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*

(Received for publication, July 21, 1999, and in revised form, October 29, 1999)

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A genetic selection method was developed for the cloning of positive-acting transcriptional regulatory genes in Saccharomyces cerevisiae. The method was applied for the isolation of activators of Trichoderma reesei (Hypocrea jecorina) cellulase genes. Activator genes were isolated from a T. reesei expression cDNA library on the basis of the ability of their translation products to activate transcription from the full-length T. reesei cbh1 promoter coupled to the S. cerevisiae HIS3 gene and to support the growth of the yeast colonies in the absence of histidine. Among the clones obtained was the ace1 gene encoding a novel polypeptide, ACEI, that contains three zinc finger motifs of Cys$_2$-His$_2$ type. Possible ACEI homologues were found among expressed sequence tags of Aspergillus and Neurospora. The ability of ACEI to bind to the cbh1 promoter was further confirmed in the yeast one-hybrid system. In vitro binding and gel mobility shift assays revealed several binding sites for the ACEI protein in the cbh1 promoter. Disruption of the ace1 gene in T. reesei resulted in retarded growth of the fungus on a cellulose-containing medium, on which cellulases are normally highly expressed.

The filamentous fungus Trichoderma reesei is well known for efficient production of cellulolytic enzymes and its powerful capacity to hydrolyze cellulose into glucose. It is an excellent cellulolytic model organism, and the cellulolytic system of T. reesei has become the best characterized among filamentous fungi in many respects. The enzymatic properties and three-dimensional structures (1–4) of T. reesei cellulases, as well as the carbon source dependent regulation of cellulase gene expression (for a recent review, see Ref. 5), have been studied in detail by several groups. The activity of cellulase genes is controlled at the level of transcription: the genes are repressed in the presence of glucose and highly induced when cellulose, glucose, or its derivatives, or certain oligosaccharides, such as sophorose, are present. Glucose repression is mediated by the CREI protein (6–8), which has been shown to act directly on the promoter of the gene encoding the major cellulase cellobiohydrolase I (CBHI) (9). CREI also mediates repression of a number of other genes coding for enzymes involved in degradation of hemicellulose and cellulose (10). In addition to cre1, no other genes for transcription factors have been described in T. reesei. Based on the gene expression data, it can be concluded that a distinct induction pathway for cellulases must exist in T. reesei that is needed for high level transcription (11, 12), but the genes responsible for transcription activation are completely unknown. Furthermore, there is lack of knowledge about possible target sequences for cellulase-specific transcription activators from any filamentous fungi. The aim of this study was to identify genes encoding transcription activators involved in regulation of the activity of cellulase genes. For this purpose, we developed a genetic selection system that allowed us to isolate novel transcription activators from T. reesei CDNA expression library based on their ability to bind and activate transcription from the T. reesei cbh1 promoter in Saccharomyces cerevisiae.

EXPERIMENTAL PROCEDURES

Strains—Escherichia coli strains JS4 and DH5α were used for library and plasmid constructions, respectively. Strain TOP10F' was used as a host for the pQEEO-1-based vectors and strain BL21(DE3)LyS was used as a host for production of glutathione S-transferase (GST) fusion proteins.

S. cerevisiae strain DBY746 (ATCC 44773, a, his3-1, leu2-3, leu2-112, ura3-52, trp1-289, cyh', cir') (D. Bothstein, Massachusetts Institute of Technology, Cambridge, MA) was used as a host for propagation of the reporter plasmids and the expression library. S. cerevisiae strain YM4271 (MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-903, tyr1-501, gal4-3512, gal80-5358, ade6::hisG) (CLONTECH Laboratories, Inc.) was the host in the one-hybrid experiment. S. cerevisiae H190 (SUC2, ade2-1, can1-100, his3-11, his3-15, leu2-3, 112, trp1-1, ura3-1, mig1-2::LEU2) was obtained from H. Ronne (Upsala, Sweden).

T. reesei strain Rut-C30 (ATCC 56765; Ref. 13) was used in the preparation of the cDNA library. The genomic cosmid library was from the strain VTT-D-80133 (14). In Southern hybridization, DNA from the cellulase-negative strains VTT-D-81512, VTT-D-81153, VTT-D-81155, VTT-D-81158, and VTT-D-81168 (cel-18, cel-1, cel-22, and cel-25 in Ref. 15, respectively), the cellulase-overproducing strain VTT-D-79125 (14), and the strain QMB9414 (ATCC 269221; Ref. 16) were used. The disruption of the ace1 gene was made into a low protease mutant strain ALKO2221 originating from VTT-D-79125.

