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Identification of the N-acetyl-D-glucosamine-inducible element in the promoter of the *Trichoderma atroviride nag1* gene encoding N-acetyl-glucosaminidase

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Abstract We have investigated the regulation by N-acetyl-glucosamine of the *nag1* gene of the mycoparasitic biocontrol fungus *Trichoderma atroviride* (= *T. harzianum* P1), which encodes a 73-kDa N-acetyl-β-D-glucosaminidase. The use of translational fusions revealed that a 290-bp fragment of the 5′ regulatory region of *nag1* is sufficient to confer inducibility on the *Aspergillus niger goxA* gene. The region between positions –150 and –290, upstream of the *nag1* coding region, was investigated using in vivo methylation protection analysis and electrophoretic mobility shift assays (EMSAs). Two neighbouring regions that interacted with regulatory proteins were identified, and bases essential for these interactions were determined in vitro. These data reveal protein binding to a CCCCT element at –240, a CCAGN$_{13}$CTGG motif at –284, and a CCAAT-box which is present in the spacer of the latter motif. Evidence for the binding of a Hap2/3/5 complex to this CCAAT motif is presented. Protein binding to all three motifs was constitutive, and no differences were observed between induced and non-induced cultures. Mutation of either the CCAGN$_{13}$CTGG or the AGGGG motif resulted in loss of inducibility of *nag1* expression by N-acetyl-D-glucosamine in vivo.

Keywords *Trichoderma* · Biocontrol · N-acetyl-glucosaminidase · Gene regulation · Protein/DNA interaction

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Introduction

Selected species of the imperfect soil fungus *Trichoderma* (e.g. *T. harzianum* and *T. atroviride*) are potent mycoparasites, acting against several economically important plant pathogenic fungi, and are therefore used in biocontrol. Biocontrol of plant pathogens is an attractive alternative to the strong dependence of modern agriculture on fungicides, which may cause environmental pollution and selection of resistant strains. Unfortunately, the successful application of biocontrol strains is often not easy to predict, and this approach is thus not yet able to compete with chemical fungicides. Consequently, knowledge of the biochemical events which determine mycoparasitism and its regulation would offer strategies for improving the reliability of *Trichoderma* as a biocontrol agent.

Several genes that are specifically induced under mycoparasitic conditions have been cloned from mycoparasitic *Trichoderma* spp. (Geremia et al. 1993; Hayes et al. 1994; Carsolio et al. 1994; Garcia et al. 1994; Limon et al. 1995; Peterbauer et al. 1996). Chitinase-encoding genes are prominent among these, and some of them have been proven to be essential for biocontrol in vivo and in vitro (Lorito 1998; Woo 1999). Overproduction of some chitinases has been shown to improve the biocontrol activity in selected cases; however, in order to enhance chitinase synthesis in these strains, the regulation of their genes needs to be understood.

Few attempts have so far been made to understand the molecular regulation of chitinase expression in *T. harzianum*. The expression of the endochitinase-encoding genes *ech42* and *chit33* has been reported by some authors to be inducible by fungal cell-walls and colloidal chitin (Carsolio et al. 1994; Garcia et al. 1994; Limon et al. 1995), whereas others failed to observe induction by chitin, and attributed transcript induction to carbon starvation (Margolles-Clark et al. 1996; Mach et al. 1999). In addition, *ech42* expression has been reported to be subject to carbon catabolite repression

(Carsolio et al. 1994; Lorito et al. 1996), and to be induced under conditions of light-induced sporulation (Carsolio et al. 1994) and by some conditions that impose physiological stress (Mach et al. 1999). In contrast, transcription of the *nag1* gene has been reported to be triggered by N-acetyl-β-D-glucosamine (Peterbauer et al. 1996; Mach et al. 1999).

The specific and rapid inducibility of *nag1* expression by N-acetylglucosamine prompted us to use this system as a model for the study of chitinase gene expression in *Trichoderma*. We have previously shown that the 830-bp segment immediately upstream of the *nag1* ORF is sufficient for this induction (Mach et al. 1999). In this study, we have used promoter deletion analysis, in vivo footprinting, EMSA, and in vivo mutagenesis to identify the precise region responsible for this induction and map the motifs involved in binding the relevant transcription factors.

Materials and methods

Strains

T. atroviride strain P1 ("*T. harzianum*" ATCC 74058) was used throughout this study and maintained on potato dextrose agar (PDA; Merck, Darmstadt, Germany). *Escherichia coli* JM109 (Yanisch-Perron et al. 1985) was used as the host for plasmid amplification.

