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Nina Aro

VTT Biotechnology

Faculty of Science
Department of Biosciences
Division of Biochemistry
University of Helsinki
Helsinki, Finland

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Abstract

Cellulose and hemicellulose are the most abundant renewable carbon sources on earth. Filamentous fungi produce a wide variety of extracellular enzymes that degrade these complex polymeric compounds and play an important role in carbon turnover in nature. The filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) is the best studied cellulolytic fungus and it is widely used by the biotechnical industry for production of hydrolytic enzymes such as cellulases and xylanases. The production of these enzymes is regulated at the transcriptional level. The cellulase genes, especially the main cellulase *cbh1*, are very highly expressed and thus their promoters are among the strongest known in nature. Despite the importance of cellulases and xylanases very little is known of the regulatory mechanism involved in the high level of expression of cellulase and xylanase genes.

In this work a novel method was developed to isolate transcription factors without previous knowledge of the important DNA sequence elements in promoters or of the nature of the activator genes and proteins. This method selects simultaneously for binding to and activation of the desired promoter in *S. cerevisiae* and is in general applicable for any organism and promoter. Using this method two new genes, *ace1* and *ace2,* encoding transcription factors binding to the promoter of the main cellulase gene *cbh1* of *T. reesei* were isolated.

ace1 encodes a protein that contains three zinc finger motifs of Cys_2-His_2 type. Amino acid sequence similarity is seen towards *A. nidulans* protein StzA and deduced *N. crassa* protein, but not to yeast proteins suggesting that *ace1* is a regulator specific for filamentous fungi. Gel mobility shift assays revealed at

least eight putative binding sites for ACEI scattered in the *cbh1* promoter all containing the core sequence 5'AGGCA. Deletion of *ace1* gene in *T. reesei* resulted in increased expression of all the main cellulase genes *cbh1*, *cbh2*, *egl1* and *egl2* and the xylanase genes *xyn1* and *xyn2* indicating that ACEI represses cellulase and xylanase expression. The results demonstrate for the first time the down regulation of cellulase and xylanase expression in inchicing conditions.

The second factor ACEII belongs to the family of zinc binuclear cluster proteins found exclusively in fungi. ACEII binds to at least one sequence *in vitro* in the *cbh1* promoter. Disruption of *ace2* results in reduced expression levels of all the main cellulase genes and the fungus showed reduced cellulase activities when grown on cellulose containing media. Also the expression of gene encoding one of the main hemicellulases, β-xylanase XYNII was reduced on cellulose medium indicating that ACEII is an activator of cellulase and xylanase genes in *T. reesei*.

Preface

Anne, Mikko and Jaana for the warm atmosphere in the lab and in the group meetings.

Finally, my parents Antti and Ulla, my sisters Jenni and Vivi deserve my thanks for love and care of me, my home and my family over the years. Thank you for just understanding me and not asking too many questions. All my friends and relatives are thanked for just being there and arranging every now and then something fun and crazy to do.

Above all, I thank you Kari for your love, confidence and understanding during this long journey.

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List of publications

This thesis is based on the following articles referred to in the text by Roman numerals I–III.

I   Saloheimo, A., Aro, N., Ilmén, M. and Penttilä, M. 2000. Isolation of the *ace1* gene encoding a Cys_2-His_2 transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. J. Biol. Chem. **275**, 5817–5825.

II   Aro, N., Saloheimo, A., Ilmén, M. and Penttilä, M. 2001. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. J. Biol. Chem. **276**, 24309–24314.

III   Aro, N., Ilmén, M., Saloheimo, A. and Penttilä, M. 2003. ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. Appl. Environ. Microbiol. **69**, 56–65.

Contents

Appendices I–III

List of abbreviations

aa   amino acid

bp   base pair(s)

CBHI   cellobiohydrolase I

CBHII   cellobiohydrolase II

cDNA   complementary DNA

DNA   deoxyribonucleic acid

EGI   endoglucanase I

EGII   endoglucanase II

GST   glutathione S-transferase

HEC   hydroxyethyl cellulose

mRNA   messenger RNA

kb   kilo base(s) or 1000 bp

MUL   methylumbelliferyl-β-D-lactoside

PCR   polymerase chain reaction

polII   RNA polymerase II

RNA   ribonucleic acid

TAF   TATA binding protein associated factor

TBP   TATA binding protein

TFIID   transcription factor IID, a complex of TBP and TAFs

TBP   TATA binding protein

1. Introduction

Cellulose is the most abundant polysaccharide in nature. It is synthesised mainly by plants and together with hemicelluloses, lignin and pectin constitutes most of the plant cell wall material. Even though approximately 4×10^9 tonnes of cellulose is formed annually, its amount on earth does not accumulate due to fungi and bacteria efficiently degrading these materials to provide themselves with carbon and an energy source for growth and recycling the carbon back into the ecosystem. The hydrolytic degradation of water-insoluble cellulose is a complex process involving the synergistic action of a number of extracellular cellulolytic enzymes.

The degradation of cellulose is a specific feature of certain microbes from various fungal and bacterial taxa capable of producing and secreting cellulose-degrading enzymes to their surroundings. These cellulolytic micro-organisms living in different habitats include the fungi *Trichoderma*, *Penicillium*, *Fusarium* and *Phanerochaete* and prokaryotes such as *Clostridium*, *Cellulomonas*, *Thermobifida* (*Thermomonospora*) and *Streptomyces*.

The cellulolytic enzyme system of many microbes is essential for the maintenance of the global carbon cycle. It provides these organisms with the means to obtain energy and nutrients. Cellulolytic enzymes are believed to participate in the plant pathogenesis by degrading cellulose within the plant cell wall (ten Have *et al*., 2002). Cellulose-hydrolysing enzymes may also play a role in the ability of antagonistic fungi to attack their target organisms by taking part in the hydrolysis of the host cell wall (Inglis *et al*., 2002). Furthermore, the wide array of cellulose- and hemicellulose-degrading enzymes are economically important in different industrial applications (de Vries *et al*., 2002; Penttilä *et al*., 2003 and references therein).

1.1 Cellulolytic filamentous fungus *Trichoderma reesei*

The best-studied cellulolytic organism, the soft-rotting deuteromycete *Trichoderma reesei*, was originally isolated from cotton fabrics degraded by fungi on the Solomon Islands. Today, *T. reesei* is considered to be an asexual clonal line of the ascomycete *Hypocrea jecorina* (Kuhls *et al*., 1996;

Leuchtmann *et al.*, 1996; Samuels, 1996). Since its isolation, *T. reesei* has become an excellent cellulolytic model organism. The enzymatic properties of many cellulases have been carefully analysed, and three-dimensional structures of the catalytic domains characterised and catalytic amino acid residues within these domains are known (Divne *et al.*, 1994; Kleywegt *et al.*, 1997; Koivula *et al.*, 1996; Rouvinen *et al.*, 1990). The secretory pathway of *T. reesei* is currently under investigation, and genes encoding the components of the secretion machinery, such as the GTP-binding protein SARI, the protein disulphide isomerase PDII and the unfolded protein-responsive transcription factor HACI, have been cloned (Saloheimo *et al.*, 2003; Saloheimo *et al.*, 1999; Veldhuisen *et al.*, 1997). The carbon source dependent regulation of cellulase production has been extensively studied in many laboratories. The regulatory mechanisms controlling the production of cellulases at transcriptional level is currently being studied and is presented in more detail in the following sections.

T. reesei is also an important organism from the biotechnological point of view. Industrial use of *T. reesei* began in the 1980s. Several applications using the enzymes produced by *T. reesei* have been developed for the pulp and paper industry, textile treatments and animal feed processing (Buchert *et al.*, 1998; Galante *et al.*, 1998a; Galante *et al.*, 1998b and references therein). Applications such as the use of xylanases in the bleaching of kraft pulp and the cellulase enzyme mixture in the enzymatic stonewashing of denim garments are currently in use (Belghith *et al.*, 2001; Miettinen-Oinonen and Suominen., 2002). The protein yields of *T. reesei* have been improved by mutagenesis and genetic engineering, resulting in strains secreting over 40 g/l of cellulases into the culture medium (Bailey and Nevalainen, 1981; Durand *et al.*, 1988; Nevalainen *et al.*, 1994, Nevalainen *et al.*, 2003). A multitude of genetically modified strains exist, which are used to produce tailored enzyme mixtures for industrial applications (Mäntylä *et al.*, 1998 and references therein). The existence of strong promoters for the expression of heterologous genes and a good secretion capacity carrying out 'mammalian-like' protein modifications makes *T. reesei* a potential host for the heterologous protein production. Heterologous proteins of fungal and mammalian origin have been successfully produced under the main cellulase promoter, cellobiohydrolase I (Keränen and Penttilä, 1995; Paloheimo *et al.*, 1993; Penttilä, 1998).

Several *Trichoderma* strains have been reported to be effective in controlling plant diseases. *Trichoderma harzianum*, a potent antagonistic fungus in the control of plant pathogenic fungi, can be used as an alternative to chemical compounds. The antifungal activity of *T. harzianum* involves production of fungal cell wall degrading enzymes, antibiotics and toxins, and competition for key nutrients (Hjeljord and Tronsmo, 1998). Several *Trichoderma* strains with improved antagonistic activity have been developed and studied in protective field tests.

1.2 Cellulose and hemicellulose breakdown

Cellulose is a chemically simple compound, consisting of β-1,4 linked D-glucose units that form linear polymeric chains of about 8 000–12 000 glucose units. In crystalline cellulose, these polymeric chains are packed together by hydrogen bonds to form highly insoluble structures. Hemicelluloses, on the other hand, have a heterogeneous composition of various sugar units. They can be classified according to the main chain sugar as xylanases, mannanases, arabinases and galactans, consisting of β-1,4 linked xylose, mannan, arabinan and galacotse units, respectively. The main chain sugars of hemicelluloses may be modified by addition of various side groups such as arabinose, galactose, 4-O-methylglucuronic acid and acetyl, making hemicelluloses branched and structurally more variable than cellulose (Timell, 1967; Wilkie, 1979).

Complete hydrolysis of cellulose to glucose requires the combined actions of multiple enzymes with different substrate specificity. The cellobiohydrolases (CBH) (1,4-β-D-glucan cellobiohydrolases) cleave cellobiose units from the ends of the polysaccharide chains. The endoglucanases (EG) (1,4-β-D-glucan-4-glucanohydrolase) cut the cellulose chains internally mainly from the amorphous regions, providing the cellobiohydrolases more ends to act upon (Teeri, 1997). And, finally, β-glucosidases hydrolyse cellobiose units and other disaccharides to glucose, which is taken up by the fungus (Béguin, 1990). With some exceptions, the fungal cellulases consist of two domains; a small C- or N-terminal cellulose-binding domain (CBD) and a catalytic domain connected together with a linker peptide.

The hydrolysis of hemicelluloses occurs by the concerted action of endo- and exo-acting enzymes and several side-chain cleaving enzymes, leading to release of various mono- and disaccharides depending on the hemicellulose type. As an example the breakdown of xylan involves at least endo-1,4-β-D-xylanase and β-xylosidases acting on the main sugar chain and the side-chain cleaving enzymes such as α-glucuronidase and acetyl xylan esterase.

Only a few micro-organisms produce a complete set of enzymes capable of degrading native cellulose efficiently. From *T. reesei* so far, nine cellulase genes have been cloned; two cellobiohydrolase-encoding genes *cbh1* (Shoemaker *et al*., 1983; Teeri *et al*., 1983) and *cbh2* (Chen *et al*., 1987; Teeri *et al*., 1987), five endoglucanase genes *egl1* (Penttilä *et al*., 1986), *egl2* (Saloheimo *et al*., 1988), *egl3* (Ward *et al*., 1993), *egl4* (Saloheimo *et al*., 1997) and *egl5* (Saloheimo *et al*., 1994), and two genes encoding β-glucosidases *bgl1* (Barnett *et al*., 1991; Mach *et al*., 1995) and *bgl2* (Takashima *et al*., 1999b). Furthermore, the *swo1* gene encoding for swollenin protein (SWOI), similar to the plant expansins, that disrupts cellulosic fibres has recently been cloned (Saloheimo *et al*., 2002). In addition, ten genes encoding the hemicellulolytic enzymes, including two xylanases *xyn1* (Törrönen *et al*., 1992) and *xyn2* (Saarelainen *et al*., 1993), have been isolated. The cellobiohdyrolase-encoding gene(s) has been cloned from several other fungi including *Penicillium janthinellum* (Koch *et al*., 1993), *Phanerochaete chrysosporium* (Covert *et al*., 1992), *Humicola grisea* (Takashima *et al*., 1996a), *Trichoderma viride* (Cheng *et al*., 1990), different *Aspergilli* species (Gielkens *et al*., 1999b; Takada *et al*., 1998), *Agaricus biosporus* (Raguz *et al*., 1992) and *Neurospora crassa* (Taleb and Radford, 1995). Cellulolytic activities have been characterised and numerous other cellulase-encoding genes have been isolated from these and other fungi not mentioned here. Among cellulolytic bacteria, *Cellulomonas fimi* is one of the most extensively studied. Hitherto, five endoglucanases and two cello-biohydrolases have been characterised at the genetic level (Coutinho *et al*., 1991; Meinke *et al*., 1991; 1993; 1994; Shen *et al*., 1995; Shen *et al*., 1994; Wong *et al*., 1986). In addition, cellobiohydrolase and endoglucanase genes have been studied in numerous other bacteria.

1.3 Production of cellulases and hemicellulases

The regulation of cellulase production has been shown to be dependent on the carbon source available. Cellulose and cellobiose generally induce cellulase production in fungi, while production is with few exceptions inhibited during growth on glucose and other readily metabolisable carbon sources such as fructose. Fungal cellulase regulation has been analysed at the molecular level mainly in *T. reesei* (described in more detail in the following sections), fungi of the *Penicillium* species (Mernitz *et al*., 1996; Rao *et al*., 1988) and the ligninolytic fungi *Phanerochaete chrysosporium* and *Acaricus biosporus* (Chow *et al*., 1994; Yague *et al*., 1997; 1994) but also in *Humicola grisea* (Takashima *et al*., 1999a; Takashima *et al*., 1996b) and *Aspergillus nidulans*, from which the first positively acting transcriptional regulator of cellulase genes XlnR, was isolated (van Peij *et al*., 1998b) (see in more detail in section 1.4.1.2).