Media and Culture Conditions—Host yeast strains were grown in yeast/peptone dextrose medium. Synthetic selection media (SC) lacking the appropriate nutrients were used for the plasmid-carrying strains (18). SC-Leu-His plates supplemented with 45 mM 3-aminotriazole were used in the one-hybrid activation test. Plates used in yeast electroporation contained 1 M sorbitol.

The culture conditions used for T. reesei Rut-C30 to induce the production of hydrolytic enzymes and the preparation of the cDNA

* Financial support was provided by the Foundation for Biotechnical and Industrial Fermentation Research, Helsinki Graduate School in Biotechnology and Molecular Biology, Roal Oy, and the Technology Development Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF190793.

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1 The abbreviations used are: GST, glutathione S-transferase; SC, synthetic complete medium; PCR, polymerase chain reaction; ACEI$_{382-582}$, ACEI DNA binding domain including amino acids 382–582 of ACEI; GAL4ad-ACEI$_{382-582}$, S. cerevisiae GAL4 activation domain-ACEI DNA binding domain fusion protein; bp, base pair(s); kb, kilobase pair(s).

‡ A. Mäntylä, unpublished data.
Electroporation with the total of 40 DBY746 yeast strain harboring the pAS3 reporter plasmid was transforming yeast, which provides expression signals for the cDNA insert. The yeast strain was used as a template. The yeast multicopy vector pAJ401 used for transformation of E. coli was constructed in the cosmid p3030 (22) as a template and sequences starting from 78 bp upstream of the initiator ATG (AAAGGAT)-HIS3 clones transformation of formants for growth on acetamide as the sole nitrogen source. transformation was done either with electroporation (Bio-Rad) or by only together with the reporter plasmid and the strains with the reporter construct and the negative control ace1 gene producing pAS3. The primers used for cDNA from rat) was ligated in front of the receptor cDNA domain was cleaved from the pAS34 plasmid by digestion with the restriction enzyme filled in with the T4 DNA polymerase (Roche Molecular Biochemicals). Other Methods—Isolation of the ace1 Gene by Genetic Selection in Yeast— (23) was excised from the p3SR2 plasmid with the appropriate restriction enzymes, gel-purified, and labeled as described above. One-Hybrid Experiment—Binding of Ace1 to a 170-bp fragment of the cbh1 promoter was assessed in in using the Matchmaker one-hybrid system (CLONTECH). For the construction of a target-reporter yeast strain, a cbh1 promoter fragment correspondng to nucleotides from –843 to –676 was amplified by PCR with primers GAG-GAGAGCGTCGAGAATCCAAACATGCGAGGCGGGACAGCTTCTGCACCTCCGAGCGGCTGAGAGAAGGCAGG (forward) and GAG-GAGAGCTCGAGAATCCAAACATGCGAGGCGGGACAGCTTCTGCACCTCCGAGCGGCTGAGAGAAGGCAGG (reverse) flanked by SacI sites (underlined). The PCR product was cloned into the SacI site of pHisi-1 (CLONTECH). The resulting plasmid, pPL2, contained two tandem copies of the insert in reverse orientation. pPL2 and pHisi-1 were linearized and transformed into the S. cerevisiae strain YM4271 by the lithium acetate method, and the transformants were selected on SC-His plates for integration of the target-reporter construct into the fungal genome, plasmid pAS38 was constructed as follows. The protein expression construct was named pARO20. Subsequently, the Leu-selectable plasmid pARO20 and pGAD10 were transformed into the pHisi-1 (CLONTECH). The resulting plasmid, pARO18 (see above) was cloned into BamHI-EcoRI cut pGAD10 expression vector (CLONTECH) of the Matchmaker two-hybrid system, which generates a hybrid protein that contains the GAL4 activation domain in the N terminus. The GAL4ad-ACEI382–582 expression construct was named pARO20. Subsequently, the Leu-selectable plasmid pARO20 and pGAD10 were transformed into the pHisi-1 (CLONTECH) plasmid pARO18 was grown to an A590 = 0.7, and the production of the GST-ACEI382–582 fusion protein was induced by addition of isopropyl-β-thiogalacto-pyranoside to 1 mM. Cells were harvested and broken by sonication. Cell debris was removed by centrifugation. The fusion protein contained in the supernatant was affinity-purified on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) and eluted in 5 mM glutathione, 50 mM Tris-HCl (pH 8.0). For some experiments, the ACEI382–582 domain was cleaved from the GST moiety by thrombin (20 units of thrombin/mg of fusion protein), and the cleavage was confirmed by SDS-PAGE.