Culture conditions

T. atroviride and recombinant strains prepared from it were grown in liquid synthetic medium (SM) containing (in g/l): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; urea, 0.6; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 2H_2O$, 0.0028; $CoCl_2 \cdot 6H_2O$, 0.0032 (pH 5.4), and supplemented with either glucose or glycerol (10 g/l), except where otherwise stated. For induction experiments, *T. atroviride* was first cultured in SM containing glycerol as the carbon source for 36 h, harvested by filtration through sterile Miracloth (Calbiochem, La Jolla, Calif.), washed with sterile tap water and transferred to 100-ml Erlenmeyer flasks containing 25 ml of SM and 0.5% (w/v) glycerol (non-inducing conditions) or 0.5% (w/v) N-acetyl-β-D-glucosamine (Sigma, St. Louis, Mo.; inducing conditions) as carbon source. For rapid testing of reporter inducibility, 12-well cell-culture plates containing 3 ml of SM per well were inoculated with fresh spores of recombinant strains, incubated overnight and induced by addition of 150 µl of 10% (w/v) glycerol or N-acetyl-β-D-glucosamine, respectively.

Plasmids and plasmid construction

Plasmid pSJ3, containing the *Aspergillus niger goxA* gene under the control of an 830-bp segment of the 5′ regulatory region of the *T. atroviride nag1* gene (Mach et al. 1999), was used for the construction of the reporter vectors with truncated promoter fragments (Fig. 1): plasmid pSJ32 was constructed by replacing the 830-bp *Xho*I-*Xba*I fragment by the 455-bp *Sal*I-*Xba*I fragment of pSJ3. pSJ33 was constructed by removing a 540-bp *Mlu*I-*Xho*I fragment from pSJ3, filling in the protruding ends with Klenow DNA Polymerase I (Promega, Madison, Wis.) and recircularizing the plasmid by blunt-end ligation. pSJ34 was constructed by amplifying a fragment of the 5′ regulatory region of *nag1* using the primers nmluF and nagR, containing internal *Mlu*I and *Xba*I restriction sites, respectively (Table 1), *Taq* polymerase (Promega) and a Biometra (Göttingen, Germany) Trio thermocycler. The amplification protocol consisted of an initial denaturation step for 1 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 59°C and 30 s at 74°C, followed by a final extension step for 5 min at 74°C. The product was digested with *Mlu*I and *Xba*I, and the resulting 210-bp fragment was inserted between the *Mlu*I site at

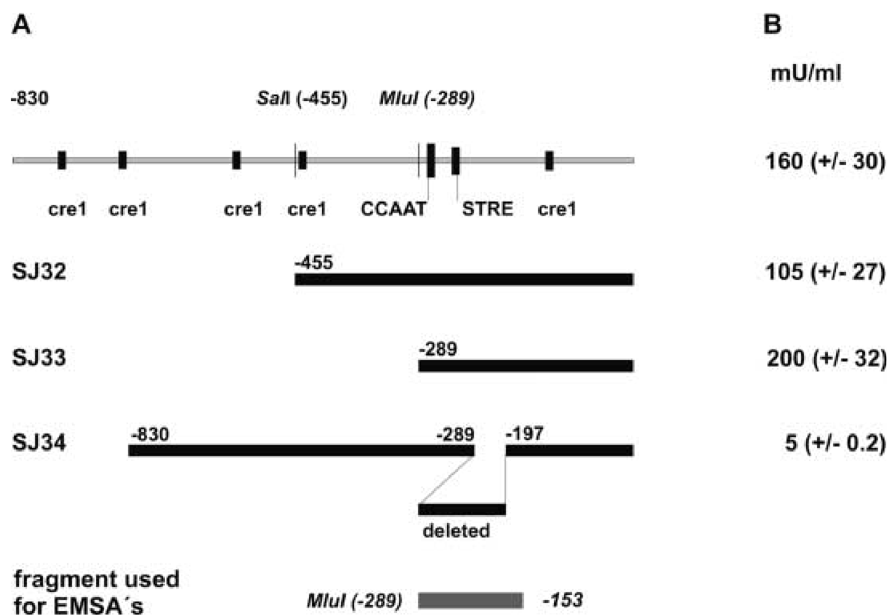

Fig. 1 A Structure of the *nag1* promoter in pSJ3, as used in the previous studies (Mach et al. 1999), and extent of the truncations/deletions used in this study. Plasmids pSJ32, pSJ33 and pSJ34 were constructed as described in Materials and methods. **B** GOX activity of reporter strains containing translational fusions of the promoter fragments depicted in **A** to the *A. niger goxA* gene. The data are derived from at least three induction experiments with at least three transformed strains each; precultures of each strain were inoculated into non-inducing and inducing medium as described in Materials and methods. The measured glucose oxidase activities were corrected for variations in biomass and copy number of the reporter constructs, as determined by Southern hybridization. Activities observed under non-inducing conditions were comparable to the activities obtained with SJ34

