Two Adjacent Protein Binding Motifs in the cbh2 (Celluliohydrolase II-encoding) Promoter of the Fungus Hypocrea jecorina (Trichoderma reesei) Cooperate in the Induction by Cellulose*  

Susanne Zeilinger‡, Robert L. Mach, and Christian P. Kubicek  

From the Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, Technische Universität Wien, Getreidemarkt 9/1725, A-1060 Wien, Austria  

The cellulase system of the filamentous fungus Hypocrea jecorina (Trichoderma reesei) consists of several celluliohydrolases, endoglucanases, and β-glucosidases, encoded by separate genes, which are coordinately expressed in the presence of cellulose or the disaccharide sophorose. Using cell-free extracts from sporohase-induced and noninduced mycelia and various fragments of the cbh2 promoter of H. jecorina in electrophoretic mobility shift assay (EMSA) analysis and performing in vitro and in vivo footprinting analysis, we detected the nucleotide sequence 5'-ATTGGGTAATA-3' (consequently named cbh2-activating element (CAE)) to bind a protein complex with different migration in EMSA of induced and noninduced cell-free extracts. EMSA analysis, employing oligonucleotide fragments containing specifically mutated versions of CAE, revealed that this protein binding requires the presence of an intact copy of either one of two adjacent motifs: a CCAAT (=ATTGG) box on the template strand and a GTAATA box on the coding strand, whereas a simultaneous mutation in both completely abolished binding. H. jecorina transfectants, containing correspondingly mutated versions of the cbh2 promoter fused to the Escherichia coli hph gene as a reporter, expressed hph in a manner parallelizing the efficacy of CAE-protein complex formation in EMSA, suggesting that the presence of either of both motifs is required for induction of cbh2 gene transcription. Antibody supershift experiments with anti-HapC antiserum as well as EMSA competition experiments with CCAAT binding promoter fragments of the Aspergillus nidulans amdS promoter suggest that the H. jecorina CCAAT box binding complex contains a homologue of HapC. The nature of the adjacent, GTAATA-binding protein(s) and its cooperation with the HapC homologue in cbh2 gene induction is discussed.

Cellulose is the most abundant renewable carbon source on earth. Its recycling in nature occurs in a variety of habitats by the action of various pro- and eukaryotic microorganisms, which hydrolyse this homopolymer with extracellular enzyme systems (1). Among the best characterized of these is the cellulase system of the saprophytic Ascomycete Hypocrea jecorina (=Trichoderma reesei), because the teleomorph form of T. reesei has now been identified by Kuhl et al. (2), we prefer to name the organism accordingly, which contains three classes of enzymes (3). 1,4-β-D-Glucan celluliohydrolases (CBH I and II; EC 3.2.1.91), which cleave cellobioyl units from the nonreducing end of cellulose chains; endo-β-1,4-glucanases (EG I, EG II, EG III, and EG V; EC 3.2.1.4), which cleave internal glucosidic bonds; and 1,4-β-d-glucosidases (BG I, BG II; EC 3.2.1.21), which cleave cellobioigosaccharides to produce glucose are the most prominent members.

H. jecorina cellulases are not formed during growth on easily metabolizable carbon sources but induced in the presence of cellulose (4). The mechanism by which this extracellular, insoluble polysaccharide triggers the biosynthesis of cellulases has been investigated for decades. Some workers claim a role for low constitutive levels of cellulases in the initial attack on cellulose, thereby releasing the inducer (5, 6), whereas others presented evidence for an involvement of carbon catabolite derepression (7) or of conidial-bound cellulases (8) in this process. The nature of the actual inducer is still unknown, but depending on the conditions, either the cellulolytic end product celllobiose itself or its transglycosylation product sophorose induces cellulase formation in pregrown mycelia (3, 9, 7). Despite the progress that has been made in the cloning of genes encoding several H. jecorina cellulases (10–14), little information is available about the transcriptional regulation of their induction. More recently, two laboratories described a nucleotide region, which is located closely upstream of the TATA box, to be necessary to trigger sophorose induction of cbh1 (celluliohydrolase I-encoding) gene transcription (15, 16), but neither the binding motif nor the proteins binding to it have as yet been characterized.