DNA-protein binding reactions were incubated for 20 min at 25 °C in 20 μL of binding buffer (50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 5 mM dithiothreit, 5 μM ZnCl2, 10% (v/v) glycerol, 100 μg/ml poly(dIdC) with 0.25–0.5 μg of the GST-ACEI382–582 fusion protein and 0.5–1 ng (about 50,000 cpm) of the labeled double-stranded DNA. In competition experiments, unlabeled DNA was added into the reaction in a 20–200-fold excess over the labeled oligonucleotide. The DNA-protein complex were separated on 6% nondenaturing polyacrylamide gels containing 10% (v/v) glycerol in 12.5 mM Tris-borate buffer (pH 8.3).

DNA Labeling for Binding Reactions—Complementary oligonucleotides were annealed producing a hybrid with a recessed 3’ end in the coding strand that was filled in with [α-32P]dCTP using the Klenow fragment of DNA polymerase. Fragments longer than 100 bp were amplified by PCR using sequence-specific primers, digested with appropriate restriction enzymes, gel-purified, and labeled as described above.

RESULTS

Isolation of the ace1 Gene by Genetic Selection in Yeast—A genetic selection method was developed for cloning of positive-transcriptional regulatory genes in S. cerevisiae, and it was applied for the isolation of activators of cellulase genes of T. reesei. First, a yeast reporter plasmid was constructed in which the promoter of the T. reesei major cellulase gene cbh1 was fused to a promoterless S. cerevisiae HIS3 gene, thus bringing HIS3 expression under the control of the cbh1 promoter. It 1.15-kb promoter fragment located immediately up-

3 Hohn and Hinnen, unpublished data.
stream of the cbh1 TATA box was inserted in front of the yeast HIS3 reporter gene, which lacked upstream regulatory sequences but contained the TATA box and the sequences downstream. The resulting LEU2-selectable single copy yeast plasmid pAS3 was transformed into the yeast strain DBY746. The reporter strain DBY746-pAS3 could not grow on media lacking histidine, showing that the *Trichoderma* promoter could not drive expression of the reporter gene by itself. In order to exclude the possibility that HIS3 was not expressed because the *S. cerevisiae* glucose repressor protein MIG1, which recognizes similar sequence elements as present in the cbh1 promoter, repressed the reporter construct, pAS3 was transformed into a mig1 deletion strain. The pAS3 construct remained silent, demonstrating that MIG1 did not repress the promoter and that yeast-encoded factors alone could not activate the reporter construct.

In order to isolate *T. reesei* cDNAs that would activate transcription from the cbh1-HIS3 construct, a cDNA of *T. reesei* was introduced into the reporter yeast. The cDNA library of *T. reesei* grown in cellulase-inducing conditions was prepared into a URA3-selectable multicyc copy yeast expression vector pAJ401 under the strong constitutive PGK promoter (19). A subset of the expression library containing 10^5 independent clones was transformed into the reporter yeast strain DBY746-pAS3, resulting in 10^6 yeast colonies growing on media lacking leucine and uracil. The colonies were scraped from the plates and screened on plates lacking histidine, leucine, and uracil. Growing colonies were detected with the frequency of 4:100 000. Plasmids were recovered from the growing colonies and retransformed into the reporter yeast strain, into the yeast strain containing the negative control plasmid pMS85, and into the host strain DBY746. Most of the plasmids supported growth of all the strains on media lacking histidine. These clones contained the *T. reesei* his3 gene as verified by partial sequencing followed by homology comparison of the open reading frame against the yeast and *Neurospora* his3 genes. 15% of the plasmids could not support growth of any of the strains and thus represented false positives. One of the remaining plasmids (pAS27) contained a 2-kb cDNA insert and supported slow growth of the reporter strain but not of the negative control or the host strain on media lacking histidine (Fig. 1). The cDNA was studied further, and the gene was named ace1 (activator of cellulase expression).