Among bacteria, particularly in the soil actinomycetes, cellulase biosynthesis has also been shown to be regulated by repression and induction depending on the carbon source. In *Thermobifida fusca* (formerly named *Thermomonospora fusca*), which produces at least six cellulases, the production of cellulases is repressed by glucose and induced by cellulose and cellobiose (Lin and Wilson, 1987). Similar carbon source dependent production of the Cel1 cellulase of *Streptomyces halstedii* has been reported (Fernandez-Abalos *et al*., 1997). The cellulase genes of *T. fusca* are controlled by the transcriptional regulator CelR and cellobiose, which acts as an inducer of cellulase expression by interfering with the CelR-promoter interaction (Spiridonov and Wilson, 1999; Spiridonov and Wilson, 2000). The synthesis of cellulase activity in the plant pathogenic enterobacterium *Erwinia carotovora* requires plant signals and is negatively regulated by the RNA binding protein RsmA (Chatterjee *et al*., 1995; Cui *et al*., 1995) and positively regulated by the RNA regulator *rsmB* which acts by neutralising the action of RsmA (Liu *et al*., 1998). An archaeon *Pyrococcus furiosus* expresses the gene coding for an extracellular β-1,3-endoglucanase, *lamA* on cellobiose, while no *lamA* transcript has been detected from pyruvate- and maltose-grown cultures (Voorhorst *et al*., 1999).

1.3.1 Regulation and induction of *T. reesei* cellulase formation

The regulation of cellulase production occurs at the transcriptional level (Abrahao-Neto *et al*., 1995; el-Gogary *et al*., 1989; Ilmén *et al*., 1997; Messner and Kubicek, 1991). The main cellulase genes of *T. reesei cbh1*, *cbh2*, *egl1* and *egl2* are thought to be regulated co-ordinately and their relative expression levels have been shown to be similar in the different growth conditions studied, *cbh1* expression always being the strongest (Fowler and Brown, 1992; Ilmén *et al*., 1997). In addition to the natural substrate of cellulases, cellulose, *T. reesei* cellulase gene expression is provoked by oligosaccharides directly or indirectly derived from cellulose such as cellobiose (two β-1,4-linked glucose units) or sophorose (two β-1,2-linked glucose units) (Fritscher *et al*., 1990; Ilmén *et al*., 1997; Mandels *et al*., 1962; Sternberg and Mandels, 1979). Furthermore, growth in the presence of several disaccharides, such as δ-cellobiono-1,5-lactone, laminaribiose, gentiobiose, lactose and xylobiose, also induce cellulase gene expression (Durand *et al*., 1988; Margolles-Clark *et al*., 1997; Vaheri *et al*., 1979). The monosaccharide L-sorbose was recently reported to induce cellulase expression in *T. reesei* (Nogawa *et al*., 2001).

T. reesei also grows on sorbitol and glycerol, but on these media, no cellulase gene transcription has been detected by Northern analysis (el-Gogary *et al*., 1989; Ilmén *et al*., 1997). These carbon sources are considered to be neutral with respect to cellulase production since addition of an inducing carbon source, *e.g.* sophorose, to glycerol or sorbitol cultivations leads to high levels of cellulase gene expression (Ilmén *et al*., 1997; Mandels *et al*., 1962; Nisizawa *et al*., 1971).

Sophorose is believed to be the most potent inducer in *T. reesei* and is also more specific for *Trichoderma* since sophorose does not induce cellulase expression in *P. janthinellum, P. chrysosporium* and *A. niger*, but does induce cellulase expression in *A. terreus* (Bisaria and Mishra, 1989; Gielkens *et al*., 1999b; Hrmova *et al*., 1991; Mernitz *et al*., 1996). Likewise, D-xylose, which induces cellulase expression in *A. niger*, does not induce *T. reesei* cellulase genes (Gielkens *et al*., 1999b; Margolles-Clark *et al*., 1997). The results, however, are not fully comparable since *A. niger* cellulases were induced with 1 mM D-xylose, while the concentration in the study of *T. reesei* cellulase expression was much higher, 4.5% at the time of analysis. The reports of the inducing power of cellobiose, which provokes cellulase production in many cellulolytic organisms,

are somewhat controversial in *T. reesei* most likely due to the different culture conditions used in the various studies. Slow feeding of cellobiose into growth media was reported to lead to the production of high levels of cellulases (Vaheri *et al.*, 1979). Northern analysis of another study showed that cellobiose induced *cbh1*, *cbh2* and *egl1* transcription to a moderate level, which was reduced at later stages of the cultivation, when glucose started to accumulate in the culture medium (Ilmén *et al.*, 1997). Cellobiose has also been reported to be a poor inducer under certain conditions (Fritscher *et al.*, 1990; Gritzali and Brown, 1979; Penttilä *et al.*, 1986; Royer and Nakas, 1990). The different induction levels in these studies possibly results from the ratio of glucose and sophorose formed from cellobiose by the action of β-glucosidase (Fritscher *et al.*, 1990; Gritzali and Brown, 1979; Ilmén *et al.*, 1997; Nisizawa *et al.*, 1971; Vaheri *et al.*, 1979).

It is generally believed that small oligosaccharides are released from cellulose and these molecules or their derivatives are taken up by the cell and act as inducers of cellulase transcription. The β-glucosidase(s) can either cleave cellobiose, the end product of cellobiohydrolase action, into glucose, which may cause repression, or transglycosylate it into sophorose. Deletion of the *bgl1* gene encoding the major extracellular β-glucosidase lead to a delay in induction of cellulase gene transcription during growth on cellulose, implying a role in cellulase induction (Fowler and Brown, 1992). The presence of the β-linked disaccharide permease was demonstrated in *T. reesei*, and at low cellobiose and sophorose concentrations their uptake into the mycelium by a permease is favoured over hydrolysis to glucose by β-glucosidase. At high cellobiose concentrations, the situation is reversed and hydrolysis into glucose occurs (Kubicek *et al.*, 1993).

Sophorose has been considered for years as the candidate for natural inducer of *T. reesei* cellulase expression. Its presence results in *cbh1*, *cbh2*, *egl1* and *egl2* expression levels comparable to those observed on cellulose cultivations (Ilmén *et al.*, 1997). Sophorose could be formed from cellobiose or other cello-oligosaccharides by the transglycosylation activity of β-glucosidase (Gritzali and Brown, 1979; Vaheri *et al.*, 1979) or EGI (Biely *et al.*, 1991; Claeyssens and Tomme, 1990). It has also been reported to be a poor substrate for β-glucosidase but readily transported into the mycelium by the above-mentioned β-linked disaccharide permease (Kubicek *et al.*, 1993). The addition of sophorose into

resting cells was also reported to increase the activity of the β-linked disaccharide permease. This increase was proposed to be due to the enhanced transcription of the gene encoding the permease (Kubicek *et al.*, 1993). The results are, however, tentative since the gene encoding the permease has not been cloned. Sternberg and Mandels (1980) failed to induce β-glucosidase and cellulase activity simultaneously by sophorose proposing that sophorose inhibited β-glucosidase activity. This inhibition of activity may increase the cellulase induction by sophorose. It thus can be concluded that the fate of the disaccharides derived from cellulose very likely plays a significant role in the cellulase induction. The mechanism for the inducer molecule transmitting the signal from the surrounding extracellular cellulose to the transcription machinery remains unknown.

Since cellulose is far too large a compound to enter the cell, an inducer capable of passing through the cell wall must be formed in the presence of cellulose. The presence of a very low level of cellulase expression is proposed to be responsible for the formation of an inducer from cellulose. El-Gogary *et al.* (1989) reported the lack of cellulose induction upon growth on cellulose when cellulase action was blocked by addition of CBHI, CBHII, EGI, EGII and β-glucosidase antibodies to the culture media prior to induction. The presence of *cbh1* and *egl1* transcripts detected by reverse transcription PCR in glycerol-grown mycelia has been reported (Carle-Urioste *et al.*, 1997). The same authors also reported that the time needed to induce the expression of *cbh1* and *egl1* genes was reduced upon addition of crude cellulase extract prior to induction to cellulose-induced cultures, indicating the need for cellulases in the process of inducer formation from cellulose. In contrast, cellulase transcripts were not detected from glucose-, sorbitol- or glycerol-grown mycelia in Northern analysis overloaded with RNA and no hybridisation signal of *cbh1*, *cbh2* or *egl1* was obtained in a slot blot analysis, where it was estimated that for *cbh1* up to 6000-fold lower levels than those seen in cellulose-induced cultivations could be detected (Ilmén *et al.*, 1997). The *T. reesei* conidia have been shown to contain cellulases, mainly CBHII, on their surface. These conidial-bound cellulases are suggested to be responsible for the initial release of an inducer from cellulose by conidia, leading to growth on cellulose (Kubicek *et al.*, 1988; Messner *et al.*, 1991). Conidia of strains deleted for *cbh1* and *cbh2* failed to grow on media containing cellulose as the sole carbon source (Suominen *et al.*, 1993).

Expression of all the main cellulase genes *cbh1*, *cbh2*, *egl1* and *egl2* is repressed to a non-detectable level during growth on glucose. Glucose repression overrides induction since addition of glucose to induced cultivations results in rapid repression of the cellulase gene expression (el-Gogary *et al*., 1989; Ilmén *et al*., 1997). The glucose repression of cellulases has been shown to be mediated by the CREI transcription factor (Ilmén *et al*., 1996b) (see more detail in section 1.4.1.1.).

Derepression of cellulase genes in glucose-grown mycelia occurs after glucose depletion. This leads to moderate expression of at least *cbh1*, *cbh2* and *egl1* genes. This expression occurs without addition of an inducer since carbon starvation as such did not lead to cellulase expression (Ilmén *et al*., 1997). It was proposed that an inducer is formed from glucose by the transglycosylation activity of a β-glucosidase (Sternberg and Mandels, 1979) or inducing oligosaccharides released or formed from the saccharides present in the fungal cell wall.

Expression studies of cellulase genes clearly show that distinct repression and induction mechanisms operate, suggesting binding of activators and repressors to the promoters. The CREI protein mediating repression of cellulase promoters and its binding to the *cbh1* promoter have been characterised (for more detail see section 1.4.1.1.). Mutation of the three 5'GTGGGG sequences in the *cbh1* promoter shown to bind CREI led to derepression of the *cbh1* promoter (Ilmén, 1997; Ilmén *et al*., 1996a). Deletion analysis of the *cbh1* promoter fused to the *lacZ* reporter gene demonstrated that a very short form of the *cbh1* promoter, containing only 161 bp upstream of the initiator ATG, was still inducible by sophorose, albeit at a lower level than the full-length promoter, suggesting that some of the cis-acting sequences needed for the sophorose induction lie within this promoter fragment (Ilmén *et al*., 1996a). Studies of the *cbh1* promoter-*E. coli* β-glucuronidase fusions led to the identification of a promoter region between -204 and -373 (relative to ATG), which was proposed to mediate cellulose-derived induction (Henrique-Silva *et al*., 1996). The same authors also report that a 72 bp region upstream of the *cbh1* promoter TATA box is needed for the basal level transcription of *cbh1*. Furthermore, DNA mobility shift assays with total protein lysates have been used in the attempt to characterise meaningful regions within cellulase promoters. However, these analyses have led only to identification of relatively large promoter fragments. Recent studies

using electrophoretic mobility shift assays and DNA footprinting analysis identified a *cbh2*-activating element (CAE) from the *cbh2* promoter. This element was proposed to consist of a partially overlapping CCAAT motif, binding the HAP2/3/5 complex of *T. reesei* and a GGGTAA sequence (HAP2/3/5 complex is discussed in detail in section 1.4.1.4). Mutations in either of the proposed binding motifs in the *cbh2* promoter caused reduction in the expression level, indicating that both sequences are essential *in vivo* for the full induction of the *cbh2* promoter (Zeilinger *et al*., 1998). Similar electrophoretic mobility shift assays with cell-free extracts from a cellulase-negative mutant of *T. reesei* produced protein complexes with altered mobility binding to oligonucleotides containing the CAE sequence (Zeilinger *et al.*, 2000). However, in addition to CREI *in vivo* or *in vitro* binding or *in vivo* function of any other transcription factor to *T. reesei* cellulase promoters has not been demonstrated.

1.3.2 Regulation of *T. reesei* xylanase encoding genes

Production of many of the *T. reesei* hemicellulases is induced during growth in the presence of cellulose, xylan, sophorose, xylobiose, and L-arabitol. However, specific induction of some hemicellulase genes occurs during growth on certain carbon sources, *e.g.* growth on galactose induces only the expression of α-galactosidase encoding genes (*agl1* and *agl2*) and acetyl xylan esterase encoding gene (*axe1*) (Margolles-Clark *et al*., 1997). Expression of most of the hemicellulase genes is repressed in the presence of glucose, and this repression has been suggested to be mediated by the glucose repressor CREI (Margolles-Clark *et al*., 1997), similar to *Aspergilli*, where xylanase expression is regulated by the carbon catabolite repressor CREA (de Vries and Visser, 2001 and references therein). In addition to carbon source dependent regulation, the production xylanases is also dependent on the pH of the culture medium. The pH-dependent regulation of xylanase gene expression in *A. nidulans* has been shown to be mediated by the transcription factor PacC (see section 1.4.1.3.).

Studies on the regulation of expression of the two *T. reesei* xylanase encoding genes *xyn1* and *xyn2* are somewhat contradictory. Margolles-Clark *et al.* (1997) showed that expression of both xylanase genes was induced in cellulose, arabitol, different xylans, sophorose and xylobiose but not on cultivations containing 4.5% xylose. Glucose depletion induced the expression of *xyn2* but

not *xyn1* at the time point analysed. Furthermore, *xyn2* expression was not seen in carbon catabolite mutant strain Rut-C30, suggesting that expression of *xyn2*, unlike *xyn1*, is not controlled by the glucose repressor CREI (Margolles-Clark *et al*., 1997). The results of Zeilinger *et al.* (1996) are somewhat different. Only *xyn2* expression was detected in cellobiose- and sophorose-induced cultivations. *xyn1* was expressed in cultivations with xylose as the carbon source. Furthermore, *xyn2* promoter was found to function at a low basal level during growth on glucose. The *xyn1* expression has been shown to be under carbon catabolite repression, but differently from the cellulase genes, since induction by xylose was reported to partially relieve *xyn1* from carbon catabolite repression (Mach *et al*., 1996). Some of the controversies in these results can be explained by the different concentrations of the carbon source or the inducer in the culture medium at the time of the analysis and that xylose at very low concentrations can act as an inducer and at higher concentrations as a repressing carbon source, as has been demonstrated in *A. niger* (de Vries *et al*., 1999).

