are uncoupled. In view of the available other data, this suggests that a protein component linking the RNA-polymerase II adaptor complex with Hap2/3/5-Ace2 is impaired in this mutant. This component still needs to be identi-

fied, however. Schmoll et al. [62] however, recently cloned a gene encoding a WD-repeat/F-box-protein, which is upregulated by the presence of cellulose and lactose in *T. reesei* QM 9414 but not in the cellulase-negative mutant, and thus could the gene encode the missing link in cellulase induction.

Carbon catabolite repression

Carbon catabolite repression is an ubiquitously occuring important regulatory mechanism that leads to the repression of genes encoding enzymes that are involved in the utilization of complex carbon sources when simple sugars like glucose are available. Such a situation is also observed with the *Trichoderma* cellulase system: in the presence of high levels of glucose, sophorose fails to induce expression of the cellulase genes, and glucose addition to already induced cultures leads to the disappearance of the transcripts of c*bh1, cbh2, egl1, egl2* and *egl5* cellulose [25]. However, this finding *per se* does not necessarily imply glucose repression, as it may well reflect impaired inducer uptake (the disaccharide permease is inhibited by glucose; [28]). Evidence for a direct repressing effect of glucose on cellulase gene expression therefore must be based on the use of null-mutants of the respective repressor gene and on cellulase promoter analysis.

The *cre1* gene encoding the general carbon catabolite repressor protein Cre1 has thus been cloned [46, 63, 64], and shown to encode a functional homologue of the CreA protein of *A. nidulans* [65]. It binds to the consensus binding site 5‘-SYGGRG-3‘. Ilmen et al. [27] mutated the consensus sites at around –700, –1000 and –1500 bp upstream of the protein coding region – which have been shown to bind recombinantly overexpressed Cre1 in *in vitro* gel shift assays [66, 67] – by replacing the native nt-sequences by the same number of nts of mutant sites in different combinations, and fused them to the *E. coli lacZ* gene as reporter. The results indicated that the mutation of the region around –700 resulted in clear derepression of *lacZ*-mRNA on glucose. Interestingly, this binding site is organized in the form of an inverted repeat, and up to date only Cre1-binding sites organized in a similar way have been shown to be functional *in vivo*. Interestingly, carbon catabolite derepression resulted only in a ten-fold lower level of transcription than upon induction [27], indicating that full expression is not simply due to antagonization of carbon catabolite repression and requires activating factors as well.

Interestingly, the commonly used *T. reesei* hyperproducer strain RutC-30, which was originally isolated by screening for growth on cellobiose in the presence of 2-deoxyglucose [68], bears a mutated *cre1* gene [46]. It has only 20 % of

the coding region remaining, and the truncated protein formed lacks all the C-terminal amino acids after the first zinc finger. Northern analysis confirmed that this strain expresses some cellulases such as *cbh1* in the presence of glucose [46] although at a low level [61], and – consistent with the above findings – was still dependent on induction.

The cellulase system was used by Cziferszky et al. [69] to study how Cre1 becomes activated in the presence of glucose. They showed that Cre1 is a phosphoprotein, identified Ser_{241} within the acidic protein region as phosphorylation target. These findings reminded the situation in yeast, where the carbon catabolite repressor protein Mig1 is phosphorylated by the Snf1-kinase [70–75]. However, in yeast Mig1 phosphorylation occurs upon glucose depletion and results in the removal of Mig1 from the nucleus and binding to the respective promoters [73]. In contrast, phosphorylation of Cre1 occurs in the presence of glucose and is required for DNA binding of Cre1 [69]. Detailed analysis showed that an $S_{241}E$ mutation mimics phosphorylation, whereas an $S_{241}A$ mutant protein shows phosphorylation-independent DNA binding activity, suggesting that phosphorylation is required to release Cre1 from an inactive conformation involving unphosphorylated Ser_{241}. Consistent with these *in vitro* data, retransformation of a *H. jecorina cre1*-non-functional mutant with Cre1-$S_{241}A$ leads to permanent carbon catabolite repression in cellobiohydrolase I expression.