We have previously identified the area between −361 and −170 in the 5′ regulatory sequences of the cbh2 (celluliohydrolase II-encoding) gene to be able in vitro to form a sophorose-specific DNA-protein complex (17). Starting from these findings, we will show in this paper that within this area, a 5'-ATTGGGTAATA-3' 9-decamer (that we will call "cbh2-activating element") (CAE)1 is responsible for the observed complex formation in vitro and essential for cbh2 gene expression in vivo. Evidence will also be presented that a CCAAT box-binding protein (complex) and another yet unknown protein bind to adjacent sites within this motif.

EXPERIMENTAL PROCEDURES

Microbial Strains and Plasmids—T. reesei (H. jecorina) QM9414 (ATCC 26921) was used throughout this study. T. reesei (H. jecorina) TU-6 (18), a pyr4 negative mutant of QM9414, was used as a recipient strain for pyr4-mediated cotransformation experiments. The strains were maintained on malt agar (containing 5 mM uridine in the case of

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‡ To whom correspondence should be addressed. Tel.: +43 1 55801 17252; Fax: +43 1 5581 62 66; E-mail: szeilinger@mail.zserv.tuwien.ac.at.

1 The abbreviations used are: CAE, cbh2-activating element; bp, base pairs; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; MES, 4-morpholineethanesulfonic acid.
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| Name          | Sequence                                                                 | Comments                  |
|---------------|--------------------------------------------------------------------------|---------------------------|
| P1c           | 5'-TGA AGC ATC ATC AGG TGG TAT T-3'                                       | (from -124 to -148)       |
| P2c           | 5'-GTA TCA GGG AGC ACA GGA TTA AGC C-3'                                   | (from -144 to -168)       |
| P3c           | 5'-GGG GCA CAG GAT AAA GCC CGG AGA GT-3'                                 | (from -151 to -176)       |
| P1n           | 5'-ATC TCT ATT GCA TTT CCC TTT T-3'                                     | (from -394 to -379)       |
| P2n           | 5'-TCT TGC CCT TCC CAC TAA ATC AGG CTT-3'                                | (from -378 to -357)       |
| P3n           | 5'-TGG CCT TCC CAC TAA ATC ATG AGG CGT C-3'                              | (from -375 to -353)       |

TABLE I

Oligonucleotides used throughout this study.

Optimal lengths of the respective promoter are given; lowercase letters indicate bases added for labeling or generating restriction enzyme sites; underlined bases indicate introduced point mutations.

| Name (from) | Sequence | Comments                  |
|-------------|----------|---------------------------|
| CKT 005     | 5'-atg gat cca TGG AAC GAC AGG GTA ATC TTA C-3'                           | (from -0 to -27)          |
| CKT 057     | 5'-att cgc TCT TTC ATT GGG TAA TAT ACA GCA AGG CGG G3'                   | (from -253 to -220)       |
| CKT 058     | 5'-tct ccc CGG CCT GCT GAT ATA TTA CCC AAT AAA GAA GC3'                  | (from -220 to -253)       |
| CKT 063     | 5'-tat ccc TCT TTC ATT GGG TAA TAT ACA GCA AGG CGG G3'                   | (from -220 to -253)       |
| CKT 065     | 5'-tat ccc CGG CCT GCT GAT ATA AAT CCC AAT AAA GAA GC3'                  | (from -220 to -253)       |
| CKT 066     | 5'-tat ccc CGG CCT GCT GAT ATA TTA CCC AAT AAA GAA GC3'                  | (from -220 to -253)       |
| CKT 067     | 5'-tat cca CTC CAT TTA AAG GGC ACT TCA ACC AGC TTC-3'                    | (from -243 to -289)       |
| CKT 068     | 5'-tat cga AGC TGG TTA AAT GGC CCT TTA ACA TGT GGA GTG-3'                 | (from -249 to -283)       |
| CKT 069     | 5'-tat cgg GTA TAC AGC CAG GCG GGG ATG AAG CTC ATT AGC C3'                | (from -242 to -284)       |
| CKT 070     | 5'-tat cgg TAC AGC CAG GCG GGG ATG AAG CTC ATT AGC C3'                    | (from -242 to -284)       |
| CKT 083     | 5'-tat ccc TCT TTC AAA GGG TTT TAT ACA GCA AGG CGG G3'                   | (from -253 to -219)       |
| CKT 084     | 5'-tat ccc CGG CCT GCT GAT ATA AAA CCC TTT AAA GAA GC3'                  | (from -219 to -253)       |
| OamDS(F)    | 5'-cat CGT ATT CGG CGA AGC GCC TCA CCA GCC GCT AGC CA3-3'                 | (from -160 to -119)       |
| OamDS(R)    | 5'-tag CTG GTC AGG CCT CTA AAC ATG AGC AAC GCC GCT G3'                   | (from -215 to -237)       |
| OxIDen1(F)  | 5'-cat ctc CGG GGC GGT TAG CAT CTA GCA TTA ACC CCC CGG A3-3'              | (from -159 to -124)       |
| OxIDen1(R)  | 5'-cat ctc CGG GGC GGT TAG CAT CTA GCA TTA ACC CCC CGG A3-3'              | (from -159 to -124)       |