1.4 Regulation of RNA polymerase II transcription

Genes transcribed by the RNA polymerase II (polII) are regulated by promoters consisting of core promoter elements and target sequences for either positively or negatively acting factors. The transcription of genes is regulated via these promoter regions by transcription factors contributing to repression or activation of the corresponding gene. The most common core promoter element is the TATA element bound by the TATA-binding protein (TBP), part of the multiprotein complex TFIID. The subunits of the polII transcription machinery, including TBP and TPB associated factors (TAFs), respond to the signals from the transcriptional activators or repressors bound at sequence-specific sites in the promoter. The interactions between the DNA-bound transcription factors and the general transcription machinery can be either direct or mediated by co-activators or corepressors (as shown in Figure 1, p. 22) such as the Ssn6/Tup1 complex of *Saccharomyces cerevisiae* that is recruited to various promoters by specific DNA-binding proteins, *e.g.* the glucose repressor Mig1 (Treitel and Carlson, 1995). Ssn6/Tup1 is believed to mediate the repression by interacting with chromatin and/or the general transcription machinery (Smith and Johnson, 2000 and references therein).
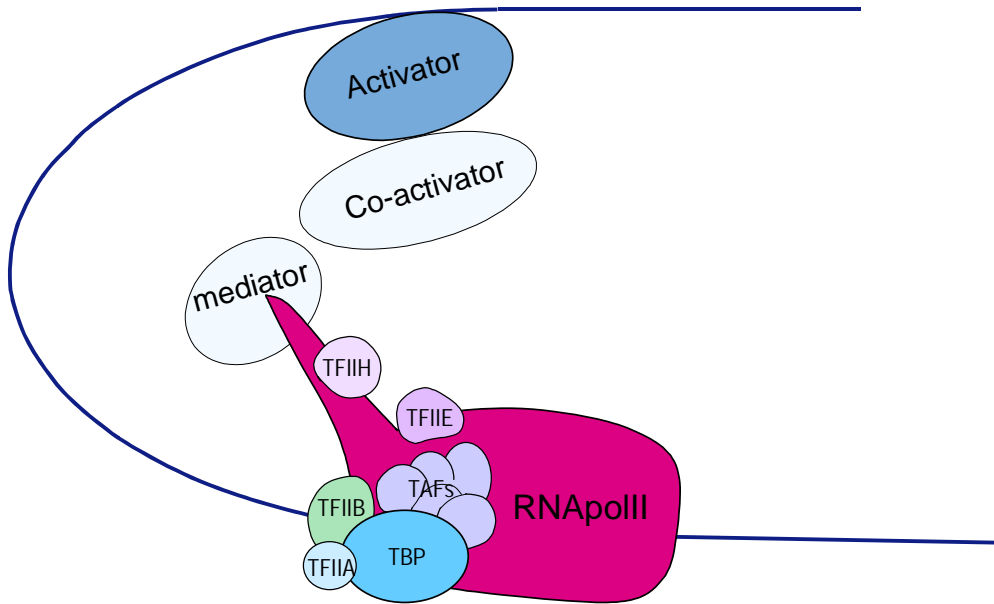
Figure 1. Schematic representation of the basal transcription machinery and a DNA bound activator together with co-activator and mediator complex that may be needed for the communication between activator and the basal transcription machinery.

Activators function in several ways to induce transcription of the corresponding gene. They can recruit co-activators or TBP, polII or other components of the general transcription machinery to the promoter or recruit or strengthen the binding of other activators to the promoter (Berk, 1999; Björklund and Kim, 1996; Carey, 1998 and references therein). Activators can also act to overcome the repressive effects of chromatin. The human transcription factor Sp1, containing two glutamine-rich activation domains, has been shown to directly contact the human TBP and the *Drosophila* TBP associated factor $TAF_{II}110$, which is a component of the TFIID complex (Emili *et al*., 1994; Gill *et al*., 1994).

Transcriptional repression can be broadly divided into 'active repression', referring to repression of transcription by specific repressor proteins when activators are present in the cell, and repression of transcription occurring due to

lack of activators. Eukaryotic cells have several ways of actively repressing expression of a specific gene or set of genes. These include interference with (co)activators or the basal transcription machinery. Similar to activation, repression is brought about by the specific DNA-bound repressors and co-repressors recruited to the promoter by the DNA-bound factors. Finally, chromatin remodelling that is brought about by the action of histone acetyltransferases and histone deacetylases recruited to the promoter by activators and repressors, respectively, modulate the acetylation status of chromatin within the promoter region, and thus, play a special role in determining the transcriptional activity of the corresponding gene (Gregory and Horz, 1998; Martienssen and Henikoff, 1999).

Most transcription factors consist of a distinct DNA-binding domain that determines the specificity of the factor and an activation or repressor domain that interacts with other proteins to either stimulate or repress transcription, respectively. Transcription factors are generally divided into separate classes according to the structures of their DNA-binding domains. The DNA-binding motifs include the zinc co-ordinating Cys_2His_2 zinc finger, basic leucine zipper (bZIP) and the fungal-specific zinc binuclear cluster ($Zn(II)_2Cys_6$). Target sequences for these binding domains can consist of specific continuous nucleotides, as in the Cys_2His_2 zinc finger containing glucose repressors CREI and CREA, or *e.g.* repeated trinucleotides with a certain spacing as in Gal4 of *S. cerevisiae*. The structure of the activation and repressor domains is more poorly understood. Activation domains have been classified into subgroups, including acidic as in VP16, proline as in AP-1, serine and glutamine rich activation domains based on their amino acid composition. The activation mechanism for some of these, *e.g.* the acidic activation domain of VP16, has been thoroughly characterised (Tumbar *et al*., 1999 and references therein). Similar to activators, some of the repressors also contain distinct domains for repressor function. Repressor domains have been loosely classified according to the predominant amino acid, *e.g.* alanine or proline (Hanna-Rose and Hansen, 1996), and more recently, according to their molecular mode of action (Thiel *et al*., 2001 and references therein).

Knowledge of the general transcription factors of filamentous fungi is practically non-existent. The cloning of the gene encoding the TBP has been reported only for *A. nidulans*. This TBP shows high sequence similarity to the few known fungal

TBPs in the 180 amino acid C-terminus and contains a 88 amino acid N-terminal domain distinct from other TBPs (Kucharski and Bartnik, 1997). The largest subunit of the RNA polymerase II (polIIL) has been cloned from *A. oryzae*. The amino acid sequence of *A. oryzae* polIIL shows high similarity to polIIL proteins from *Schizosaccharomyces pombe* and *S. cerevisiae* (Nakajima *et al*., 2000).

Transcriptional regulation involves integration of signals from various factors that in turn respond to environmental and developmental signals. The combined action of several factors determines the level of transcription of the corresponding gene.

1.4.1 Regulatory proteins affecting cellulase and hemicellulase expression

The synthesis of the cellulase system is an energy-consuming but important process for cellulolytic fungi. Therefore, transcription of cellulase and hemicellulase genes is carefully regulated so that the fungus can react and benefit from the changes in the environment to obtain energy and carbon from the most suitable substrate and to avoid the production of cellulase and hemicellulase enzymes in vain. Recently, knowledge of the factors regulating cellulase and hemicellulase synthesis in filamentous fungi has expanded, a number of different factors are known to affect the activity of cellulase and/or hemicellulase gene expression. These factors and their effect on cellulase and xylanase expression are discussed in the following sections and summarised in Figure 2, p. 31.

1.4.1.1 Glucose (carbon catabolite) repression

The presence of easily metabolisable and energetically favourable glucose in the microbial surroundings results in the repression of various genes needed for the use of other carbon sources. The mechanism controlling this preferential use of glucose over other alternative carbon sources is called glucose (carbon catabolite) repression. In filamentous fungi, glucose repression is mediated by the transcription factor CREA/CREI. Genes encoding the glucose repressors have been isolated from numerous filamentous fungi including *Trichoderma* and *Aspergillus* species (Dowzer and Kelly, 1991; Drysdale *et al*., 1993; Ilmén *et al*.,

1996b; Strauss *et al*., 1995; Takashima *et al*., 1996b) and gene bank accession numbers CAB89774 and BAA75519), *Humicola grisea* (Takashima *et al*., 1998), *Sclerotinia sclerotiorum* (Vautard *et al*., 1999) and *Botrytis cinerea* (gene bank accession number O94130). The CREI/CREA proteins contain two almost perfectly conserved C_2H_2 zinc fingers responsible for the DNA binding. The binding consensus motif for *A. nidulans* CREA was determined to 5'-SYGGRG and the binding was shown to be context dependent (Cubero and Scazzocchio, 1994; Espeso and Peñalva, 1994; Kulmburg *et al*., 1993). In addition to the zinc finger region, the CREI/CREA proteins carry other conserved sequences such as the proline serine threonine-rich region of unknown function. Phosphorylation of a serine in a conserved short stretch within an acidic region of CREI has been demonstrated to be required for CREI binding to DNA. Based on the sequence of the phosphorylation site, the authors proposed that the phosphorylation involves a casein kinase II-like enzyme (Cziferszky *et al*., 2002). The expression of *A. nidulans creA* and *T. reesei cre1* is regulated depending on the carbon source, expression being higher in the presence of a derepressing /inducing carbon source than in the presence of glucose (Ilmén *et al*., 1996b; Strauss *et al*., 1999), suggesting that the functioning of the CREI/CREA proteins in carbon catabolite repression is regulated at the posttranscriptional level and/or by the presence of other regulatory factors. The finding that in *A. nidulans* CREA-DNA complex was formed *in vivo* more efficiently under repressing than derepressing conditions further supports this idea (Strauss *et al*., 1999). The same authors also show that the repression of an induced alcohol dehydrogenase gene *alcA* by CREA requires *de novo* protein synthesis, indicating that the higher amount of *creA* mRNA in the presence of inducing carbon source does not correlate with the functional CREA protein and suggesting that CREA production could also be regulated at the translational level. Furthermore, *creA* transcription in *A. nidulans* was shown to be autoregulated via two closely spaced CreA binding sites in the *creA* promoter (Strauss *et al*., 1999).

On the other hand, the CREI of phytopathogenic fungus *Sclerotinia sclerotiorum* was demonstrated to be localised in the nucleus during growth on glucose and transported into the cytoplasm when glucose was removed from the culture medium, implying that the activity of the glucose repressor is controlled by its nuclear translocation (Vautard-Mey *et al*., 1999). Furthermore, mutation of the same serine of *S. sclerotiorum* CREI that was phosphorylated in *T. reesei* CREI abolished its repressor activity but not its nuclear localisation. The

phosphorylation site is consistent with the cyclic AMP-dependent protein kinase (AMPK)-like phosphorylation motif, and the authors suggest that AMPK protein kinase may be involved in the regulation of the function of *S. sclerotiorum* CREI (Vautard-Mey and Fevre, 2000). The glucose repressor Mig1 of *S. cerevisiae* is translocated into the nucleus in the presence of glucose, and the activity and localisation of Mig1 is regulated by phosphorylation mediated at least by the protein kinase Snf1 and possibly the phophatase complex Reg1-Glc7 (De Vit *et al.*, 1997; Östling and Ronne, 1998; Treitel *et al.*, 1998). Recently, a *T. reesei snf1* gene encoding for a protein similar to *S. cerevisiae* Snf1 protein kinase was deposited into the database (gene bank accession number AAK69569).

Since the expression of *cre1* of *T. reesei* was found to be higher on an inducing carbon source, cellulose, than in glucose-grown mycelia, CREI might have an additional role beyond acting as a repressor for glucose-repressed genes. Other roles have also been proposed for *A. nidulans* CREA (Dowzer and Kelly, 1991). Furthermore, the glucose repressor Mig1 has been reported to function as an activator when the interaction with the co-repressor complex Ssn6(Cyc8)-Tup1 is disrupted (Treitel and Carlson, 1995). CreC protein, having weak similarity to Tup1, has been characterised from *A. nidulans* and shown to be involved in carbon catabolite repression (Todd *et al.*, 2000). The third gene identified in genetic screening designed for isolation of regulatory proteins affecting carbon catabolite repression is the *creB* gene of *A. nidulans*, which encodes a deubiquinating enzyme (Lockington and Kelly, 2001). In *A. nidulans*, a shift form glucose to derepressing conditions results in degradation of CREA suggesting that ubiquitin processing may play a role in carbon catabolite repression (Strauss *et al.,* 1999). Interestingly, activation domains of certain transcription factors have recently been demonstrated to serve as direct ubiquitylation targets, and modulation of activation domains by ubiquitylation levels has been hypothesized to provide an important mechanism for the regulation of gene transcription (Salghetti *et al.*, 2001).

A hypercellulolytic *T. reesei* strain Rut-C30, in which the cellulase and most of the hemicellulase genes are expressed to some extent during growth on glucose, contains a truncated *cre1* gene. Transformation of a full-length *cre1* gene into this strain restored the glucose repression of cellulase and hemicellulase genes, indicating that the glucose repression of cellulase and most of the hemicellulase genes is mediated by CREI in *T. reesei* (Ilmén *et al.*, 1996b; Margolles-Clark *et*

al., 1997). In the *cbh1* promoter, mutation of three 5'GTGGGG sites between –726 and –685 relative to ATG, has been demonstrated to lead to derepression of the *cbh1* promoter during growth on glucose indicating functionality of at least one of the sites (Ilmén *et al*., 1996a; Ilmén *et al*., 1998). In addition, CREI has also been shown to bind *in vitro* to all the three sites mentioned above and to a 5'GTGGGG site at –1510 in the *cbh1* promoter (Ilmén, 1997). 5'GTGGGG sequence fits well to the consensus binding sequence 5'SYGGRG described for *A. nidulans* CREA. CREI binding *in vitro* to *cbh2* and *xyn1* promoter fragments has also been demonstrated (Strauss *et al*., 1995; Takashima *et al*., 1996b). Studies of a derepressed mutant strain *creA^d*30 and the wild type *creA* strain of *A. nidulans* established that the expression of *xlnA* encoding the major xylanase is derepressed in the presence of glucose in the *creA^d*30 strain. The mutation of a CREA binding site in *xlnA* promoter also leads to derepression of the *xlnA* expression. Furthermore, the activity of the *xlnA* promoter is higher under inducing conditions in the *creA^d*30 strain, and the authors thus suggest that the function of CREA involves, in addition to this direct mechanism, an indirect mechanism where CREA represses formation of a transcriptional activator or synthesis of an inducer (Orejas *et al*., 1999). CREA has been shown to regulate the expression of the *A. niger* hemicellulase genes *xlnB*, *xnlD*, *faeA* and *aguA*. Expression of these genes is suggested to be regulated by the balance of CREA and the activator XlnR since in inducing conditions *creA* mutant strain expresses all of the above-mentioned genes at a higher level than the wild-type strain during growth on various xylose concentrations (de Vries *et al*., 1999). The use of *creA* mutant strains has indicated that the carbon catabolite repression of the hemicellulase genes of *A. niger afbA*, *afb, abnA*, and *A. nidulans afbB* is also regulated by CREA (Gielkens *et al*., 1999a; Ruijter *et al*., 1997).