**ace1 cDNA Codes for a DNA-binding Protein**—Sequencing of the ace1 cDNA from the pAS27 plasmid revealed a 1943-bp cDNA with an open reading frame of 491 amino acids starting from the first ATG codon in the insert. Northern hybridization using the cDNA as a probe gave two signals of about 3.2 and 3.0 kb in length (data not shown) and thus showed that the cDNA was not full-length. Therefore, the full-length cDNA of the *ace1* gene was isolated from a library prepared in AZAP from the same induced *Trichoderma* cDNA as used in the initial screening. A 300-bp PCR fragment from the 5′ end of the original cDNA was used as a probe in plaque hybridization. The resulting plasmid pAS28 contained a cDNA insert of 3223 bp, which is in good accordance with the estimated size of the longest mRNA. The open reading frame of 733 amino acids starting from the first ATG codon of the cDNA maintains the frame of the original open reading frame and contains 242 additional amino acids. There is a rather long, 611-bp, untranslated 5′ leader sequence in the cDNA. The existence of three in-frame stop codons before the first ATG in the cDNA confirms that the plasmid contains the whole protein coding sequence of the *ace1* gene.

The *ace1* gene sequence and the deduced protein sequence are shown in Fig. 2. Amino acids 387–403 form a putative bipartite nuclear targeting signal RRKKNATPDVPAPKKCR (basic residues are shown in boldface), fitting well to the consensus (29). Partially overlapping with the nuclear targeting signal follows an area containing three zinc fingers of the Cys3-His2 type. The original PROSITE pattern C^2^H^2^ recognizes the first Cys2-His2 finger of ACE1, and an extended pattern C^2^H^2^C (Ref. 30) recognizes the third finger that contains a serine instead of the conserved hydrophobic amino acid of the original pattern. The middle finger of ACE1 has a 15-amino acid-long loop instead of the usual 12 amino acids between the conserved zinc-coordinating second Cys and first His. The distribution of acidic and bulky nonpolar residues between amino acids 373–388 and 666–682 suggests that amphipatic a-helices characteristic of several transcription activation domains may be formed (31, 32).

**Sequence Comparisons of the ACE1 Protein**—The deduced ACE1 protein sequence was compared with the sequence data banks by using the BLAST program. The first finger showed highest similarity to zinc fingers of fungal origin, e.g. to those of the *S. cerevisiae* sulfite resistance gene FZF1 (33), the Aspergillus developmental regulator BRLA (34), homologues of the *Crea* glucose repressor from several fungi, the meiotic regulator RIM101/RIM101 of *S. cerevisiae* (35), and RIM101 of *Yarrowia lipolytica* (36) that shows homology to the *A. nidulans* pH-regulator PACC (37). The second finger showed similarity to the meiotic inhibitor RME1 from yeast (38). RME1 also contains three zinc fingers, the second of which has a 15-amino acid central loop as ACE1, but the amino acid sequences of the fingers are quite different. The third finger showed similarity to the MSS1 protein of *S. cerevisiae* (39) involved in glucoamylose gene expression. Outside the zinc finger region, no meaningful similarities with known proteins could be detected. DNA and protein sequence comparisons against the *A. nidulans* and *Neurospora crassa* expressed sequence tag data bases detected four Aspergillus clones (n8h08a1.r1, c3b04a1.r1, w4e06a1.r1, g6h12a1.r1), three of which are partially overlapping, and one *Neurospora* clone (b306ne.f1). All five of these clones have clear similarity with *T. reesei* ACE1, suggesting that homologues of *ace1* are expressed in other filamentous fungi. Amino acid sequence alignment of these clones with *T. reesei* ACE1 is shown in Fig. 3.

**Characterization of the Genomic ace1 Locus**—Chromosomal DNA isolated from different *T. reesei* strains, including hypercellulolytic and cellulase-negative strains (15, 14), was subject to Southern hybridization in stringent conditions using the full-length cDNA of *ace1* as a probe (data not shown). *ace1* appeared to be a single-copy gene in the genome. Identical

![Figure 1](http://www.jbc.org/)
bands were detected from all the strains studied (see under "Experimental Procedures"), indicating that deletion, duplication, or any major rearrangement of the ace1 activator locus is not responsible for the different amounts of cellulase enzymes produced by the cellulase-negative or -overproducing strains.