Also the kinase phosphorylating Cre1 is different from that phosphorylating Mig1: the amino acid sequence surrounding Ser*241* (HSNDEDD) suggests that phosphorylation may occur by a casein kinase II-like protein, which is supported by a mutation of $E_{244}V$ which leads to loss of phosphorylation, loss of DNA binding, and gain of carbon catabolite derepression. In addition, an overexpressed catalytic domain of *H. jecorina* Snf1 did not phosphorylate *H. jecorina* Cre1 but readily phosphorylated yeast Mig1 [76]. *H. jecorina* Snf1 also phosphorylated peptides comprising the strict Snf1 consensus, but notably did not phosphorylate peptides containing the regulatory serine residue in Cre1 identified by Cziferszky et al. [69]. Thus the Snf1 kinase appears not to be involved in Cre1-mediated carbon catabolite repression in *H. jecorina*. These findings are also supported by recent results from *A. nidulans* [77] which showed that another gene whose mutation leads to a carbon catabolite derepressed phenotype – *creB* – encodes an ubiquitin carboxy-terminal hydrolase [78]. The authors proposed that under carbon catabolite repressing conditions, CreB removes the ubiquitin chains from CreA that would otherwise lead to its degradation by the proteasome. Thus, glucose signalling to carbon catabolite repression seems to operate via different ways in filamentous fungi and yeasts.

Signalling pathways

While the data reviewed above clearly show that the cellulase genes are dependent on induction, neither the nature of the inducer nor the pathway of its signalling are known. In another fungus – *Cryphonectria parasitica* – Wang and Nuss [79] provided evidence for the involvement of a G_α-protein in the induction of *cbh1* by cellulose. For *T. reesei*, Sestak and Farkas [80] reported that the efficacy of sophorose induction could be doubled by the addition of permeable cyclic-AMP derivatives or by the inhibitors of cyclic-AMP phosphodiesterase, hence suggesting that the signalling by the inducer involves a cyclic-AMP dependent protein kinase. A sequential action of a G-protein and cyclic AMP would not be without precedent in fungi, and has been reported e.g. for signalling the presence of a host plant to *Ustilago maydis* [81].

Towards an elucidation of how cellulose signals its presence, we have recently made use of the cellulase negative mutants of *T. reesei* using subtraction hybridization [82]. This led to the identification of several genes which were differentially expressed in the cellulase negative mutant QM9978 and the wildtype strain QM9414. Northern analyses proved that some of them are indeed regulated by the presence of cellulose. Among them, the most interesting gene is *envoy*, which encodes a PAS-domain-containing protein, and which is strongly upregulated in the presence of cellulose. It displays a transcript of smaller size in the wildtype, and the mutant also lacks the upregulation by cellulose. Another gene called *csi1* (cellulase signalling 1) is strongly transcribed upon induction by sophorose in the wildtype but not in the mutant, but is less abundant in the wildtype than in the mutant on cellulose. Knock-out mutants in these two genes are currently prepared in our lab to learn about their role in cellulose signalling.

Concluding Remarks

Figure 3 summarizes the current state of knowledge, as outlined above, about the regulation of expression of *H. jecorina cbh1* and *cbh2*. The availability of now at least five different transcription factors and two signalling components and respective gene knock-out mutants for cellulase regulation offers an excellent basis for entering the genomic age. This will undoubtely lead to the discovery of other components either directly or indirectly involved in the cellulose signalling circuits. The knowledge of these mechanisms will not only lead to an academic understanding of how "an insoluble molecule like cellulose triggers its own break-