1.4.1.2 Xylanolytic activator XlnR

The first transcriptional activator controlling the expression of genes encoding xylanolytic and cellulolytic enzymes in filamentous fungi was isolated from *A. niger* by complementation of a mutant strain carrying the bidirectional selectable marker *pyrG* under the upstream activating region of *xlnA* gene of *A. tubingensis* (van Peij *et al*., 1998b). XlnR has a zinc binuclear cluster DNA-binding domain, and it was shown to bind *in vitro* to the 5'-GGCTAATAA sequence in the *xlnA* promoter. The binding site is proposed to contain a core of 5'-GGCTAA that is found in most hemicellulase and cellulase gene promoters of *A. niger* (Gielkens

et al., 1999b; van Peij *et al.*, 1998a; van Peij *et al.*, 1998b). The expression of two endoxylanases (*xlnB* and *xlnC*), a β-xylosidase (*xlnD*), two endoglucanase-encoding genes (*eglA* and *eglB*), two cellobiohydrolase genes (*cbhA* and *cbhB*) and several genes encoding the side chain cleaving hemicellulases are co-ordinately regulated by the xylanolytic activator XlnR (Gielkens *et al.*, 1999b; van Peij *et al.*, 1998a; van Peij *et al.*, 1998b). *xlnR* loss of function mutation leads to reduced or complete loss of induction by xylose or xylan of the regulated genes. The expression of some of the genes controlled by XlnR was increased by *xlnR* overexpression (Gielkens *et al.*, 1999b; van Peij *et al.*, 1998a). The fact that both hemicellulase- and cellulase-encoding genes are regulated by XlnR indicates that, in addition to carbon catabolite repression, the transcriptional activation mechanism is at least partially shared between cellulases and hemicellulases.

The role of XlnR in *A. niger* seems not to be restricted to regulation of genes encoding secreted polysaccharide-degrading enzymes but also to an intracellular enzyme involved in D-xylose catabolism. D-xylose reductase, *xyrA*, which catalyses the first step of D-xylose catabolism, reduction of xylose to xylitol, was shown to be regulated by XlnR (Hasper *et al.*, 2000). A similar situation where the same transcriptional regulator controls the expression of genes encoding intracellular and extracellular enzymes has been characterised for *S. cerevisiae* GAL4, which regulates the expression of secreted α-galactosidase and several genes of the galactose pathway. Recently a gene encoding *T. reesei* Xyr1 has been deposited into the database (AF479644). Xyr1 has 49% similarity to the XlnR of *A. niger*. The gene encoding XlnR has also been isolated from *A. oryzae* and AoXlnR was demonstrated to control the expression of the xylanase-encoding *xynF1* and *xynG2* genes (Aburatani *et al.*, 2000; Marui *et al.*, 2002). In addition, the databases contain a sequence of the XlnR homologue from *A. kawachii* (AB064658).

1.4.1.3 Wide domain regulator PacC

In nature, the enzymatic breakdown of cellulose and xylan can occur in variable conditions such as in different surrounding pH. Thus, a mechanism is needed to ensure that only enzymes functional in a certain pH range are produced with respect to the ambient pH of the culture habitat. The regulatory mechanism controlling pH-dependent transcriptional regulation has been analysed in detail

in *A. nidulans*, and a major role has been demonstrated for zinc finger transcription factor PacC (Caddick *et al*., 1986; Tilburn *et al*., 1995), which acts as an activator for alkaline-expressed genes and prevents expression of acid-expressed genes. PacC contains a DNA-binding domain with three Cys_2His_2 zinc fingers and it binds to promoter sites containing the core hexanucleotide 5'GCCARG sequence (Caddick *et al*., 1986; Espeso *et al*., 1997; Tilburn *et al*., 1995). In response to a signal transduced by the *pal* genes, PacC is proteolytically processed to its functional form (Arst *et al*., 1994; Denison *et al*., 1995; Negrete-Urtasun *et al*., 1997; Orejas *et al*., 1995). This proteolytic cleavage removes ~420 C-terminal amino acids that contain the negative-acting C-terminal domain (Mingot *et al*., 1999; Orejas *et al*., 1995). Conformation of the full-length PacC alternates between a protease-resistant and protease-sensitive form which is cleaved to the active form in alkaline conditions. The genes regulated by PacC or its homologues include enzymes involved in the synthesis of biologically active metabolites such as penicillin (Espeso *et al*., 1993; Tilburn *et al*., 1995), proteases (Madzak *et al*., 1999) and xylanases (MacCabe *et al*., 1998). Studies with alkaline- and acidic-mimicking mutants of PacC showed that two genes, *xlnA* and *xlnB*, encoding xylanases in *A. nidulans* are also regulated by the PacC. *xlnB* encoding a minor xylanase is expressed under conditions of acidic ambient pH, and *xlnA* encoding a major xylanase at alkaline ambient pH. Using the same mutants, Gielkens *et al.* (Gielkens *et al*., 1999a) also established the pH-dependent regulation of the *abfB* gene encoding α-L-arabinofuranosidase, a secreted enzyme releasing L-arabitol from polysaccharides. PacC homologues mediating pH regulation in *A. niger* (MacCabe *et al*., 1996) and *Penicillium chrysogenum* (Suarez and Peñalva, 1996) have been characterised. In addition, the RIM101 protein that is activated by proteolytical processing and contains a similar DNA-binding domain as PacC has been identified from yeasts *S. cerevisiae* and *Yarrowia lipolytica* (Lambert *et al*., 1997; Li and Mitchell, 1997; Tilburn *et al*., 1995).

1.4.1.4 Protein complex binding to CCAAT sequence

CCAAT sequences are found in the 5' regions of approximately 30% of the eukaryotic genes. A conserved multimeric protein complex recognises and binds to this sequence. The first CCAAT-binding complex described, designated as the Hap complex, consisting of Hap2, Hap3, Hap4 and Hap5 proteins, was identified from *S. cerevisiae* (McNabb *et al*., 1995; Olesen and Guarente, 1990; Pinkham

and Guarente, 1985). Since then, homologues of the Hap protein complex, excluding Hap4, have been found from various organisms, from human to the *Xenopus* (Mantovani, 1998 and references therein). In filamentous fungi, Hap2/3/5 from *T. reesei* (Zeilinger *et al*., 2001), AnCF from *A*. *nidulans* (Papagiannopoulos *et al*., 1996; Steidl *et al*., 1999) and a Hap5 homologue AAB1 from *N. crassa* (Chen *et al*., 1998) have been cloned. In each Hap2/3/5 subunit, the segment needed for the formation of the protein-DNA complex and/or subunit association is conserved between the different species, suggesting conservation in the mode of action. In *S. cerevisiae*, the Hap complex is required in various functions, *e.g.* for the positive regulation of many respiratory genes involved in oxidative phosphorylation in response to growth on non-fermentable carbon sources (Gancedo, 1998; Marui *et al*., 2002). In *A. nidulans*, the deletion of any of the genes encoding AnCF subunits results in slowly growing and weakly conidiating phenotypes (Papagiannopoulos *et al*., 1996; Steidl *et al*., 1999).

Mutation of the CCAAT motif or deletions of the subunits generally reduce the expression level of the gene studied, the reduction occurring either at the basal expression level or in the response to specific stimulatory signal, indicating that the CCAAT motif co-operates with other specific elements to induce transcription (Papagiannopoulos *et al*., 1996; Steidl *et al*., 1999). The AnCF complex has subsequently been shown to have a role in the formation of an open chromatin structure in the promoter region of the *amdS* and *fmdS* genes of *A. nidulans*, indicating that CCAAT binding complexes are necessary for the full transcriptional activation of certain promoters (Narendja *et al*., 1999).

In the *T. reesei cbh2* promoter, the mutation of a CCAAT element has been reported to reduce the activity of the *cbh2* promoter by approximately 20% when the promoter is induced by the addition of sophorose (Zeilinger *et al*., 1998). The binding of another factor to a site overlapping the CCAAT box was proposed and mutation of this site together with the CCAAT element abolishes *cbh2* promoter activity (Zeilinger *et al*., 1998). The same authors have also proposed a role for the CCAAT element in the regulation of xylanase promoters *xyn1* and *xyn2*. In these studies, a protein or protein complex was demonstrated to bind to promoter fragments containing the CCAAT motif. This binding complex can be competed out by an oligonucleotide containing the CCAAT motif (Zeilinger *et al*., 1996).
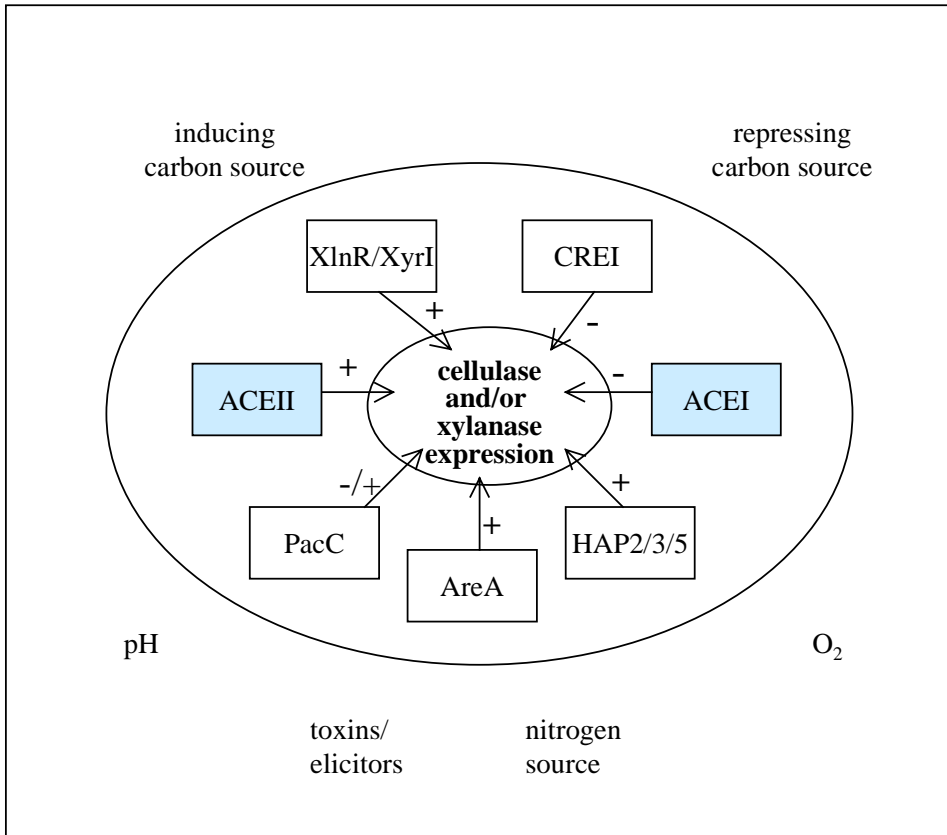
Figure 2. Schematic representation of the different fungal transcriptional regulators affecting cellulase and/or xylanase expression. The carbon catabolite repressors CREI/CREA (discussed in 1.4.1.1.), activator XlnR (1.4.1.2.), HAP2/3/5 complex (1.4.1.3.) and PacC mediating pH dependent control (1.4.1.4.). First indications of the involvement of the global nitrogen metabolism regulator AreA in the regulation of cellulase expression in A. nidulans *were recently reported by Lockington* et al. *(2002). ACEI and ACEII are the regulators isolated in this work. The effect on the expression of cellulase and xylanase expression is indicated with + or – symbols. + indicates positive effect and – negative effect on expression. Outside the circle are listed some known and possible environmental factors affecting cellulase and xylanase expression.*

1.5 Aims of the study

Several lines of evidence suggest that a distinct induction mechanism for cellulase gene expression must exist. On sorbitol or glycerol cultivations, no cellulase mRNAs are detected, but addition of an inducer, *e.g.* sophorose, into these cultures provokes strong induction of cellulase expression. In addition, the mutation of neither the *cre1* gene (Rut-C30 strain) nor the CREI binding sites in the *cbh1* promoter allows the *cbh1* gene to be expressed at levels comparable with the expression seen under inducing conditions, indicating that simply the release from glucose repression is insufficient for full induction of cellulase genes. Instead activation is needed. Several groups have attempted to characterise the sequences within cellulase promoters mediating the strong induction and expression occurring in inducing conditions. However, sequence comparisons, DNA binding analysis with whole cell and nuclear extracts and studies of promoter deletions have not led to identification of the precise promoter sites binding activators and mediating induction.

At the beginning of this work, no transcriptional activators involved in the activation and strong expression of the cellulase and hemicellulase genes had been characterised from any filamentous fungi. The aim of this work was therefore to develop a mechanism for isolation of these factors from one of the best-known cellulolytic fungi, *T. reesei*, and to characterise them to elucidate the regulatory mechanism of cellulase expression.

2. Materials and methods

All the materials and methods used, except those described in section 2.1 are described in detail in the original papers (I–III).

2.1 Analysis of genes encoding two intracellular reductases

2.1.1 Growth conditions

The Δ*ace1* (VTT-D-01850) and the host strain ALKO2221 (Mäntylä, unpublished) were cultivated in *Trichoderma* minimal medium, pH 4.8 (Ilmén *et al*., 1997), supplemented with either 2% glycerol or 2% sorbitol as the carbon source. A total of 8 x 10^7 spores were inoculated into 400 ml of culture medium in two parallel 2-litre shake flasks and incubated in a rotary shaker at 250 rpm at 28°C. Mycelial samples for RNA isolation were collected by filtration through glass microfibre filter after two and four days of growth. pH of the culture medium was measured every day to follow the growth of the two strains.

2.1.2 RNA isolation and Northern analysis

Total RNA was isolated with the Trizol reagent according to the manufacturers instructions (Life Technologies, Inc.) The probes for Northern analysis were the entire cDNAs of *xrd1* encoding xylose reductase (M. Saloheimo, pers. comm.) and *lxr1* encoding L-xylulose reductase (Richard *et al*., 2002) released from vector sequences. As an internal loading control, the membranes were hybridised with an actin (*act1*)-encoding cDNA fragment. The probes were labelled using the random primed DNA labelling kit (Roche Molecular Biochemicals) and [α-^{32}P]dCTP (Amersham Biosciences). The amount of the analysed mRNAs were quantified from phosphor screen autoradiographs using the ImageQuant software of Phosphoimager SI (Molecular Dynamics) and normalised for the total amount of mRNA by using the actin mRNA as a loading control.

3. Results

Production of cellulases and hemicellulases is regulated at the transcriptional level, and both repression and activation mechanisms seem to underlie this process. Different conditions where cellulase and hemicellulase genes are induced have been characterised but the general picture of the molecular mechanism involved in the activation process is largely unknown. Transcription factors involved in the positive regulation of cellulase and hemicellulase expression have not been identified mainly due to the lack of suitable methods for their isolation. A new method was developed to isolate novel transcription factors of *T. reesei*, and the function of these new factors was studied in a hypercellulolytic and low protease producing *T. reesei* strain, ALKO2221 (Mäntylä, unpublished).