In order to clone the genomic copy of the ace1 gene, a chromosomal cosmid library of T. reesei was screened using a PCR fragment of the coding sequence as a probe in colony hybridization. The genomic gene (Fig. 2) was subcloned, and sequencing revealed three introns of 229, 63, and 65 bp. The first intron is located 5' to the predicted protein coding region. The 5' noncoding region of ace1 does not appear to contain a TATA box, but a CT-rich sequence is present immediately upstream of the cDNA start site. Nine putative binding sites for the glucose repressor protein CREI, 5' SYGGRG, are present within the sequenced 1-kb promoter region.

ACEI Protein Binds to the cbh1 Promoter in Vitro and in Vivo—Because the cbh1 promoter region used in the initial screening was about 1.15 kb, further experiments were required to map more precisely the region to which ACEI binds. The putative DNA binding domain of ACEI (ACEI382–582) was produced in E. coli as a GST fusion for in vitro protein-DNA binding studies. At the beginning, four cbh1 promoter fragments about 300 bp each were amplified by PCR and assayed for binding to the GST-ACEI382–582 fusion protein. GST-ACEI382–582 bound to fragments from 2133 to 2392 (fragment 1), 2621 to 2941 (fragment 2), and 21116 to 21420 (fragment 4), but not to 21177 to 2886 (fragment 3). The best results were obtained with the region 2621 to 2941 (fragment 2) (data not shown). Based on DNA sequence features, it was assumed that this region in the cbh1 promoter could be especially important for the regulation of the activity of the cbh1 promoter.

**FIG. 2.** DNA sequence of the ace1 gene and the deduced amino acid sequence. The sequences found in the cDNA are in uppercase, and the nontranscribed regions and introns are in lowercase. The amino acids corresponding to the three zinc fingers are underlined, and the zinc-coordinating Cys and His residues are double underlined. The predicted bipartite nuclear targeting signal is indicated (*). The regions predicted to be α-helical with the possibility of forming an amphipatic region are indicated by dotted underlining. The first methionine, Met263, in the original ace1 clone sufficient for activation in yeast is shown in boldface. The first intron contains an ATG codon and an open reading frame corresponding to 32 amino acids. The nucleotide sequence appears in the GenBank sequence data base with the accession number AF190783.
because it contains repeated nucleotide motifs that are possible targets for transcription factors. There are, for example, three GTGGGG repeats that are binding sites for CREI and mediate glucose repression (9), three CCAAT repeats, two GGCAA repeats, and three GGCTAA repeats. The following gel mobility shift assay results indicated that the GST-ACEI382–582 protein bound in vitro to the 170-bp cbh1 promoter fragment 2P, corresponding to the region between −843 and −676, and that an overlapping fragment, 2K, corresponding to sequences −763 to −676, did not compete for binding (Fig. 4). It was also shown that GST-ACEI382–582 bound to a 120-bp fragment 2A from −843 to −726 (data not shown). Based on these data, it was concluded that a binding site for GST-ACEI382–582 would be localized between nucleotides −843 and −763.

After having roughly identified regions recognized by GST-ACEI382–582, oligonucleotide probes were prepared for binding assays (Table I). A series of 30–40 bp double-stranded oligonucleotides covering the whole 170-bp 2P region was made. Among these, ACEI382–582 bound to the 36-mer oligonucleotide 151GGCAAA, fragment 1D, but not to any of the four (g/c/t)GGGAAA sequences (Table I, oligonucleotides 3A, 1B, 2J, and 2K; data not shown). The hexanucleotide −151GGGAAA was mutated into −151AATCCC, which abolished binding of GST-ACEI382–582 (Fig. 6, lane 10) indicating that the −151GGGAAA repeat is critical for binding of ACEI382–582. A mutation generated elsewhere in the oligonucleotide did not affect binding of ACEI382–582 (Fig. 6, lane 12). Competition assays with the native and mutated oligonucleotides confirmed the result (Fig. 6, lanes 3–8). Furthermore, ACEI382–582 did not bind to any of the five GGCTAA sequences present in the 1.15-kb cbh1 promoter fragment (Table I, oligonucleotides 1H, 2B, 2D, and 2E; data not shown). A summary of ACEI-binding and nonbinding sequences is shown in Fig. 7.