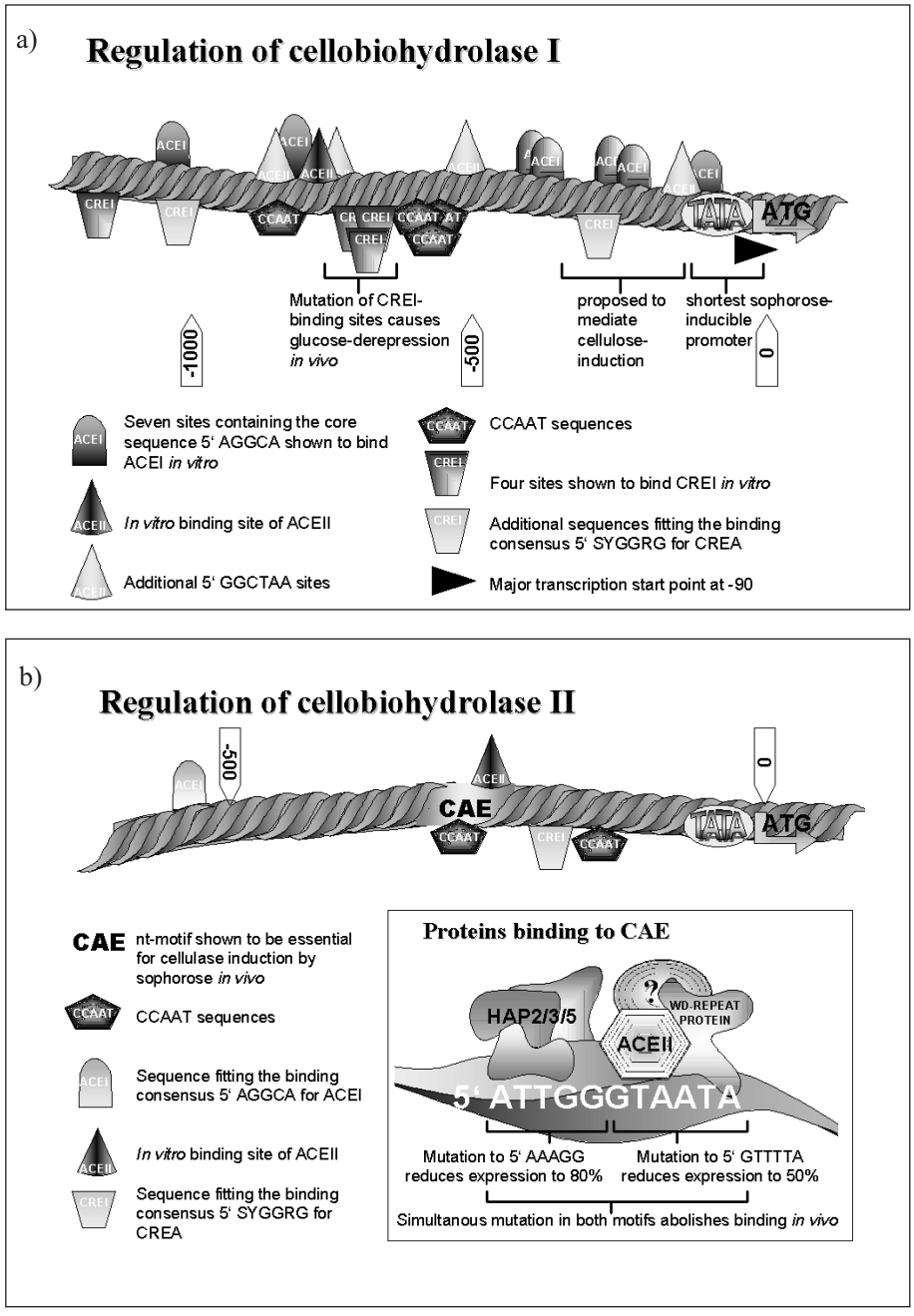

Figure 3. Summarizing scheme of the regulation of the (a) *cbh1* and (b) *cbh2* promoter

down", but is also likely to open new avenues for the improvement of the cellulase promoters. In addition, *H. jecorina* cellulases may become a model system for the regulation of synthesis of extracellular hydrolytic enzymes, which are important traits in understanding fungal plant pathogenesis, by other pyrenomyceteous fungi such as *Fusarium, Claviceps, Verticillium* and others.

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