3.1 Isolation of transcriptional activators of the *cbh1* promoter (I)

A novel method was set up to isolate transcriptional activators using *Saccharomyces cerevisiae* as a host (Fig. 3, p. 36). This method selects simultaneously for binding and activation of the promoter of interest. In this work, the method was applied to the *T. reesei cbh1* promoter. Previous work by Ilmén *et al.* (1996a) had shown that the *cbh1* promoter containing only 1.15-kb region upstream from TATA behaves as the wild type promoter suggesting that the main regulatory regions are located within this region. A reporter plasmid construct was first made by fusing the TATA and sequences downstream of *HIS3* yeast marker gene to the 1.15-kb fragment of the *cbh1* promoter upstream from TATA, resulting in the plasmid pAS3. This construct was made in a yeast single copy plasmid to minimise the possible leakage from the construct. A negative reporter construct containing non-relevant DNA fused to the *HIS3* gene was also constructed since a plasmid containing only the *HIS3* gene was able to support growth of the yeast in the absence of histidine, suggesting that some vector sequences were able to promote *HIS3* expression. An auxotrophic yeast strain, DBY746, was transformed with the reporter plasmid pAS3 and the negative reporter plasmid. The yeast reporter strain containing the reporter plasmid was unable to grow in the absence of histidine, indicating that the *cbh1* promoter alone could not promote the expression of *HIS3*. pAS3 plasmid was

also transformed to a *mig1*-mutant strain to determine whether the *cbh1* promoter is repressed by Mig1 protein binding to the sites in the *cbh1* promoter. However, because the *mig1*-mutant strain transformed with pAS3 was unable to grow in the absence of histidine, this was not the case (I).

A *T. reesei* cDNA library was prepared under cellulase-inducing conditions, and a subset of this yeast expression library was transformed to the reporter strain. cDNA library plasmids were isolated from the colonies growing in the absence of histidine. These cDNA plasmids were transformed to the yeast strain containing a negative control plasmid, where *cbh1* promoter was replaced with nonsense DNA, the DBY746 strain, and back to the reporter strain to identify the true positives of the screening. Of the transformed plasmids, 15% were false-positives and did not support growth of any of the strains, 75% promoted growth of all the strains and were found to contain the *his3* gene of *T. reesei* and 10% only promoted growth of the reporter strain containing the plasmid pAS3 and were true-positives. The translation products of these library plasmids were able to bind to and activate the *T. reesei cbh1* promoter in yeast (Fig. 3, p. 36). Among the positive library plasmids were pAS26 (II, Fig. 1) and pAS27 (I, Fig. 1). The genes in these plasmids were named *ace1* and *ace2* (<u>a</u>ctivator of <u>c</u>ellulase <u>e</u>xpression) for pAS27 and pAS26, respectively.
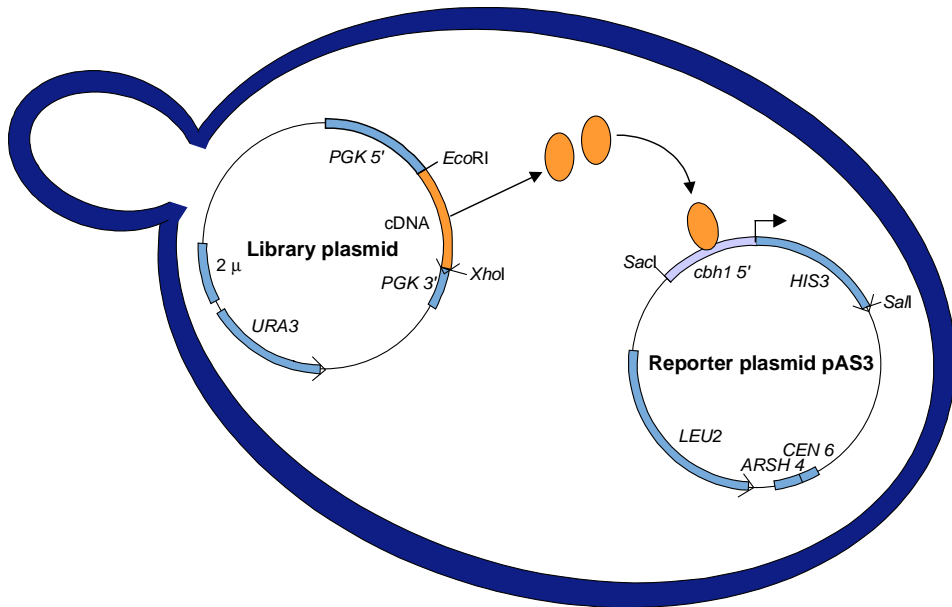
Figure 3. Schematic representation of the isolation method for genes encoding transcriptional activators. The host S. cerevisiae *strain DBY746 (-his3, -ura3, -leu2) was transformed consecutively with two plasmids, the reporter plasmid containing the promoter of interest, which controls expression of the* S. cerevisiae HIS3 *gene, and the cDNA expression library plasmid containing cDNA insert. When an activator (orange sphere) binding to the promoter of interest is encoded by the cDNA in the library plasmid, it activates the transcription of the* HIS3 *gene and supports growth of the yeast in the absence of histidine.* URA3, LEU2 *are selection marker genes and* ARSH4 *and 2 µ serve as origin of replication and* CEN6 *represents centromere sequence.*

3.2 Characterisation of the novel transcriptional activators (I, II, III)

3.2.1 *ace1* encodes for a Cys2-His2 type DNA-binding protein (I, III)

The first characterised library plasmid pAS27 that promoted growth of the pAS3 yeast on media lacking histidine by activating the *cbh1* promoter contained a cDNA with an open reading frame of 491 amino acids. The full-length cDNA isolated later was named *ace1* and it encoded for a protein of 733 amino acids. The deduced ACEI protein contains three zinc fingers of the Cys_2-His_2 type, one typical and two more unusual ones. The zinc finger region partially overlaps with a bipartite nuclear targeting signal, suggesting that ACEI is localised in the nucleus as expected for transcriptional regulators (I, Fig. 2).

ACEI shows 30% sequence identity and 46% sequence similarity to a recently identified *A. nidulans* protein encoded by the *stzA* gene that was deposited into the database as a gene alleviating sensitivity to salt and DNA-damaging agents (accession number AF202995). A sequence similarity search in the database containing the full *N. crassa* genome identified a gene encoding a deduced protein with 48% identity and 70% similarity to ACEI. Moreover, the genome sequence of the white rot fungus *Phanerochaete chrysosporium* contains a sequence that when translated shows 14% identity and 45% similarity to ACEI. Partial amino acid sequence of ACEI of *Talaromyces emersonii* (AAL69549) has recently been deposited in the NCBI data bank. This sequence shows 77% identity to the ACEI of *T. reesei*. No sequence similarities except to the zinc finger region were found from any other databases, which suggests that ACEI is a factor specific to filamentous fungi. Multiple alignment (III, Fig. 5) of the ACEI with the StzA and the deduced *N. crassa* protein identify several conserved regions within the tree proteins, suggesting functional importance of these regions (III, Figure 5). All of the conserved regions are located N-terminal to the three zinc fingers, and thus, were missing from the 491 amino acid protein encoded by the cDNA library plasmid in yeast.

3.2.2 Target sequence of ACEI (I)

Because the *cbh1* promoter used in the initial screening was about 1.15 kb in length, further experiments were needed to identify the target sequence of ACEI. The putative DNA-binding domain of ACEI (amino acids 382–582) was produced in *E. coli* as a GST fusion protein, and DNA mobility shift assays were carried out together with labelled *cbh1* promoter fragments. Consecutive gel mobility shift analysis with continuously shorter *cbh1* promoter fragments localised one ACEI binding site between nucleotides –843 and –763 (I, Fig. 4). This site was further narrowed by analysing the binding to a set of overlapping oligonucleotides covering the sequence between the –843 and –763 nucleotide. ACEI bound to a 36-bp oligonucleotide containing the promoter sequence from –818 to –780 (2C). Mutations introduced into this oligonucleotide identified the 5'TTTAGGCAT sequence necessary for the full binding of ACEI (I, Fig. 5), suggesting that ACEI is in contact with some of these bases. Since 13 copies of the 5'GGC(T/A)AA sequence can be found from the 1.15 kb *cbh1* promoter, binding of ACEI was analysed in all of them. ACEI bound to four oligonucleotides containing the sequence 5'GGCAAA but not to any of the nucleotides containing 5'GGCTAA. Comparisons of the binding and non-binding oligonucleotides identified the 5'AGGCA sequence as a feature common to all of the binding oligonucleotides. Therefore binding to the remaining 5'AGGCA sites in the *cbh1* promoter was analysed. Alltogether, ACEI bound to oligonucleotides representing eight different sites in the *cbh1* promoter (I, Table 1) Based on the binding data, it can be concluded that the 5'AGGCA sequence is required but not sufficient alone for ACEI binding since one of the sites containing 5'AGGCA did not bind ACEI. Figure 7 in Appendix I summarises the sequences binding and not binding ACEI and their location of the binding sites within the *cbh1* promoter is shown in Figure 5, p. 45.

The binding of ACEI to the *cbh1* promoter was also studied *in vivo* by the yeast one-hybrid assay. A 170-bp fragment corresponding to the *cbh1* promoter sequences between –843 and –676 was amplified by PCR and cloned in front of the *HIS3* gene in the one-hybrid reporter vector pHisi-1. The resulting construct, pPL2, was integrated to the YM4271 yeast genome. A pARO20 plasmid expressing a GAL4 activation domain-ACEI DNA-binding domain fusion protein was transformed to the pPL2 yeast and to the pHisi-1 yeast containing the empty vector sequence. The pPL2 yeast transformed with the pARO20 was able to grow in the absence of histidine, whereas the strain containing either the empty pHIisi-1 vector or the plasmid expressing only the GAL4 activation domain (pGAD10) did not grow on plates lacking histidine (I, Fig. 8). This was in accordance with results obtained from DNA mobility shift assays and further supports the finding that ACEI binds to the *cbh1* promoter sequence from –843 to –676.

3.2.3 Function of ACEI

3.2.3.1 Growth of Δ*ace1* strain on inducing, repressing and neutral carbon sources (I, III)

The role of ACEI in the regulation of cellulase gene expression was studied by constructing a strain deleted for the *ace1* gene. A disruption cassette, where the coding region of *ace1* was replaced with the *amdS* marker gene, was transformed to the hypercellulolytic *T. reesei* strain ALKO2221 (I). The transformants were screened by Southern analysis for the replacement of *ace1* gene, and two transformants, VTT-D-01850 and VTT-D-01849, were chosen for further studies (Δ*ace1* strain). Our approach was to compare the growth of the Δ*ace1* strain to that of the strain ALKO2221 on different carbon sources with respect to cellulase production. On cellulose, where cellulase genes are transcribed at a high level growth of the Δ*ace1* strain was faster than that of the host strain. On glycerol, a carbon source considered "neutral", where cellulase genes are neither repressed nor induced, growth of the two strains was similar. On glucose, where cellulase transcription is repressed by CREI, growth of the Δ*ace1* strain was slightly slower than that of the host strain (III, Fig. 1). The growth was also assayed by plating single spores on plates containing glucose and cellulose as the sole carbon sources. Somewhat contradictorily, the colony

growth of the Δ*ace1* strain was reduced on cellulose plates when compared with the growth of the host strain, forming colonies with approximately 40% reduced diameter. On glucose plates, growth was unaffected by *ace1* deletion (I, Fig. 9).

3.2.3.2 Expression of cellulases in Δ*ace1* strain (III)

To study whether the *ace1* deletion affects the expression of the cellulase genes, the Δ*ace1* strain and the host were cultivated under two different types of inducing conditions. Northern analysis was performed to analyse the expression of all the main cellulases (*cbh1*, *cbh2*, *egl1* and *egl2*) and enzyme activity measurements using 4-methylumbelliferyl-β-D-lactoside (MUL) as a substrate were carried out to measure the amount of cellulases produced by the fungus. In the first approach, two parallel shake flasks of each of the Δ*ace1* strains and the host strain were cultivated on glycerol medium for three days, whereafter 1 mM sophorose was added to induce cellulase expression. Mycelial samples were collected 0, 1, 2, 3, 6 and 12 hours after sophorose addition and RNA was isolated. Northern analysis showed that all the cellulases were induced faster and at a higher level in the Δ*ace1* strain as compared with the ALKO2221 strain (III, Fig. 3). The quantified and normalised *cbh1* mRNA signals of the Δ*ace1* strain were 1.3 to 4.3 times higher than those of the host strain. Differences in *cbh2* and *egl1* mRNA signals were of a similar range (III, Fig 3B).

To study the effect of *ace1* deletion on cellulase expression in cellulose-induced cultivation, the Δ*ace1* strains and the ALKO2221 strain were cultivated on glycerol for three days, after which the mycelia was harvested by filtration and transferred to a culture media containing Solka floc cellulose as the sole carbon source (cellulose transfer assay). The preculturing of the strain enabled the study of the early time points in cellulose-induced cultures since when inoculated directly onto cellulose media sufficient amount of mycelia for RNA isolation is obtained at the earliest after approximately 36 hours. However, with the biomass obtained from the preculturing, samples could be taken at very early time points. Mycelial samples were collected for RNA isolation 0, 6, 9, 12, 15, 18, 21 and 33 hours after the transfer. Northern analysis of the isolated RNA showed that expression of all the main cellulases (*cbh1*, *cbh2*, *egl1* and *egl2*) occurred earlier and at a higher level in the Δ*ace1* strain than in the host strain. The mRNA signals were quantified and normalised to actin mRNA. At the early time points (6 to 12 hours), the cellulase mRNA amounts were 2 to 20 times higher in the

Δ*ace1* strain than in the ALKO2221 strain. In the later time points (15 to 21 hours), the Δ*ace1* strain expressed cellulases at approximately the same level as the host strain (III, Fig. 2). Together these results of the Northern analyses show that *ace1* deletion leads to faster induction and a higher level of expression of all the cellulase genes in cellulose- and sophorose-induced cultivations, thus indicating that ACEI acts as repressor of cellulase genes rather than an activator.

We also analysed whether the difference in expression of the cellulases could be seen in the amount of enzymes produced into the culture medium. Four parallel shake flasks of both of the Δ*ace1* strain (VTT-D-01850 and VTT-D-01849) and two of the host strain were cultivated on cellulose, and after 3, 4, 5 and 6 days, the amount of cellulases produced was measured. On the average, the Δ*ace1* strain produced approximately two times more MUL activity, reflecting the amount of CBHI and EGI in the culture supernatant (III, Fig. 1B). In the transfer assay the Δ*ace1* strain produced, on the average, two times more MUL activity than the host strain ALKO2221 (III, Fig. 2C). These results further support the finding that ACEI down-regulates cellulase gene expression in the conditions studied here.