Table 1 List of oligonucleotides used in this study

Name	Sequence (5′→3′)[a]	Location
nmluF	CTTTGGGACGCGTGTCGATTTGAGAAATCCG	–205 to –175
nagF	GCTGATATGGCCGCTCGAGTACCTAGATC	–848 to –820
nagR	CCTTGGGCAGCATCTAGAACGACCGAGG	–15 to + 13
npr1	AGCCAAATACTTAAATACCTGGCCC	–139 to –115
npr2	CACGACGCGATTCATTGAAGGGAT	–171 to –148
npr3	CGCGATTCATTGAAGGGATGTGCGG	–177 to –153
npf4	AGAACGCCACAGCTTCACTATCAA	–439 to –416
npf5	GACTCCTCTATCTTCGGAGCGCAT	–408 to –384
npf6	CCTCTATCTTCGGAGCGCATGCAGC	–404 to –380
ckp0	acgtGCCAGGTAGCATTGGCCACTGGATCATC GACCTTGTCGCGTGTCAGGGGGCCGTGC	–284 to –228
ckp1	acgtGCCAGGTAGCATTGGCCACTGGATC	–284 to –260
ckp2	acgtGTCGCGTGTCAGGGGGCCGTGCT	–250 to –228
ckp3	acgtCCCGTCCAGCTGTGGGCTGCTTCTTTGGG	–227 to –199
ckp1m38	CAGGTCACGCGTGCC**CT**GTAGCATTGGCCA**AGT**GATC	–296 to –260
ckp2m4f	GTCAG**TTT**GCCGTGCTCCCGTCC	–243 to –221
ckp2m4r	GGACGGGAGCACGGC**AAA**CTGAC	–243 to –221
CKT67	gatcCACTCCACATGTTAAAGGCGCATTCAACCAGCTTC	Nonspecific competitor

[a] Complementary oligonucleotides (used for EMSAs) are not given. *Lower case letters* indicate bases added for labeling. Mutated bases are shown in *bold*, newly generated restriction sites are *underlined*

position –289 and the *Xba*I site at + 1 of the *goxA* coding region, thereby deleting a 92-bp segment from the 5′ non-transcribed region of *nag1*.

Reporter plasmids with mutations in the 5′ upstream region of *nag1* were constructed as follows: a 310-bp fragment of the region was amplified as described above for pSJ34, using the primers ckp1m38 (containing mutations as indicated in Table 1) and nagR, and an annealing temperature of 62°C. After purification by gel electrophoresis, the amplified product was digested with *Mlu*I and *Xba*I, and inserted in place of the *Mlu*I (–289)-*Xba*I (+ 1) fragment of *goxA*, to yield plasmid pN1M38. Plasmid pN2M4 was constructed by Fusion PCR: a 628-bp promoter fragment was amplified with the primers nagF and ckp2m4r, and a 256-bp fragment was synthesized with primers ckp2m4f and nagR (amplification protocol as for pSJ34 with an annealing temperature of 62°C, mutations as indicated in Table 1). Both amplicons were purified, combined and used as the template for amplification with primers nagF and nagR. The resulting 860-bp fragment was digested with *Xho*I and *Xba*I, and the corresponding fragment of plasmid pSJ3 was replaced by this mutated derivative. All constructs were checked by restriction analysis and sequencing (LI-COR 4000).

Fungal transformation

Plasmids were introduced into *T. atroviride* by cotransformation with plasmid pHATα, which confers hygromycin B resistance (Herrera-Estrella et al. 1990), as described previously (Mach et al. 1999). Ten micrograms of the *goxA*-bearing plasmids and 2 μg of pHATα were used per 5×10^7 protoplasts. Protoplasts were regenerated on PDA supplemented with 1 M sorbitol and 100 μg/ml hygromycin B (Calbiochem, La Jolla, Calif.). Mitotically stable transformants were obtained by performing at least three sequential transfers of conidia from non-selective to selective medium.