Construction of pSMZ Reporter Plasmids—The pSMZ reporter plasmids were developed from plasmid pLRM330 and contain the E. coli *hph* (hygromycin B-phosphotransferase-encoding) gene fused to the *H. jecorina* cbh2 promoter and 249 bp of the *cbh2* promoter fragment to create a more optimal reporter plasmid. The resulting plasmids were verified by double strand sequencing by the dideoxynucleotide chain termination method. All restriction enzyme sites were digested with appropriate restriction endonucleases and the plasmids were circularized by using T4 DNA ligase. The resulting plasmids were electroporated into E. coli DH5α and transformed into *H. jecorina* by using the electrochemical method of Grabau and Mandels (4). Mycelia were grown on a 1-liter Erlenmeyer flask containing a defined medium lacking carbon source to give a final density of 0.7–1.5 g dry weight/liter. Either soxophore (final concentration 2 mM) or gentamicin (final concentration 1%, w/v) was added, and incubation was carried out for further 3 or 5 h, respectively.

From the resulting plasmids, the cbh2 promoter fragments were excised using restriction enzymes *XhoI* and *BstXI* and inserted into pSMZ1, thereby generating the cbh2 wild-type promoter sequences. All vectors were verified by double strand sequencing by the dideoxynucleotide chain termination method (23), and the restriction enzyme sites generated were proven via restriction enzyme analysis.

**Isolation and Manipulation of Nucleic Acids—** Genomic DNA and mRNA were isolated as described by Gruber et al. (18) and Chomczynski and Sacchi (24), respectively. After electrophoretic separation, RNA was blotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech) and hybridized according to standard protocols (25) at 42°C for 20 h. Washing was performed with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) + 0.1% SDS at 42°C (2× 15 min). Standard methods were used for plasmid isolation, restriction enzyme digestion, random priming and Southern analysis (25). All PCR amplifications were performed by the aid of Taq polymerase (Promega, Madison, WI) in a Biometra thermocycler.

**DNA Transformations—** *E. coli* transformations were carried out according to standard techniques (25). Transformation of *T. reesei* (*H. jecorina*) TU-6 was carried out as described by Gruber et al. (26) using co-transformation of pFG1 with linear 2.6-kb *HindIII/EcoRI* fragments of the pSMZ vectors (Fig. 4A).

**Determination of Gene Copy Number and Integration Locus in Transformants—** Southern hybridization was conducted as described by Sambrook et al. (25). Chromosomal DNA was digested with EcoRI, and hybridization was performed using a 862-bp *EcoRI* fragment of the vector pSMZ1 bearing 613 bp of the *cbh2* promoter and 249 bp of the *hph* gene labeled with α-32PdCTP. Signals observed upon fluorography for the single-copy gene *cbh2* and for intact, integrated vector copy numbers, respectively, were calculated by image analysis. Copy numbers were corrected by including the length of the labeled probe. The copy number was divided by the values observed by image analyses through the length of the labeled probe. To confirm the point mutations in the *cbh2* promoter (generating a Dral restriction enzyme site) in transformants bearing the pSMZ2 and pSMZ4 plasmids, chromosomal DNA was digested with Dral/XhoI/ClaI, and hybridization signals were observed on autoradiograms as shown in Fig. 4A, which were directly cloned in vector pCR 2.1 using the TA Cloning Kit (Invitrogen). From the resulting plasmids, the cbh2 promoter fragments were excised using restriction enzymes *XhoI*/*BstXI* and inserted into pSMZ1, thereby generating the cbh2 wild-type promoter sequences. All vectors were verified by double strand sequencing by the dideoxynucleotide chain termination method (23), and the restriction enzyme sites generated were proven via restriction enzyme analysis.
was performed with a 2.6-kb XhoI/HindIII fragment of the vector psMZ1 bearing the \( cbh2 \) promoter and terminator sequences and the \( hph \) structure gene labeled with [\( ^{32}P \)]dCTP.