3.2.3.3   Growth on sorbitol (III)

On sorbitol, another carbon source considered to be neutral with respect to cellulase gene expression, growth of Δ*ace1* strains was reduced. The Δ*ace1* strain accumulated only half of the biomass of ALKO2221 during the six days of growth (III, Fig. 1). Since no cellulases are needed for growth on sorbitol media, this result implies that in addition to regulation of cellulase genes ACEI functions in the regulation of other genes as well. This defect in growth caused by *ace1* deletion seems to be limited to sorbitol since growth on other polyol sugars, such as arabitol and xylitol, was not affected by *ace1* deletion. Nor was any effect seen in growth on fructose, the product of sorbitol dehydrogenase (data not shown).

3.2.3.4 ACEI regulates the expression of genes encoding intracellular enzymes (unpublished data)

Genes encoding for factors involved in the utilisation of sorbitol as a carbon source, *e.g.* polyol transporters or sorbitol dehydrogenase, have not been characterised from *T. reesei*. To identify the other genes regulated by ACEI, glycerol and sorbitol cultivations were carried out for Δ*ace1* and host strain ALKO2221, and expression of two genes encoding for intracellular reductases was studied. *xdr1* encodes a xylose reductase (M. Saloheimo, pers. comm.) and *lxr1* encodes a L-xylulose reductase that can use xylitol, D-sorbitol or D-mannitol as a substrate (Richard *et al.*, 2002). The Δ*ace1* strain and the host strain were grown in two parallel shake flasks and mycelial samples were collected after two and four days of growth for mRNA analysis. Figure 4 shows Northern analysis of *xdr1* and *lxr1* expression in the Δ*ace1* and ALKO2221 strains. At the different time points of the glycerol culture, *xdr1* and *lxr1* are expressed approximately at the same level in the two strains analysed (Fig. 4). In sorbitol culture, expression of *lxr1* is reduced in the strain deleted for the *ace1* gene, indicating that ACEI is needed for the normal expression of *lxr1* during growth on sorbitol but not on glycerol. In the Δ*ace1* strain, the expression of *lxr1* is 24% to 38% of that seen in the host strain after two and four days of growth, respectively. Expression of *xrd1* is similar in host and the Δ*ace1* strain during growth on glycerol and slightly increased in the Δ*ace1* strain during growth on sorbitol. These results indicate that ACEI is involved in the regulation of genes encoding for certain intracellular enzymes as well as the extracellular cellulase and xylanase enzymes. Furthermore, the decreased expression of the *lxr1* gene in the Δ*ace1* strain suggests that ACEI may also act as an activator of transcription of certain genes.

GLYCEROL   SORBITOL
       ALKO   ALKO
Δace1 2221 Δace1 2221
 2   4   2   4   2   4   2   4

lxr1

xrd1

act1
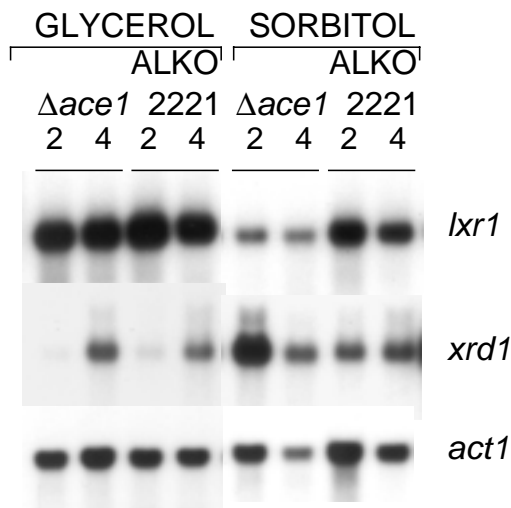
Figure 4. Northern analysis of lxr1 *and* xrd1 *mRNA and actin mRNA as a control from glycerol and sorbitol cultivations. The strains and the time points (days) are indicated on the top.*

3.2.4   ACEII, novel zinc binuclear cluster protein (II)

The second library plasmid, pAS26, obtained in the search of *cbh1* promoter activators contained cDNA with an open reading frame of 341 amino acids. The isolated gene was named *ace2*. Sequence analysis of the deduced ACEII protein revealed a zinc binuclear cluster (Zn(II)Cys6) domain in the N-terminus. The DNA-binding domain of ACEII is most similar to the Zn(II)Cys6 domains of ACU-15 and FacB, the factors involved in the regulation of acetate utilisation in *N. crassa* (accession number P87000) and *A. nidulans*, respectively (Todd *et al*., 1997) (II, Fig. 2). The Zn(II)Cys6 domain is followed by a region rich in proline and a region where the His-X sequence is repeated seven times. Similar regions with unknown function are found in other Zn(II)Cys6 factors, *e.g.* in the AflR protein of *A. niger* encoding a factor regulating aflatoxin production (Yu *et al*., 1996). ACEII also contains a region rich in glutamine that resembles the glutamine-rich activation domains found in the human transcription factor Sp1 and Oct1 (Gill *et al*., 1994). The Coils programme (Lupas *et al*., 1991) predicts possible leucine zipper-like heptad repeat motifs, coiled coils, after the region rich in proline and glutamine and in the C-terminal region of ACEII. However,

the score of the prediction is relatively low, 0.49 and 0.36 for these two regions, respectively, as compared, for example, with the 0.98 and 0.55 of GAL4 protein, activator of *S. cerevisiae*, where the coiled coil domain has been shown to mediate dimerisation (Hidalgo *et al*., 2001). Coiled coil motifs are predicted in a number of other Zn(II)2Cys6 proteins, and in certain proteins they have been shown to form coiled coil structures involved in protein-protein interactions (Lupas, 1996; Lupas *et al*., 1991; Todd and Andrianopoulos, 1997). Many DNA-binding proteins containing coiled coil structures have been shown to bind to palindromic DNA sites as dimers *e.g.* GAL4 and PPR1 activators of *S. cerevisiae* (Carey *et al*., 1989; Hidalgo *et al*., 2001; Marmorstein and Harrison, 1994). In addition, some Zn(II)Cys6 proteins contain a region of weak homology, called a middle homology region (MHR), that is suggested to contribute to DNA target discrimination (Schjerling and Holmberg, 1996). Based on amino acid sequence comparisons, no obvious MHR region is found in ACEII. It is noteworthy that no clear sequence similarities to regions of ACEII other than the Zn(II)Cys6 domain are found from the databases at NCBI or from databases containing the full genomic sequence of *N. crassa* or the EST sequences of *A. nidulans*, although similar cellulose-induced cellulase production has been reported in these fungi (Chikamatsu *et al*., 1999; Eberhart *et al*., 1977; Yazdi *et al*., 1990).

3.2.5 Target sequence of ACEII (II)

To characterise the target sequence of ACEII, the Zn(II)Cys6 domain of ACEII (amino acids 1-58) was produced as a fusion protein in *E. coli*, and DNA mobility shift assays were performed with labelled *cbh1* promoter fragments. The strongest specific binding was observed to a fragment encompassing *cbh1* promoter sequences from -621 to -941 (data not shown). Short 30-bp oligonucleotides covering this region were designed and binding of GST-ACEII$_{1-58}$ was tested. ACEII bound to an oligonucleotide 2D, corresponding to *cbh1* promoter sequences from -789 to -764 (Fig. 5). This oligonucleotide was mutated in triplets and binding was established to nucleotides 5'GGCTAATAA. Mutation of the GGC triplet in the binding site abolished binding (II, Fig. 3). A 5'GGCTAATAA sequence in another part of the *cbh1* promoter did not bind GST-ACEII$_{1-58}$, indicating that surrounding sequences contribute to the binding (data not shown). Most of Zn(II)Cys6 proteins bind as dimers to repeated

sequences (Todd and Andrianopoulos, 1997). However, well-studied examples of monomer binding exist, such as the AlcR, which regulates the genes involved in the utilisation of ethanol *A. nidulans* (Lenouvel *et al.*, 1997). Interestingly, the binding site of ACEII was found to be almost identical to the binding site of the Zn(II)Cys6 protein XlnR (5'GGCTAA), the positive regulator of cellulase and xylanase expression in *A. niger*. Although the two factors seem to bind to similar sites, the sequence similarity within the Zn(II)Cys6 domain is low as compared with other Zn(II)Cys6 proteins (I, Fig. 2).
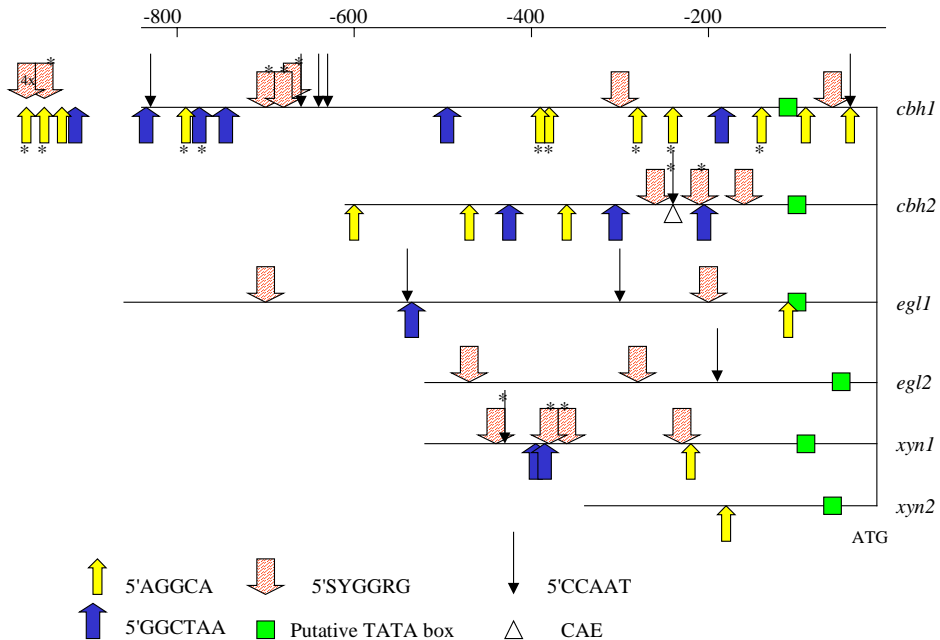
Figure 5. Schematic representation of the localisation of putative transcription factor binding sites and the TATA box within the cellulase and xylanase promoters of T. reesei. *5'AGGCA, 5'GGCTAA and 5'SYGGRG represent the binding sequences of ACEI, ACEII and CREI, respectively. 5'CCAAT is the binding sequence of the HAP2/3/5 complex. CAE represents the* cbh2-*activating-element identified by Zeilinger* et al. *(1998). The sites to which in vitro binding has been reported either with purified transcription factors or with total protein lysates are indicated with asterisk (*). Promoters were analysed by using the Regulatory sequence analysis tools at http://embnet.cifn.unam.mx/rsa-tools/.*

3.2.6 ACEII function

3.2.6.1 Growth on different carbon sources and cellulase production (II)

To elucidate the role of ACEII in the regulation of *cbh1* and other cellulase-encoding genes, a strain deleted for the *ace2* gene was constructed. The ALKO2221 strain was transformed with a reporter cassette that replaces the *ace2* coding region with a hygromycin selectable marker *hph*. Series of cultivations were carried out with the three following transformants deleted for the *ace2* gene: VTT-D-99729, VTT-D-99757 and VTT-99758 (Δ*ace2* strains), and the host to establish the role of ACEII in cellulase gene regulation. The growth of the Δ*ace2* strain and the host ALKO2221 was analysed on glucose, cellulose, glycerol and sorbitol by pH measurements of the culture media. The growth of the Δ*ace2* strain was normal on all other carbon sources except cellulose, where growth was slightly retarded (data not shown). The amount of cellulase activity was measured from the culture supernatants of cellulose cultivations, and the Δ*ace2* strain was found to have approximately 20% to 55% of the MUL activity of the host, indicating that the Δ*ace2* strains produce less cellulases than the host (II, Table 1).

3.2.6.2 ACEII and the regulation of cellulase genes in inducing conditions (II)

The function of ACEII in regulation of cellulase gene expression was studied under two different inducing conditions in cultivations similar to those carried out for the *ace1*-deleted strains. In the cellulose transfer assay, the expression of all the main cellulases (*cbh1*, *cbh2*, *egl1* and *egl2*) was reduced and induction delayed in the Δ*ace2*-deleted strain. Expression occurred at a lower level in the Δ*ace2* strains than in the host strain (II, Fig. 4). To make sure that the difference in cellulase expression did not only occur early in the induction of cellulase transcription, Δ*ace2* strains were grown on cellulose for six days after which the cellulase expression was analysed. mRNA amounts of *cbh1*, *cbh2* and *egl1* were found to be approximately 70% of those of the host. Cellulase genes were also induced by addition of sophorose to glycerol cultivations, and the effect of *ace2* deletion was studied. Unexpectedly, *cbh1* (II, Fig. 5), *cbh2* and *egl1* (data not shown) mRNA amounts were the same in the Δ*ace2* strain as in the host strain,

indicating that ACEII does not function in the sophorose-mediated induction of cellulase genes.

3.3 Expression of xylanase encoding genes *xyn1* and *xyn2* in Δ*ace1* and Δ*ace2* strains (II, III)

We studied the effect of *ace1* and *ace2* deletion on the expression of *T. reesei* xylanase genes *xyn1* and *xyn2* to determine whether the regulatory mechanisms involving ACEI and ACEII are shared between xylanases and cellulases. The expression of *xyn1* and *xyn2* was studied by Northern analysis from the samples taken from the cellulose transfer assays. Deletion of *ace1* increased the expression of both xylanase genes. The amount of *xyn1* mRNA was 2 to 4 times higher in the samples collected between 12 and 21 hours after transfer to cellulose media in the Δ*ace1* strain (III, Fig. 2). The expression of *xyn2* was advanced and occurred at a higher level in the *ace1*-deleted strain. These results demonstrate that ACEI is a negative regulator of *xyn1* and *xyn2* genes. The deletion of *ace2* reduced the expression of *xyn2*. Signal quantification reveals that the *xyn2* signal in the Δ*ace2* strain is 30% to 45% of that of the host after 15, 18 and 32 hours of cultivation on cellulose. No meaningful difference in the *xyn1* mRNA amounts between the Δ*ace2* and host strains could be seen (II, Fig. 4). Deletion of either *ace1* or *ace2* resulted in similar changes in the expression of both cellulases and xylanases, suggesting that cellulase and xylanase expression is co-ordinately regulated, at least in the conditions studied in this work.