Most binding reactions described here were performed with both the GST-ACEI382–582 fusion and with the thrombin-cleaved protein preparation releasing the ACEI382–582 from the GST fusion partner. The thrombin-cleaved ACEI382–582-DNA complexes migrated faster in the gel than the larger GST fusions; otherwise, identical binding results were obtained with both protein preparations.

In parallel with the in vitro binding experiments, we applied the yeast one-hybrid system to assess whether ACEI binds in vivo to the defined 170-bp cbh1 promoter region between −843 and −676. This fragment was amplified by PCR, and it was cloned in front of the HIS3 gene (Fig. 8). The resulting target-reporter construct pARO20 expressing the GAL4ad-ACEI382–582 was then transformed into the reporter yeast. The pARO20 yeast transformed with pARO20 grew on SC-Leu-His plates, indicating that the GAL4ad-ACEI382–582 fusion protein bound to the cbh1 promoter target (−843–686) and activated transcription of the HIS3 gene (Fig. 2—continued
8). This result is in accordance with the in vitro binding data. The pPL2 reporter yeast transformed with the control vector pGAD10 expressing the GAL4ad without any DNA binding domain did not grow on the test plates, nor did the negative control strains containing the integrated construct pHisi-1 and pARO20 or pGAD10 (Fig. 8).

Effect of the Disruption of the ace1 Gene—In order to study the role of ace1 in Trichoderma, a strain deleted for the ace1 gene was constructed. The ace1 disruption cassette contained in pAS38 included 2.5 kb of the 5' region and 2.5 kb of the 3' region of the chromosomal ace1 gene, but the protein coding region (except for the last 150 bp) was removed and replaced by the A. nidulans amdS gene. The disruption cassette was transformed into T. reesei, and the transformants were screened for replacement of ace1 by the amdS gene by Southern analysis (data not shown). The desired gene replacement occurred at a frequency of 50%, which indicated that ace1 is not an essential gene.

Whether disruption of ace1 affects the ability of the fungus to grow on cellulose was studied by plating the ace1 disruptants and the host ALKO2221 as single spore colonies on minimal medium containing either glucose or cellulose as the carbon source. The disruptants grew normally on glucose medium as compared with the host, but on cellulose medium, the diameter of ace1 disruptant colonies was smaller than that of the host strain (Fig. 9). Even though the effect of ace1 disruption was observed on cellulose, it is clear that ace1 disruption did not render Trichoderma completely incapable of using cellulose as a carbon source under the conditions tested.

DISCUSSION

We have shown earlier that the production of cellulolytic enzymes from the fungus T. reesei is subject to transcriptional regulation by the available carbon source (12). In glucose-containing media, cellulase genes are repressed by CREI (7, 10). In media containing cellulose or its derivatives, cellulase transcription is very strongly induced. The observations that
on sorbitol or glycerol alone, no cellulase mRNAs were detected and that the addition of small amounts of the known inducer of cellulase genes, sophorose, into sorbitol or glycerol cultures caused strong induction of the genes suggested that a distinct induction mechanism must exist. This prompted us to proceed toward isolation of genes mediating this regulation.

From our previous deletion analysis of the cbh1 promoter, one could not clearly define regions required for activation because no dramatic differences between different deletion derivatives with respect to their inducibility by sophorose were observed (9). Because there was no knowledge of components involved in activation of cbh1 transcription, a method was required that would allow isolation of transcription activator genes without any previous knowledge of the important DNA sequence elements or of the nature of the activator genes and proteins. Our approach selects for both binding and activation properties of the trans-acting factor and uses a large promoter fragment as the target. This enables simultaneous isolation of activators with different binding specificities. Furthermore, the method is likely to yield activators but not repressors or other DNA-binding proteins without activation properties, which is an advantage as compared with alternative screening methods that are based on binding only, such as one-hybrid or Southwestern, and that also require detailed knowledge on the DNA target. A further complication of one-hybrid screening may be caused by the fact that the GAL4 activation domain is at the N terminus of the fusion, and if the activator cDNA contains an in-frame translation stop codon upstream of the first ATG of the cDNA, those clones will be missed. However, the probability of getting such transcription factors, the function of which requires more than one polypeptide encoded by different genes, is very low using our method or the one-hybrid system. We have demonstrated that a full-length promoter can be used in cloning of regulatory genes. The method should be generally applicable for different promoters and organisms. Limitations may be encountered in such cases, where the promoter in question contains functional target sequences for some yeast-encoded factors that dominate the regulation in yeast in such a