**Hygromycin B Resistance Assay—**Transformants were analyzed for hygromycin B resistance by means of a plate assay essentially as described by Mueller and Wold (30).

**Electrophoretic Mobility Shift Assay (EMSA)—**DNA fragments used for EMSA were generated as follows. A \( cbh2 \) fragment containing the regulatory nucleotide regions from \(-263 \) to \(-170 \) was prepared by PCR amplification of a 613-bp fragment of the \( cbh2 \) upstream regulatory sequences using primers pbchI2F and pbchR2 and subsequent digestion with \( HpaII \). A 94-bp fragment was isolated after end-labeling with [\( ^{32}P \)]dCTP using Sequenase version 2.0 (Amersham Pharmacia Biotech). After purification by nondenaturing polyacrylamide gel electrophoresis, binding was achieved by incubating 100 ng of cell-free extracts with 5 ng of labeled fragment on ice for 10 min. Preparation of cell-free \( H. \) jecorina extracts following growth on various carbon sources was carried out as described previously (17). Because maximal accumulation of \( cbh1 - \) and \( cbh2 \) mRNA was observed after 5 h of incubation with sophorose, we prepared protein extracts from mycelia harvested after 3 h. The binding assay and acrylamide gel electrophoresis have been described previously (17). For competition experiments, synthetic oligonucleotides were used in a 10-, 50-, or 150-fold molar excess. The oligonucleotides were annealed with the complementary oligonucleotide as described by Strauss et al. (28). After annealing, double strands were filled in using Sequenase version 2.0 (Amersham Pharmacia Biotech). Oligonucleotides used for EMSA were also annealed with their complementary oligonucleotides and end-labeled using Sequenase version 2.0 and [\( ^{32}P \)]dCTP as described above. The resulting double-stranded oligonucleotides were purified by nondenaturing polyacrylamide gel electrophoresis, and binding was achieved by incubating 100 ng of cell-free extracts with 25 ng of labeled oligonucleotide. For supershift assays 5 \( \mu \)l of polyclonal anti-HapC antiserum were added to the binding reaction.

**In Vitro Footprinting Procedure—**In vitro methylation protection and hydrazine interference footprinting was performed with a 191-bp DNA fragment containing upstream regulatory sequences of the \( cbh2 \) gene from \(-367 \) to \(-171 \). The fragment was generated by digestion of a 613-bp PCR product with \( HpaII \), and the resulting 333-bp fragment was isolated and end-labeled on the coding strand using Sequenase version 2.0 (Amersham Pharmacia Biotech) and [\( ^{32}P \)]dATP (Amersham Pharmacia Biotech). After nondenaturing polyacrylamide gel electrophoresis, binding was achieved by incubating 100 ng of cell-free extracts with 25 ng of labeled oligonucleotide. For supershift assays 5 \( \mu \)l of polyclonal anti-HapC antiserum were added to the binding reaction.

**In vitro** methylation protection footprinting was performed by incubating \( 3 \times 10^{9} \) counts of end-labeled DNA fragment with 100 ng of cell-free extract as described above, methylating with dimethylsulfate, and subjecting the mixture to EMSA analysis. After separation by native gel electrophoresis, complexes were visualized by autoradiography, excised, eluted, purified, and cleaved with piperidine (G) or 0.5 M HCl, 0.1 M NaOH, 1 mM EDTA (A/G) as described previously (29). After resuspension in loading buffer, the samples were applied to denaturing polyacrylamide gel electrophoresis on a 6% sequencing gel as described by Strauss et al. (28).

In vitro hydrazine interference footprinting was performed by incubating 300,000 counts of end-labeled, hydrazine-treated DNA fragment (25) with 100 \( \mu \)g of cell-free extract and separating the bound and free probes by native polyacrylamide gel electrophoresis. After elution and purification, the samples were cleaved with piperidine, suspended in loading buffer, and loaded on a 6% sequencing gel.

**In Vivo Genomic Footprinting via Ligation-mediated PCR—**To precisely identify the protein binding sequence, we performed **in vivo** footprinting with cell-free extracts of \( H. \) jecorina: methylation protection demonstrated protein contact with G\( ^{236} \) of the coding strand (Fig. 2C). In addition, the involvement of T\( ^{238} \), T\( ^{239} \), and T\( ^{240} \) on the template strand was shown by hydrazine interference (Fig. 2, A and C). Interestingly, despite the different mobility of the protein-DNA complexes from induced and noninduced mycelia, essentially the same protection/interference pattern was observed under both conditions.