3.4 Effect of simultaneous deletion of *ace1* and *ace2* (III)

To investigate the possible synergism between the actions of ACEI and ACEII, the *ace2* gene was disrupted in the Δ*ace1* strain (VTT-D-01850). The Δ*ace1* strain was transformed with the same *hph*-reproter cassette as was used in the generation of the Δ*ace2* strain, which replaced the coding region of *ace2* with the selectable marker. The growth of the Δ*ace1*Δ*ace2* strain was similar to that of the Δ*ace1* strain on the cellulose, glycerol, sorbitol, glucose and fructose cultivations studied (III, Fig. 1). The expression of cellulase genes was examined in cellulose transfer assay, and samples taken 6, 9, 12, 15, 18, 21 and 32 hours

after the transfer of mycelia to cellulose medium were analysed for cellulase and xylanase expression. Similar to the Δ*ace1* strain, all the cellulase and xylanase genes investigated were induced earlier and at a higher level in the Δ*ace1*Δ*ace2* strain than in the host ALKO2221 (III, Fig. 4). The difference in expression was more marked at the early time points as compared with the late time points, where expression was approximately the same in the two strains. We also studied the effect of simultaneous deletion of *ace1* and *ace2* on the sophorose-mediated induction of cellulase expression. All the cellulase genes were induced earlier and at a higher level in the Δ*ace1*Δ*ace2* strain than in the host strain (III). These results demonstrate that the increased cellulase and xylanase gene transcription seen in the Δ*ace1* strain is not dependent on the action of ACEII, but most likely yet another cellulase and xylanase activator.

3.5 Expression of *ace1*, *ace2* and *cre1* (III, unpublished data)

The expression of *ace1*, *ace2*, and *cre1* genes of *T. reesei* was studied by Northern analysis in the different strains constructed in this work, i.e. the Δ*ace1*, Δ*ace2* and Δ*ace1*Δ*ace2* strains, the host strain ALKO2221 and in the Rut-C30 strain expressing the truncated *cre1* gene and VTT-D-96482 (Rut-C30 transformed with the full-length *cre1* gene). Since the cDNA library from which *ace1* was isolated was made from mRNA under cellulase-inducing conditions, it was of interest to see whether *ace1* expression behaves in accordance with cellulase expression. Northern analysis of RNAs isolated from different carbon sources indicated that *ace1* is expressed during growth on all of the carbon sources studied. Slight induction in the expression of *ace1* is seen when the glycerol-grown mycelia are transferred onto cellulose medium (III. Fig. 2, 4). *ace1* expression was unaffected by the deletion of the *ace2* gene in cellulose- and sophorose-induced cultivations and by the lack of functional CREI protein during growth on cellulose, glycerol and sorbitol. These results suggest that *ace1* expression is not regulated by ACEII or CREI. Expression of *ace2* was too low for detection by Northern analysis. Expression of *cre1* appeared to be similar in the strains with the deletions of *ace1* or *ace2* gene and the host strain ALKO2221 suggesting that neither ACEI or ACEII regulates expression of *cre1* under the conditions studied.

4. Discussion

4.1 Isolation of transcriptional regulators ACEI and ACEII

Isolation of transcriptional activators by traditional methods, such as the Southwestern analysis, requires previous knowledge of their target sequences within the promoter of interest. Cloning based on sequence similarities is feasible only when homologous factors exist, and isolation methods based on protein-protein interactions require knowledge of the other factors involved in the same regulatory pathway. Since the description of the two-hybrid system in 1989 by Fields and Song (1989), yeast expression systems have been used to identify subunits involved in protein-protein interactions and later in the identification of genes encoding for proteins binding to specific DNA sequences (the one-hybrid system) (Li and Herskowitz, 1993).

In the absence of previous knowledge of the regulatory sites within the cellulase promoters, a novel screening method was developed that allowed the isolation of genes encoding for activators without knowledge of their binding sites. This method selects simultaneously for binding to and activation of the promoter of interest in *S. cerevisiae*. A great advantage of this method, as compared with the yeast one-hybrid system, is that it allows the use of the whole promoter (up to 1.1 kb in the study presented here) in the screening, thus bypassing the need for the previously identified regulatory regions within the promoter and allowing isolation of factors acting through different parts of the promoter in the same experiment. Concurrently with our work, longer promoters have been used in other yeast expression screenings successfully (Park *et al*., 1999). As a yeast-based *in vivo* screening method, it is particularly useful for proteins that are difficult to detect biochemically or for which starting material for purification is difficult to obtain due to, for instance, strong proteolytic activity of cell lysates. Furthermore, the method is designed to yield only activators since the protein encoded by library cDNA needs to be capable of both binding to and activating the promoter of interest, thus leading to transcription of the marker gene. The activation domain must be such that it functions in yeast, and in our particular case, with the *HIS3* TATA element. Examples exist where mammalian activators in a certain context fail to activate transcription in yeast or are very weak activators in yeast (Escher *et al*., 2000; Ponticelli *et al*., 1995), and thus making recognition of true positive clones more difficult and growth of the

positive clones much slower than in the one hybrid system, where strong activation domains, such as that of GAL4, are used (Li and Herskowitz, 1993). Our screening method also requires a 'better' cDNA library in the sense that the length of the cDNAs must be sufficiently long to contain both the DNA-binding domain and the activation domain which may be localised at opposite ends of the encoded polypeptide. The presence of in-frame stop codons in the 5'-terminus of the cDNAs does not impede the isolation in this method as it does in those one-hybrid systems where the cDNA is fused to the 3'-terminus of the activation domain-encoding sequence. As an example, the *ace2* gene isolated in this work would not most likely have been isolated by one-hybrid screening since the *ace2* cDNA contains an in-frame stop codon at position -8 from ATG, and a shorter cDNA would not have contained the DNA-binding domain that is encoded by nucleotides onwards from position +17 relative to ATG. Activation domains consisting of several different polypeptides and activators functioning only in synergy with other factors are most likely not isolated by our method or the yeast one-hybrid system. Furthermore, it is possible that certain sequences within the promoters, such as those that act as binding sites for yeast repressors, impede with the use of some promoters in this system. However, the presence of putative binding sites for the *S. cerevisiae* Mig1 repressor in the promoter used in this study did not interfere the with screening (I).

To examine the events of cellulase gene induction, this method was applied to the promoter of the main cellulase, *cbh1*, of *T. reesei*. One successful screening led to the isolation of two genes named *ace1* and *ace2* for <u>a</u>ctivators of <u>c</u>ellulase <u>e</u>xpression. The result of the screening shows that long promoter regions can fruitfully be used in yeast expression-based screenings. After the retransformation of the plasmids to the yeast strain containing the *cbh1-HIS3* reporter plasmid and to yeast containing the negative control plasmid, no false-positives were obtained. Therefore, the plasmids supporting growth of the pAS3 yeast, and not the yeast containing the negative control plasmid, all contained a cDNA encoding for a DNA-binding regulator (Fig. 3, p. 36). Thus it seems that simultaneous selection for DNA binding and activation is so strict that it greatly reduces the amount of false positives often obtained in expression-based screenings.

4.2 The novel transcriptional regulators ACEI and ACEII

The *ace2* gene encoded for a novel zinc binuclear cluster DNA-binding protein that shared no overall sequence similarity to the other sequences in the databases. In the DNA mobility shift assay, GST-ACEII$_{1-58}$ bound to the 5'GGCTAATAA sequence in the *cbh1* promoter at –779 relative to ATG. Mutation of the GGC triplet abolished binding, indicating that at least one of the bases is in contact with the Zn(II)2Cys6 domain of ACEII. The binding site of ACEII is similar to the suggested binding consensus of the XlnR of *A. niger* (5'GGCTAA) (van Peij *et al*., 1998a). However, ACEII did not bind to the identical XlnR binding sequence present in the *cbh1* promoter at –827 relative to ATG (5'GGCTAAA), to which XlnR binding has been analysed (van Peij *et al*., 1998b). The lack of sequence similarity outside the DNA-binding domain and the difference in size of the ACEII and XlnR proteins implies that although binding to similar sequences and regulating cellulase and xylanase expression in *T. reesei* and *A. niger* they are not homologous factors. ACEII was found to regulate four cellulase promoters and the *xyn2* promoter. However the 5'GGCTAATAA sequence is found only in the *cbh1* and *cbh2* promoters, thus suggesting that some heterogeneity exists in the target sequence. The promoter comparisons are, however, impeded by the short length of the promoter sequence available for *cbh2*, *egl2* and the xylanase promoters (Fig. 5, p. 45). The binding site of ACEII appears not to be a repeated sequence, as has been shown for most factors having the zinc binuclear cluster DNA-binding domain (reviewed by Todd and Andrianopoulos, 1997). However, for some Zn(II)2Cys6 proteins binding sites, that lack repeated triplets have been identified, *e.g.* AlcR and AmdR of *A. nidulans* and XlnR of *A. niger* (Lenouvel *et al*., 1997; Richardson *et al*., 1992; van Peij *et al*., 1998b). Due to the poor stability of different GST-ACEII fusion proteins in *E. coli*, the fusion protein used in the binding studies contained only the Zn(II)Cys6 domain and lacked the linker region C-terminal to the Zn(II)Cys6 domain. Linker regions have been shown to contribute to the binding specificity of some Zn(II)Cys6 proteins (Reece and Ptashne, 1993). Thus, it is possible that the GST-ACEII$_{1-58}$ does not exhibit exactly the same DNA binding properties as the full-length protein. Further studies are needed to determine the correct consensus binding sequence and additional binding sites of ACEII in the different promoters.

Eukaryotic transcriptional activation domains are classified according to the predominant amino acids (Triezenberg, 1995). Based on this classification and sequence comparisons with known activation domains, the activation domain of ACEII may involve the glutamine-proline rich region from amino acids 82 to 99. Glutamine-rich activation domains have been shown to interact directly with the TBP and the general transcription factors cross-species (Emili *et al*., 1994; Hoey *et al*., 1993).

The *ace1* gene encoded for a zinc finger protein that bound *in vitro* to eight sites in the *cbh1* promoter. ACEI recognised all 5'AGGCAAA sites and some 5'AGGCA proceeded by a sequence rich in A-T. In the sequenced promoter regions available, the 5'AGGCAAA sequence is found, in addition to the *cbh1* promoter, only in the *xyn1* promoter. However, sequences having the core 5'AGGCA are found in all the cellulase- and xylanase-promoters regulated by ACEI except the 525-bp *egl2* promoter (Fig. 5, p. 45). In the *cbh1* promoter, five of the ACEI binding sites are located relatively close to the TATA box, with the first site only 15 bp upstream, proposing that ACEI might act by interfering directly with the basal transcription machinery. On the other hand, as shown in Figure 5 (p. 45), one site is located only 20 bp apart from the ACEII binding site and the CCAAT-element and this might imply that direct or indirect contact between ACEI and ACEII or HAP 2/3/5 complex occurs if factors are bound to the promoter at the same time.

The putative ACEI protein of *N. crassa* and *T.reesei* ACEI contain nearly identical zinc fingers. Promoters of putative *N. crassa* cellulase and xylanase genes retrieved by sequence similarity to *T. reesei* enzymes from the *N. crassa* genome database at the Whitehead Institute/MIT Center for Genome Research also contain 5'AGGCA sequences, suggesting that a factor homologous to ACEI could regulate the expression of cellulase and xylanase genes in *N. crassa* as well.

The truncated ACEI expressed in yeast lacked 242 amino acids from the N-terminus and functioned as an activator in yeast, while the full-length protein appears to be a repressor. The repressor domain of ACEI is most likely located in the N-terminal third of the ACEI protein. The strong similarity found exclusively between the N-terminal regions of ACEI and StzA of *A. nidulans* and the deduced *N. crassa* sequence further suggest that regions important for

function reside in the region N-terminal to the zinc fingers (III, Fig. 7). However, no sequence similarity to these regions was found in other proteins in the databases, and no obvious repressor domain was identified in the ACEI amino acid sequence based on characterisation of repressor domains according to the predominant amino acids (Hanna-Rose and Hansen, 1996). The C-terminal part of ACEI, by contrast, is acidic and contains a region rich in asparagine and glutamine ($_{668}$EXDEX$_2$DEX$_4$DX$_2$D) that is predicted to form an amphipathic alpha helix (I, Fig. 2). This region could function as an acidic activation domain. The increased or decreased expression of the cellulase and xylanase genes or the reductase gene studied in this work together with the analysis of the amino acid sequence of ACEI suggests that ACEI has potential for dual repressor-activator function.

4.3 Expression of the transcription factors in *T. reesei*

The expression of *ace1* and *ace2* genes was studied by Northern analysis. The expression of *ace2* was too low for detection by the methods used in this work. *ace1* was found to be expressed in glycerol, sorbitol, glucose cultures and in cultures induced by cellulose or sophorose (III and data not shown). The amount of *ace1* mRNA was slightly increased upon transfer of glycerol-grown mycelia to cellulose media and by the addition of sophorose to glycerol-grown mycelia (III). The constitutive expression of transcription factors is not uncommon since their activity can be controlled at various different levels, *e.g.* nuclear localisation, availability of cofactors, phosphorylation and post-translational modification.

The availability of genes encoding three factors regulating cellulase and hemicellulase expression in *T. reesei* enables the study of possible cross-regulation between these factors. The *ace1* promoter contains three sites fitting to the binding consensus for CREI. Northern analysis of a strain containing a truncated *cre1* gene and this strain transformed with a full-length *cre1* gene (Ilmén *et al*., 1996b), expressed *ace1* similarly in all the conditions studied, indicating that the expression of *ace1* is not affected by the *cre1* mutation (data not shown). Furthermore, the expression of *ace1* in the strain deleted for *ace2* was similar to that in the host strain, suggesting that ACEII does not regulate the expression of *ace1* under the conditions used in the cellulose transfer and

sophorose-induction experiments carried out in this work. This data suggest that *ace1* is expressed constitutively and that its activity may be regulated by an yet unknown manner. Although the promoter of the gene encoding for the glucose repressor CREI contains three copies of the core-binding sequence for ACEI, 5'AGGCA, deletion of the *ace1* gene did not affect the expression of *cre1* during growth under the inducing conditions studied here. Similarly, the deletion of *ace2* or both *ace1* and *ace2* did not influence the expression of *cre1*.