DNA manipulations

Chromosomal DNA was isolated by the CTAB (cetyltrimethylammonium bromide) method (Ausubel 1990) from mycelia grown for 36 h at 28°C in potato dextrose broth (PDB; Difco Laboratories, Detroit, Mich.). Plasmid DNA was isolated using a midiprep kit (Qiagen, Chatsworth, Calif.) as recommended by the manufacturer. Other molecular techniques were performed according to standard protocols (Sambrook 1989; Ausubel 1990).

Glucose oxidase assay

Glucose oxidase activity in culture supernatants was quantified spectrophotometrically as described previously (Mach et al. 1999). One unit (1 U) of activity is defined as the amount of enzyme required to oxidize 1 μmol of glucose per min at pH 5.8 and 25°C.

In vivo genomic footprinting via ligation-mediated PCR

A pre-culture of *T. atroviride* was grown and used to inoculate non-inducing and inducing medium as described above; 18-ml aliquots of the cultures were incubated in 100-ml Erlenmeyer flasks in a shaking water bath at 28°C. In vivo methylation of genomic DNA with dimethyl sulfate (Merck, Darmstadt, Germany) was carried out 40, 90 and 120 min after inoculation of the media. Methylation, extraction of methylated DNA and cleavage at adenine and guanine bases with HCl and NaOH was carried out as described previously (Wolschek et al. 1998; Zeilinger et al. 1998). In vitro methylation and cleavage of genomic DNA for control samples was performed as described by Mueller and Wold (1989). Ligation-mediated PCR was performed as described by Garrity and Wold (1992) and modified by Wolschek et al. (1998), using Vent Polymerase (New England Biolabs, Beverly, Mass.) and the primer sets npr1, npr2 and npr3 to visualize the coding strand and npf4, npf5 and npf6 for the non-coding strand of the 5′ region of *nag1*.

Electrophoretic mobility shift assay (EMSA)

DNA fragments for EMSA were obtained as follows. A 160-bp segment of the *nag1* 5′ regulatory region (–153 to –312) was amplified by PCR using primers npf2 and npr3, and cut with *Mlu*I. The resulting 137-bp fragment was end-labeled with [α-^{32}P]dCTP using Sequenase Version 2.0 (USB, Cleveland, Ohio). Annealing and end-labeling of synthetic oligonucleotides used for EMSAs was done as described by Strauss et al. (1995). All labeled probes were purified by non-denaturing polyacrylamide gel electrophoresis. Binding assays were performed for 5 min on ice with 4 ng of the labeled 137-bp fragment and 25 μg of cell-free extract, or 4 ng of labeled oligonucleotide and 100 μg of cell-free extract. To prepare the extracts, a pre-culture of *T. atroviride* was grown and inoculated into inducing and non-inducing medium as described above, and mycelia were harvested after 90 min. Preparation of cell-free extracts, binding assays and non-denaturing polyacrylamide gel electrophoresis were done as described previously (Zeilinger et al. 1996). For competition experiments, synthetic oligonucleotides

were annealed and filled in using Sequenase Version 2.0 as described above, and used in 10-, 50-, or 150-fold molar excess. Supershift experiments were done as described by Zeilinger et al. (1998), by adding 1- or 5-µl aliquots of anti-HapC antibody (Kato et al. 1998) to the binding assay.

Results

In vivo identification of the upstream region of *nag1* responsible for its inducibility by N-acetyl-D-glucosamine

To determine which portions of the *nag1* 5′ region are required for its induction by N-acetyl-glucosamine, we first constructed a series of recombinant *T. atroviride* strains which carried the *A. niger goxA* gene as reporter under the control of *nag1* promoter fragments of varying lengths (Fig. 1A). The resulting strains were tested for the presence and copy number of the reporter constructs (data not shown), and strains with copy numbers comparable to those used in the previous studies (1–3; Mach et al. 1999) were selected for analysis. Induction experiments with N-acetyl-β-D-glucosamine revealed that the first 289 bp of the 5′-upstream region of the *nag1* gene conferred the same response as the entire 830-bp fragment, consequently the stretch between –830 and –289 contains neither positive nor negative *cis*-acting elements. Elimination of the segment between –289 and –197, however, resulted in complete loss of inducibility by N-acetyl-β-D-glucosamine (Fig. 1B). We therefore conclude that this 92-bp fragment includes the promoter regions essential for induction of *nag1* by N-acetyl-β-D-glucosamine. Visual inspection of the sequence revealed a CCAAT box on the template strand (5′-ATTGG-3′) as well as the motif 5′-AGGGG-3′. The former has been reported to bind the Hap protein complex in *Aspergillus nidulans* and *Trichoderma reesei*, and is involved in the regulation of several genes in filamentous fungi (Papagiannopoulos et al. 1996; Brakhage et al. 1999; Zeilinger et al. 2001). The complement of the latter motif corresponds to the consensus sequence for the *Saccharomyces cerevisiae* stress response element (STRE; Marchler et al. 1993).