![TABLE I](http://www.jbc.org/)

Regions of the cbh1 promoter used in gel mobility shift assays

The 5' and 3' end points in the cbh1 promoter relative to the initiator ATG and the entire coding strand sequences in case of short oligonucleotides are shown. Changes introduced to the oligonucleotides as compared to the native sequence are underlined. Binding to the GST-ACEI<sub>382–582</sub> protein is indicated (+).

| Name | 5' | 3' | Sequence | ACEI binding |
|------|----|----|----------|--------------|
| 1    | −392 | −133 |            | +            |
| 2    | −941 | −621 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 3    | −117 | −886 | TTAATACGACTATAGAGAAGAACGATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 4    | −142 | −1116 |            | +            |
| 2P   | −843 | −676 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2A   | −843 | −726 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2K   | −763 | −667 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2C   | −818 | −780 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 1D   | −172 | −133 | TTAATACGACTATAGAGAAGAACGATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 1C   | −269 | −523 | TTAATACGACTATAGAGAAGAACGATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 1A   | −416 | −378 | TTAATACGACTATAGAGAAGAACGATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 3B   | −1039 | −1001 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 1B   | −337 | −299 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2J   | −722 | −691 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2H   | −754 | −722 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2A   | −1081 | −1043 | CAGTGATGATATAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 1F   | −302 | −264 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 1E   | −395 | −357 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 3D   | −970 | −932 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 4A   | −1235 | −1198 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 4H   | −292 | −184 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 5A   | −532 | −494 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2G   | −772 | −737 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2D   | −789 | −764 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |
| 2B   | −843 | −809 | AATGACATAGTAATGCACATTAGGAGTTGCCTCGACGGTTGCAATGCAG + |

Mutated oligonucleotides
way that leakage from the promoter occurs or that the activator function of the searched component is prevented.

ace1 was isolated on the basis of the ability of its translation product to activate transcription from the cellulase cbh1 promoter in yeast. In addition to ace1, a second activator gene, ace2, was isolated using the same approach.4 ace1 is a novel gene encoding a DNA-binding protein that belongs to the Cys2-His2 class of transcription factors. It is the first reported Cys2-His2 zinc fingers of higher eukaryotes, which often contain few of them contain more than two fingers, in contrast to Cys2-His2 proteins that have Cys2-His2 zinc fingers and two more unusual ones. The yeast genome contains approximately 50 proteins that have Cys2-His2 zinc fingers (30), and relatively few of them contain more than two fingers, in contrast to Cys2-His2 proteins of higher eukaryotes, which often contain several fingers. The yeast genome did not appear to contain a homologue of ace1. However, ace1 homologues were identified among A. nidulans and N. crassa expressed sequence tag sequences, suggesting that ACEI might be a regulatory protein specific for filamentous fungi.

According to the in vitro binding data, there are at least eight binding sites for ACEI in the cbh1 promoter. ACEI recognized all AGGCCAAA sites and some AGGCA sites preceded by a relatively A-T rich region. It is possible that other variants of the binding sequence exist and will be found. One or very closely related sequences occur, e.g. in the promoters of T. reesei cellulase genes egdl, egl5, and cbh2 and the xylanase gene xynl. Our results also showed that the disruption of the ace1 gene caused retardation in the radial growth of the colonies on cellulose-containing plates representing conditions under which cellulosytic enzymes need to be formed in order to enable growth. However, growth was not totally prevented, which indicates that significant cellulase expression occurs even in the absence of ace1. It is not possible to conclude yet to what extent ace1 regulates cellulase expression and what is the contribution of each of the several putative binding sites in the cbh1 promoter. The data are combined from gel shift assays. The GGC triplet found in all oligonucleotides in one strand is aligned centrally, and the 5' and 3' end points relative to the initiator ATG are shown. G and C nucleotides are shaded in dark gray, A nucleotides in medium gray, and T nucleotides in light gray. The complete sequences of the oligonucleotides are shown in Table I.
Cellulase Regulator ace1 of T. reesei

Acknowledgments—We warmly thank Seija Nordberg for skilled technical assistance. We also thank Pia Mannström for help in the construction of the pPL2 yeast and Markku Saloheimo for the pMS95 plasmid.

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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*

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*J. Biol. Chem.* 2000, 275:5817-5825.  
doi: 10.1074/jbc.275.8.5817

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