4.4 Regulation of genes encoding extracellular hydrolases

Although some mutant data propose that separate regulations exists for cellobiohydrolases and endoglucanases, ACEI and ACEII were found to regulate these genes to the same relative extent (I, III). ACEI and ACEII regulated both genes encoding cellulases and xylanases as does CREI of *T. reesei* and XlnR of *A. niger* as well. That the same factors regulate cellulase- and xylanase-encoding genes is logical since both cellulose and hemicelluloses are often encountered in nature simultaneously. The deletion of the *ace2* gene in *T. reesei* resulted in reduction of cellulase and xylanase gene expression when cellulase genes were induced in the presence of cellulose. The expression of the main cellulase genes (*cbh1*, *cbh2*, *egl1* and *egl2*) was reduced on the average 25% to 75% (II, Fig. 4), while the production of cellulases, measured as HEC and MUL activity found in the medium, was reduced 45% after six days of cellulose cultivation (II, Table 1). The strains deleted for ACEII also grew slightly slower on cellulose cultivations, most likely due to the reduced amount of mono- and disaccharides released from cellulose. These results show that *ace2* encodes the first transcriptional activator of cellulase and xylanase gene expression isolated from *T. reesei*. Previously, only one other fungal cellulase and xylanase activator had been isolated, the XlnR from *A. niger*.

The reduction in cellulase expression was of the same relative magnitude in all the cellulase genes studied, although the binding sequence of ACEII (5'GGCTAA) was not found in all the cellulase promoter sequences known to date. ACEII was shown to regulate *xyn2* but no effect on *xyn1* expression was seen under the conditions studied here. Different regulatory mechanisms have

been suggested for these two xylanases (Zeilinger *et al*., 1996) and different expression patterns of the two xylanase genes are seen in the cellulose transfer assays of this study as well (II, Fig. 4 and III, Figs. 2, 4). Although *xyn1* was not shown to be regulated by ACEII, the result does not exclude this possibility because the time points of the Northern analysis were not optimal for *xyn1* expression (II, Fig. 4). The *xyn1* promoter contains two putative ACEII binding sites in the promoter region from –538 to –321. In promoter deletion studies, the deletion of this region abolishes the activity of the *xyn1* promoter on xylan, xylose and cellulose plates, indicating that regions important for full activation of the promoter reside in this region (Zeilinger *et al*., 1996). The 330-bp sequence available for the *xyn2* promoter does not contain the 5'GGCTAA sequence.

The induction of cellulase expression upon transfer to cellulose-containing culture media was delayed in the *ace2*-deleted strain (II). A special feature of ACEII is that it does not seem to have a role in induction when sophorose is used as an inducer of cellulase expression since cellulase induction by sophorose was unaffected by *ace2* deletion (II). These findings strongly suggest that cellulose and sophorose induction of cellulase expression are mediated by at least partially separate mechanisms. This hypothesis is further argumented by the studies on different promoter deletions where certain *cbh1* promoter deletions are inducible by sophorose but not induced upon growth on cellulose. A strain transformed with a *cbh1* promoter-*lacZ* expression construct, where a *cbh1* promoter region upstream of –187 was removed, was inducible by sophorose but not by cellulose (Ilmén *et al*., 1998 and M. Ilmén, unpubl. results).

The deletion of the *ace2* gene reduced but did not completely abolish the expression of cellulase and *xyn2* genes, indicating that other factors contribute to the activation of these genes as well. These factors may include the recently isolated XlnR homologue of *T. reesei*, Xyr1 (R. Mach, pers. comm.) and/or the recently cloned leucine-zipper type DNA-binding protein that binds *in vivo* to the *cbh1* promoter (Aro *et al.*, unpubl. results). The finding that simultaneous deletion of *ace1* and *ace2* genes resulted in a similar increase in expression as that seen in the strains deleted only for the *ace1* gene (III) further supports the hypothesis that activators in addition to ACEII contribute to the high level of cellulase- and xylanase-gene expression seen in the cellulose- and sophorose-induced cultivations. The CCAAT element binding HAP 2/3/5 complex that has

recently been cloned from *T. reesei* (Zeilinger *et al*., 2001) most likely contributes to the regulation of both cellulase and xylanase genes. The HAP 2/3/5 complex might play a more general regulatory role in the organisation of chromatin structure within promoters and in the formation of the transcriptional complex, as has been suggested for the various CCAAT binding complexes (Mantovani, 1999; Narendja *et al*., 1999 and references therein). The *A. nidulans* CCAAT binding protein demonstrated to affect the activation of the *amdS* promoter by the pathway-specific zinc binuclear DNA-binding protein AmdR. The authors suggest that the CCAAT binding protein of *A. nidulans,* AnCF, facilitates the binding of AmdR to its binding site near the CCAAT box in the *amdS* promoter (Steidl *et al*., 1999). Similar positioning of the ACEII binding site and a CCAAT box was proposed for the CAE (*cbh2*-activating-element) in the *cbh2* promoter of *T. reesei* (Zeilinger *et al*., 1998). The ACEII binding site proposed by Zeilinger *et al*. (1998) is not a 5'GGCTAA but a 5'GGGTAA. Binding to this site in the *cbh2* promoter has not been studied, but a 5'GGGTAA sequence in the *cbh1* promoter oligonucleotide failed to bind ACEII$_{1-58}$-GST. However, a similar region of an adjacent CCAAT and putative ACEII binding site (5'GGCTAA) can also be found in the *cbh1* promoter at the position -825 from the ATG and the *egl1* promoter at position -550 from ATG (Fig. 5, p. 45).

Even though ACEI was originally isolated as an activator of the *cbh1* promoter, ACEI was found mediate repression of cellulase and xylanase expression in *T. reesei*. Strains deleted for the *ace1* gene expressed cellulases at a higher level under the inducing conditions studied here. The deletion of *ace1* increased expression of all the main cellulases, *cbh1*, *cbh2*, *egl1* and *egl2* and the two xylanase genes, *xyn1* and *xyn2*. These results demonstrate for the first time that down-regulation of cellulase and xylanase genes exists under inducing conditions and that the expression of cellulase and xylanase genes can still be increased under these conditions. It is possible that ACEI responds to some unknown change in the environment or to the intracellular balance between transcription of cellulase genes and translation, post-translational modification and/or secretion, and modulates the cellulase and xylanase expression, either directly or indirectly, according to these changes. Under inducing conditions, nearly all of the secreted protein constitutes of cellulases, most of it CBHI, encoded by a single gene, therefore, it is possible that a feedback loop from secretion down-regulates cellulase and xylanase mRNA production and in this way maintains a balance between expression of a gene encoding for secreted

protein and translation or secretion efficiency. Down-regulation of *cbh1* expression in response to a secretory block has been demonstrated in *T. reesei* (T. Pakula, pers. comm.).

The full role of ACEI in the regulation of cellulase gene expression requires further investigation. Deletion of the *ace1* gene leading to an increase in cellulase expression indicated that ACEI functions as a repressor of cellulase transcription under the conditions studied. The activator function revealed in yeast and the fact that *ace1* deletion actually led to decreased expression of *lxr1* encoding L-xylulose reductase in *T. reesei* suggest that ACEI can also act as an activator. The *in vitro* binding data and the fact that ACEI bound to the *cbh1* promoter in yeast propose that ACEI would have a direct role as a regulator of the genes (at least *cbh1*). However, it still remains possible that the effect of ACEI is mediated through an indirect mechanism, ACEI regulating the expression of genes encoding factors functioning in the signalling cascade involved in the induction of genes encoding extracellular enzymes. At present, it is justified to say that the regulation of cellulase gene expression is complex, and the level of cellulase gene expression is likely to be modulated by the synergistic action of several regulatory factors, of which some are shown in Figure 2, p. 31.

4.5 Role of ACEI in the regulation of genes encoding intracellular enzymes

Deletion of *ace1* led to a clear reduction in growth in the sorbitol cultivations. Since the growth of the fungus on sorbitol is not dependent on cellulase activity, this growth defect suggests that ACEI has a role in the regulation of other genes apart from cellulases and xylanases. Growth on fructose, the end product of the action of sorbitol dehydrogenase, was unaffected (III). Genes encoding enzymes involved in sorbitol utilisation have not been cloned from *T. reesei*. To address the reason for the poor growth, we tested the effect of *ace1* deletion on the two relevant genes available encoding for intracellular enzymes. The *lxr1* gene was originally isolated as a gene involved in the L-arabinose utilisation of *T. reesei*. It encodes for a L-xylulose reductase, with activity also on D-arabinitol and D-sorbitol (Richard *et al.*, 2002). The *xrd1* gene encodes for a xylose reductase (M. Saloheimo, unpubl. data). The effect of *ace1* deletion on the expression of *lxr1* and *xrd1* was examined in the Δ*ace1* and the ALKO2221 strain. In the glycerol

cultivation, the expression of the *lxr1* was similar between the two strains, whereas in the sorbitol cultivation, the deletion of the *ace1* gene led to a decrease in the expression of the *lxr1* gene (Fig. 4, p. 43). The expression of the *xrd1* gene was increased in the Δ*ace1* strain after two days of growth on sorbitol. On glycerol, the expression of *xrd1* was similar in the two strains studied (Fig. 4, p. 43). *ace1* deletion leading to a decrease in *lxr1* expression and an increase in *xrd1* expression indicates that the deletion of *ace1* affects the expression of genes encoding intracellular enzymes in addition to those encoding cellulases and xylanases. The results appears to suggest a role for ACEI in balancing the intracellular and extracellular pathways underlying in carbon metabolism.

The finding that the same factor regulates expression of extracellular and intracellular catabolic enzymes has been demonstrated for the *A. niger* XlnR. *xlnR* deletion has been reported to abolish the expression of *xyrA*, which encodes a xylose reductase (Hasper *et al.*, 2000). However, the extent of the effect of *xlnR* deletion on the regulation of *xyrA* is difficult to reconcile with the report of de Groot, where the *xyrA* gene is expressed similarly in the wild-type and *xlnR*-disrupted strains (de Groot *et al.*, 2001). In *A. niger*, co-ordinated induction of the extracellular arabinose-releasing enzymes and the enzymes involved in L-arabinose catabolism has been proposed (de Vries and Visser, 2001; vanKuyk *et al.*, 2001). This induction is suggested to be mediated by yet unknown factors AraA and AraB and not by XlnR (de Groot *et al.*, 2001).

Sorbitol is known to act as an osmolyte, counteracting water loss in response to osmotic shock. A protein with high similarity to ACEI, the StzA of *A. nidulans*, is thought to encode a factor involved in salt sensitivity (GeneBank accession number AAF15889). Since no *T. reesei* genes involved in the osmoregulation were available for further studies, the results presented here are only indicative and further studies are needed to fully understand the role of ACEI in pentose utilisation and stress reactions of the cell.

The finding that *lxr1* expression was decreased in the Δ*ace1* strain suggests that ACEI acts as an activator of *lxr1*. Transcription factors that activate in one circumstance and repress in another have been documented, and the molecular basis of their transitions is quite diverse (Roberts and Green, 1995). The truncated form of ACEI activating the *cbh1* promoter in yeast further leads support to the possibility that ACEI may also function as an activator, possibly

depending on the promoter context and on the presence and function of other factors as well. Taken together, the findings presented here suggest a role for ACEI as a multifunctional DNA-binding protein capable of alternately activating or repressing genes involved in carbohydrate metabolism.

4.6 Future perspectives

The novel factors ACEI and ACEII control a set of genes that function in the breakdown of cellulose and hemicelluloses. A more detailed analysis is needed to establish their role during growth on various carbon sources such as xylan where xylanases are needed for growth. To fully understand the regulatory mechanism utilised by ACEI and ACEII, *in vivo* the regulatory regions within ACEI and ACEII proteins should be identified by deletions or point mutations. Also binding of the factors to cellulase and xylanase promoters in *T. reesei* should be studied and promoter mutations made to characterise the functional binding sites within the promoters. However, this is impeded due to the high number of ACEI binding sites in the *cbh1* promoter. It is now apparent that other factors apart from ACEI and ACEII are involved in the positive regulation of (hemi)cellulase genes. Their cloning and characterisation and their synergism with ACEI and ACEII await further studies.

The role of ACEI in down-regulation of cellulase expression under inducing conditions represents a novel feature in the regulation of cellulase expression. From the biotechnological point of view, deletion of ACEI and overexpression of ACEII represent a promising way to increase the production of cellulases, xylanases or any protein expressed under the cellulase and xylanase promoters.

Finally, a full understanding of the regulation of (hemi)cellulase genes will require not only a more complete analysis of the multiple factors involved in the positive regulation of these genes but also an investigation of the possible interactions between these factors and elucidation of the signalling pathways leading to the induction of (hemi)cellulase gene expression.

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Author(s)
Aro, Nina

Title

Characterization of novel transcription factors ACEI and ACEII involved in regulation of cellulase and xylanase genes in *Trichoderma reesei*

Abstract

Cellulose and hemicellulose are the most abundant renewable carbon sources on earth. Filamentous fungi produce a wide variety of extracellular enzymes that degrade these complex polymeric compounds and play an important role in carbon turnover in nature. The filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) is the best studied cellulolytic fungus and it is widely used by the biotechnical industry for production of hydrolytic enzymes such as cellulases and xylanases. The production of these enzymes is regulated at the transcriptional level. The cellulase genes, especially the main cellulase *cbh1*, are very highly expressed and thus their promoters are among the strongest known in nature. Despite the importance of cellulases and xylanases very little is known of the regulatory mechanism involved in the high level of expression of cellulase and xylanase genes

In this work a novel method was developed to isolate transcription factors without previous knowledge of the important DNA sequence elements in promoters or of the nature of the activator genes and proteins. This method selects for simultaneous binding and activation of the desired promoter in *S. cerevisiae* and is in general applicable for any organism and promoter. Using this method two new genes, *ace1* and *ace2,* encoding transcription factors binding to the promoter of the main cellulase gene *cbh1* of *T. reesei* were isolated.

ace1 encodes a protein that contains three zinc finger motifs of Cys$_2$-His$_2$ type. Amino acid sequence similarity is seen towards *A. nidulans* protein StzA and deduced *N. crassa* protein, but not to yeast proteins suggesting that *ace1* is a regulator specific for filamentous fungi. Gel mobility shift assays revealed at least eight putative binding sites for ACEI scattered in the *cbh1* promoter all containing the core sequence 5'AGGCA. Deletion of *ace1* gene in *T. reesei* resulted in increased expression of all the main cellulase genes *cbh1, cbh2, egl1* and *egl2* and the xylanase genes *xyn1* and *xyn2* indicating that ACEI down regulates cellulase and xylanase expression.

The second factor ACEII belongs to the family of zinc binuclear cluster proteins found exclusively in fungi. ACEII binds to at least one sequence *in vitro* in the *cbh1* promoter. Disruption of *ace2* results in reduced expression levels of all the main cellulase genes and the fungus showed reduced cellulase activities when grown on cellulose containing media. Also the expression of gene encoding one of the main hemicellulases, β-xylanase XYNII was reduced on cellulose medium.

Keywords

Trichoderma reesei, *Hypocrea jecorina*, ACEI, ACEII, transcriptional regulation, activators, repressors, cellulase, xylanase, transcription factors, isolation

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