In order to map more precisely the regions within the 92-bp segment which are responsible for the transcriptional regulation of the *nag1* gene, we performed in vivo footprinting by methylation protection, and protected regions were visualized using ligation-mediated PCR; isolated genomic DNA of *T. atroviride* methylated in vitro was used as a control. The results of this investigation are shown in Fig. 2: protection was observed for all 5 bp of the putative STRE motif (–240 to –236), while flanking guanine residues (at –245 and –232, respectively) were hypersensitive to digestion. In addition, adenine –277 and guanine –247 were protected against methylation (Fig. 2).

In vitro analysis of the *T. atroviride nag1* promoter

To confirm and extend these observations, electrophoretic mobility shift analysis (EMSA) was performed. First, a 137-bp fragment of the *nag1* promoter including the above-mentioned 92-bp segment was incubated with cell-free extracts from *T. atroviride,* prepared from mycelia grown under *nag1*-inducing and non-inducing conditions. Formation of defined protein-DNA complexes was observed under both conditions (Fig. 3A) and their specificity was confirmed by competition experiments using nonspecific competitor DNA (from calf thymus or *E. coli*) and BSA as a nonspecific control protein (data not shown). No difference in migration

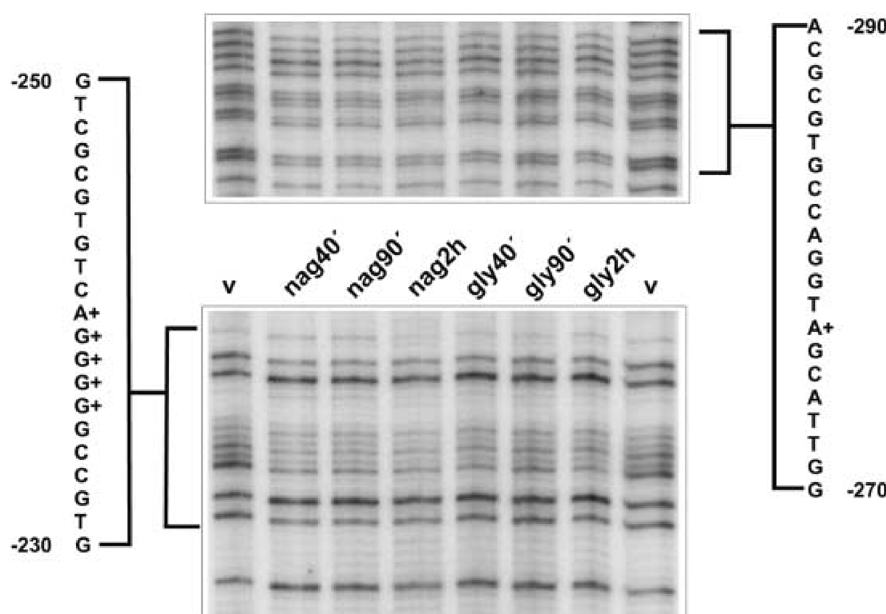

Fig. 2 In vivo methylation protection analysis using Ligation-Mediated PCR. Precultures of *T. atroviride* were inoculated into non-inducing (samples gly) and inducing (samples nag) medium as described in Materials and methods; in vivo methylation was carried out after 40, 90 and 120 min. Lanes v contain control samples of in vitro methylated DNA. Nucleotides or motifs that are protected from methylation are marked +