To investigate whether the observed binding in **in vitro** actually reflects the binding conditions in **in vivo**, we performed **in vivo** genomic footprinting by the aid of ligation-mediated PCR of the respective area of the \( cbh2 \) upstream regulatory region. To this end, methylated DNA was isolated from mycelia, treated with HCl, and cleaved with NaOH (29) before the respective PCR amplification steps. Thereby, several purine bases on the coding strand within and in the direct neighborhood of the region previously identified in **in vitro** were identified to be involved in protein-DNA contact (Fig. 2B); on the template strand, no protection of As within the identified motif could be detected. Consistent with the results from **in vitro** footprinting, essentially the same protection pattern was observed under all conditions tested.

Summarizing the **in vitro** and **in vivo** data, we therefore propose that the nucleotide area between −246 and −236 is

**RESULTS**

**Delimitation of the \( cbh2 \) Promoter Area, Which Binds a Protein Complex Unique for Sophorose Induction—**When \( H. \) jecorina is pregrown on a carbon source not inducing cellulase formation (glycerol) and then replaced to a medium containing the inducer sophorose, a transient accumulation of \( cbh1 \) and \( cbh2 \) mRNA is triggered (Fig. 1A). This pattern of expression is also paralleled by that of transcript formation of other cellulase-encoding genes (e.g. eglI and egl2; data not shown). Fusion of 613 bp of the upstream regulatory region of the \( cbh2 \) gene to the \( E. \) coli hygromycin B phosphotransferase (\( hph \)) gene as a reporter mimicked this induction (Fig. 1A), thus providing evidence for a sophorose-responding element within these 613 bp. Within these 613 bp, a 191-bp fragment has previously been identified to form a unique complex with proteins isolated from sophorose-induced or glucose-grown mycelia (17). To further delimit this area, we prepared extracts from cultures replaced on glucose or sophorose and subjected them to EMSA with three smaller DNA fragments spanning this area. Hereby, the binding ability was retained by a 94-bp fragment, spanning from −263 to −170 (Fig. 1B and C). Consequently, this fragment was further analyzed by competition experiments using three synthetic oligonucleotides spanning overlapping parts of this region (Fig. 1, B and C). Competition was only achieved with CKT 057, whereas oligonucleotides CKT 067 and CKT 069 were unable to specifically compete (Fig. 1, B and C), suggesting that the area between −250 and −243 was essential for complex formation. Hence sophorose-specific protein binding seems to be confined to a relatively short motif within the 613 bp.

**Identification of the CAE—**To precisely identify the protein binding sequence, we performed **in vitro** footprinting with cell-free extracts of \( H. \) jecorina: methylation protection demonstrated protein contact with G\( ^{244} \) of the coding strand (Fig. 2C). In addition, the involvement of T\( ^{238} \), T\( ^{239} \), and T\( ^{240} \) on the template strand was shown by hydrazine interference (Fig. 2, A and C). Interestingly, despite the different mobility of the protein-DNA complexes from induced and noninduced mycelia, essentially the same protection/interference pattern was observed under both conditions.

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Summarizing the **in vitro** and **in vivo** data, we therefore propose that the nucleotide area between −246 and −236 is
responsible for the binding of the protein complex.

CAE Consists of Two Adjacent, Cooperating Protein Binding Motifs—Visual inspection of the identified nucleotide region revealed no major similarities to known protein binding motifs with two exceptions: one is a CCAAT box on the template strand (=ATTGG, at −246 to −242); the other is the sequence GGTTTAAT (at −243 to −237), which bears some similarity to the 5′-GGCTAAA-3′ motif recently shown to bind the xylanase regulator XinR of Aspergillus niger (33). To test whether any of these two motifs may be involved in protein binding, we introduced point mutations into these two motifs and compared the effect of these mutations on protein binding by EMSA (Fig. 3, A–D). To this end, we replaced the two As in the CCAAT box by Ts, because this mutation has been shown to impair binding of the Hap complex to the CCAAT motif in the A. nidulans amdS promoter (34). The GGGAATTAAT motif, on the other hand, was mutated to GGTTTT because of the strong protection of the two As in footprinting experiments. Interestingly, competition experiments with either of the single mutated oligonucleotides alone (CKT 063 and CKT 065) still exerted a very strong effect on binding of cell-free extracts to the wild-type 94-bp promoter fragment. A significant reduction in competition could only be observed when both motifs were simultaneously mutated (CKT 083; Fig. 3A). Consistent results were also obtained when oligonucleotide CKT 057 was used as a labeled probe in EMSA with the same competitors as in Fig. 3A (Fig. 3B). To directly compare the effect of these mutations on protein binding, all four oligonucleotides were labeled and directly used as probes for EMSA (Fig. 3C). In these experiments, two specific DNA-protein complexes were detected with the wild-type promoter (CKT 057). Substitution of CCTTTT for GGAA (Fig. 3A) resulted in a complete loss of specific protein-DNA complex formation (Fig. 3C). It is intriguing that the separation into two defined complexes, which is because of the shorter DNA fragment used, was not observed with the 94-bp promoter fragment. Combining the results from Fig. 3, A, B, and C, we conclude that CAE is simultaneously contacted by several DNA-binding proteins binding to two different cooperating motifs.

Mutations Impairing Protein Binding in Vitro Lead to a Loss of cbh2 Gene Transcription in Vivo—To analyze whether the identified motifs actually confer inducibility of cbh2 transcription in vivo, a set of reporter constructs consisting of the 613-bp of the 5′-noncoding region of the cbh2 promoter fused to the hph structural gene from E. coli were made. The different constructs were essentially identical with the exception of the point mutations introduced, which exactly resembled those used for the analysis of protein-DNA complex formation in vitro (pSMZ2 = CKT 065; pSMZ4 = CKT 083; pSMZ5 = CKT 063; cf. Figs. 3D and 4A). Reporter cassettes derived from plasmids pSMZ1–pSMZ5 were introduced into H. jecorina by cotransformation with pFG1 (26). For each construct, 30 pyr+ transformants were purified to mitotic stability, and their DNA was isolated and examined for integration and copy number of the respective reporter cassette by Southern analysis. Cotransformation frequency varied between 30 to 50%, leading to 9 (pSMZ1), 10 (pSMZ2), 13 (pSMZ4), and 15 (pSMZ5) stable transformants per construct, respectively. All obtained transformants exhibited copy numbers from 1 to 4 copies, where the reporter cassette had integrated into ectopic loci. They were examined for the expression of hygromycin B resistance on plates containing cellulose or glucose as carbon sources, respectively. The observed results, taking into account the copy number of the integrated reporter cassette, pointed to a weak influence of mutations present in pSMZ2 and pSMZ5 (less than 20%), whereas the mutation of both motifs in pSMZ4 led to a complete loss of inducibility of the hph reporter gene (data not shown).

To directly demonstrate the effect of these mutations on the induction of transcription from the cbh2 promoter, selected transformants were induced by sophorose and subjected to Northern analysis. Consistent with the results from hygromycin-
Fig. 2. Identification of nucleotides contacted by DNA-binding proteins using in vitro and in vivo footprinting techniques. A, in vitro footprinting analysis using hydrazine interference technique of the template strand with cell-free extracts from cultures grown in the presence of glucose (Glu) or sophorose (Sop) and harvested 3 h after the addition of sophorose and 5 h after the addition of glucose. F(G/T) indicates DNA not incubated with cell-free extract. Bases involved in protein/DNA contact are indicated by +. B, in vivo genomic footprinting of the coding strand with DNA methylated in vivo and treated with HCl and cleaved with NaOH from cultures grown in the presence of glycerol (Gly), glucose (Glu), or sophorose (Sop). Control DNA methylated in vitro and cleaved with piperidine or HCl/NaOH are given as F(G) and F(A/G), respectively. Protected bases involved in protein/DNA contact are indicated by +, C, summary of contacted bases on both strands. Bases found by in vitro footprinting techniques to be involved in protein/DNA contact are indicated by + (hydrazine interference) and Δ (methylation protection), whereas bases found to be protected by in vivo footprinting technique are indicated by +.

Fig. A

A

B

C

Does a XlnR homologue Bind to the 3′ Motif of CAE?—The demonstration of binding of a HapC homologue to the 5′ part of CAE supports the assumption that the observed binding involves two different proteins rather than a single one contacting the whole area. As the 3′ area, as explained before, bears some resemblance to the proposed binding sequence for the xylanase regulator XlnR (33), we also tested whether a respective H. jecorina homologue may be involved in this binding. To this end, we tested whether the binding of protein extracts to oligonucleotide CKT 057 could be competed by a respective fragment of the A. niger xlnD promoter (xlnD(wt)), for which XlnR binding has been demonstrated in vivo and in vitro (33) and which, with exception of the GGCTAAA sequence, does not contain any nucleotide similarity to CKT 057. However, even at a high excess (50-fold) of xlnD(wt), only a low degree of competition was observed (Fig. 6). To test whether this low degree of competition was specific at all, we repeated the experiment with a mutated version of the competing oligonucleotide, for which a loss of XlnR binding had been demonstrated (33). In our case, we again observed a weak competition at a 50-fold excess of the competitor xlnD(mut), (Fig. 6) and thus, the mutation has no effect. From these results we conclude that binding to the 3′ area of CAE is not because of a protein with binding characteristics similar to XlnR.

**DISCUSSION**

In this study, we have used in vitro and in vivo techniques to identify the nucleotide area in the cbh2 promoter with the sequence 5′-ATTGGCAATTA-3′ (CAE), which is responsible for binding of protein complexes from cellulase-forming (induced) and nonforming (not-induced) mycelia of H. jecorina and which is essential for induction of gene expression by cellulase and sophorase in vivo. Results from the application of different in vitro and in vivo techniques point to this region to be essential for this process. To the best of our knowledge, this is the first time that a cellulase-responsive nucleotide region has been verified in any cellulase-encoding gene, and these results could therefore become a basis for deeper investigations into the

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**Fig. A**

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**DISCUSSION**

In this study, we have used in vitro and in vivo techniques to identify the nucleotide area in the cbh2 promoter with the sequence 5′-ATTGGCAATTA-3′ (CAE), which is responsible for binding of protein complexes from cellulase-forming (induced) and nonforming (not-induced) mycelia of H. jecorina and which is essential for induction of gene expression by cellulase and sophorase in vivo. Results from the application of different in vitro and in vivo techniques point to this region to be essential for this process. To the best of our knowledge, this is the first time that a cellulase-responsive nucleotide region has been verified in any cellulase-encoding gene, and these results could therefore become a basis for deeper investigations into the

**Fig. 2. Identification of nucleotides contacted by DNA-binding proteins using in vitro and in vivo footprinting techniques.** A, in vitro footprinting analysis using hydrazine interference technique of the template strand with cell-free extracts from cultures grown in the presence of glucose (Glu) or sophorose (Sop) and harvested 3 h after the addition of sophorose and 5 h after the addition of glucose. F(G/T) indicates DNA not incubated with cell-free extract. Bases involved in protein/DNA contact are indicated by +. B, in vivo genomic footprinting of the coding strand with DNA methylated in vivo and treated with HCl and cleaved with NaOH from cultures grown in the presence of glycerol (Gly), glucose (Glu), or sophorose (Sop). Control DNA methylated in vitro and cleaved with piperidine or HCl/NaOH are given as F(G) and F(A/G), respectively. Protected bases involved in protein/DNA contact are indicated by +, C, summary of contacted bases on both strands. Bases found by in vitro footprinting techniques to be involved in protein/DNA contact are indicated by + (hydrazine interference) and Δ (methylation protection), whereas bases found to be protected by in vivo footprinting technique are indicated by +.

**Fig. A**

A

B

C

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molecular biology of cellulase induction.

Results from mutational analysis of CAR are consistent with the assumption that it consists of two adjacent and functionally cooperating binding sites for transcriptional activators. Indirect evidence suggests that one of these involves a H. jecorina homologue of the CCAAT box binding protein HapC. The
CCAAT motif is a common cis-acting element found in the promoter and enhancer regions of a large number of genes from eukaryotes, including filamentous fungi, and a number of different nuclear proteins have been found to interact with the CCAAT sequence (40). However, with respect to fungi, the only CCAAT box binding protein characterized in detail is the *Saccharomyces cerevisiae* Hap complex, which consists of at least three subunits: Hap2p, Hap3p, and Hap5p (41, 42). In filamentous fungi, *A. nidulans* hapC and *Neurospora crassa* aab-1, which exhibit significant similarity to the *S. cerevisiae* HAP3 and HAP5 genes, respectively, are so far the only functionally homologous genes known (37, 43). HapC has been shown to bind to CCAAT boxes present in the promoters of genes *amdS*, *gatA*, *taaG2*, *yA*, *pcbAB*, and *pcbC* (37–39, 44, 45). It is interesting to note that functional inactivation of the CCAAT motif in these promoters has been leading to an altered efficacy of expression of the respective genes but not to a loss of their regulation. HapC has therefore been considered to be only responsible for basal expression (37). On a first glance, this compares well with the results from the present study, in which a mutation in the CCAAT box alone only slightly decreased transcription from the *cbh2* promoter. However, our data with *H. jecorina* show that the presence of the CCAAT box becomes essential for transcription if the adjacent, 3'-located protein binding motif is mutated, and vice versa, a mutation in the CCAAT element is balanced by binding via the adjacent protein binding motif. The fact that a mutation in the CCAAT box of *H. jecorina cbh2* does not lead to a change in the size of the bound

**Fig. 4.** Effect of mutations in the *cbh2* activating element on induction of *cbh2* gene expression. A, schematic representation of the reporter cassette used for transformation. Point mutations are indicated by *arrows*. B, Northern analysis of hygromycin B phosphotransferase (*hph*) gene transcript formation in SMZ1, SMZ2, SMZ4, and SMZ5 strains grown in the presence of glucose (G) or sophorose (S); C, densitometric calculation of formed transcript of the *hph* reporter gene in relation to *act1*. 20 μg of total RNA were loaded. Hybridization with the actin gene (*act1*) and the ethidium bromide-stained gel are given as loading controls. The same blot was used for both hybridizations after stripping the membrane.

**Fig. 5.** The CCAAT motif is bound by a protein complex containing a HapC-like component. A, effect of anti-HapC antiserum (38) on the mobility of protein-DNA complexes obtained with the labeled 94-bp *cbh2* promoter fragment and cell-free extracts from cultures grown in the presence of glucose (G) or sophorose (S). The addition of 5 μl of antiserum is indicated by +. B, EMSA analysis using labeled oligonucleotide CTK 057 and cell-free extracts are as described in panel A. In competition experiments, unlabeled oligonucleotide OamdS (Table I) derived from the promoter region of the *amdS* gene of *A. nidulans*, containing a CCAAT box that was shown to bind an *Aspergillus* Hap-like complex (34, 37), was added to the binding assay in a 50-fold molar excess.

**Fig. 6.** Analysis of the involvement of a XlnR-like factor in binding to the *cbh2* activating element. EMSA analysis with labeled oligonucleotide CTK 057 as a probe and 100 μg of cell-free extract of *H. jecorina* cultures grown in the presence of glucose (G) or sophorose (S) is shown. In competition experiments a 10- or 50-fold molar excess of unlabeled oligonucleotide xlnD(wt) and xlnD(mut), respectively, was added to the binding reaction where indicated. F, free probe.
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proteins indicates an interaction between the proteins binding to the CCAAT box and the GTAAT motif, stressing that binding to either of these motifs is apparently sufficient for functional complex formation. Whether this mechanism may be a general feature of fungal CCAAT boxes remains a matter of speculation so far. However, the presence of a binding site for a transcriptional regulator adjacent to the CCAAT box as observed in this study has been shown in the A. nidulans amdS, gataA, and pcbAB/pebC promoters (34, 45). In higher eukaryotes, the Hap homologue NF-Y was in some cases shown to promote transcription by stabilizing or recruiting the binding of additional factors to adjacent promoter or enhancer elements. This function seems to be brought about by NF-Y-induced DNA distortions at the CCAAT box (46). Such a mechanism would also be considered possible for the cbh2 promoter.

With respect to the nature of the protein contacting the 3′-adjacent area within CAE, a sequence comparison showed identity in 5 of the 7 nucleotides with a box identified to bind the A. niger transcriptional regulator of xylanase gene expression, XlnR (33), hence raising the possibility that a H. jecorina homologue of XlnR could bind to the 3′ motif in CAE. However, the results obtained did not support this hypothesis. While this paper was prepared for submission, we became aware of the cloning of two putative regulatory genes (Ilmen, M., Onnela, M. L., Klemsdal, S., Keraänen, S., and Penttilä, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7755–7760). Kubicek, C. P., and Penttilä, M. (1998) in Trichoderma and Gliocladium (Harman, G. K., and Kubicek, C. P., eds) Vol. 2, pp. 49–67, Taylor & Francis Ltd., London. 35. Sternberg, D., and Mandels, G. (1979) J. Bacteriol. 139, 761–769

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Two Adjacent Protein Binding Motifs in the cbh2(Cellobiohydrolase II-encoding) Promoter of the Fungus Hypocrea jecorina (Trichoderma reesei) Cooperate in the Induction by Cellulose

Susanne Zeilinger, Robert L. Mach and Christian P. Kubicek

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