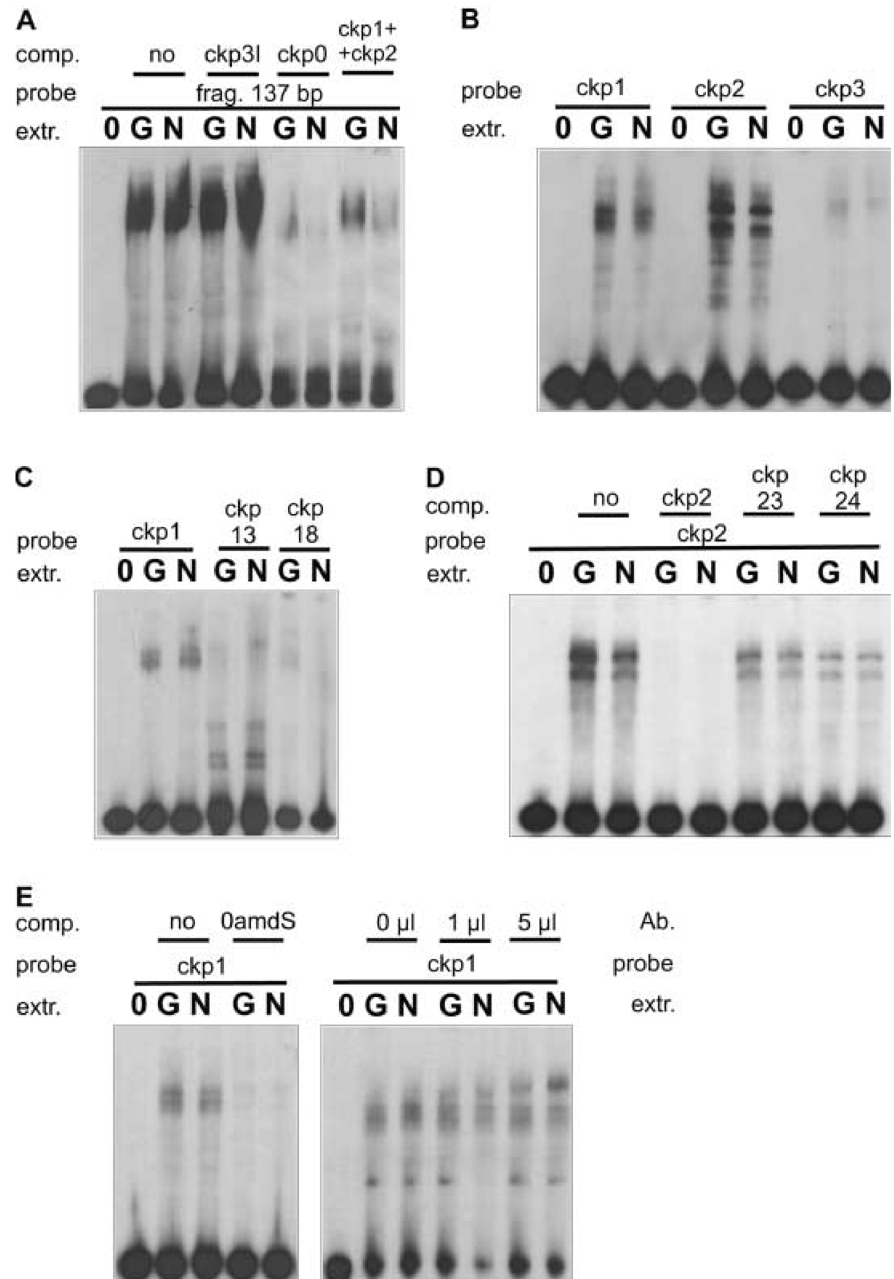

Fig. 3A–E Electrophoretic mobility shift assay of the *nag1* promoter. **A** EMSA using the 137-bp *nag1* promoter fragment (see Materials and methods) and 25-µg aliquots of cell-free extracts (extr). G, extract obtained from mycelium grown in medium containing glycerol as carbon source; N, extract from mycelium grown into medium containing N-acetyl-glucosamine as carbon source; 0, free probe; comp., oligonucleotides used as specific competitors (in 50-fold molar excess) where indicated (for sequences, see Table 1). **B** EMSA using the synthetic oligonucleotides ckp1, 2 and 3 (Table 1) and 100-µg aliquots of cell-free extract as described in **A**. **C** Analysis of mutations within the region covered by oligo ckp1. in ckp13 positions –281/-280 are mutated (AG→CT, Table 2); in ckp18 bases –266 to –264 (CTG) are changed to AGT (Table 2). **D** Competition experiments with oligo ckp2. ckp23 is mutated at position –247 (G→T), ckp24 carries a mutation in the AG-GGG element (AGTTT; Table 2)**E** Involvement of the Hap complex in the binding of the CCAAT-motif in oligo ckp1 is shown by quantitative competition by the oligonucleotide OamdS (Table 2; added in 50-fold molar excess), and by a supershift experiment: 0, 1 and 5 µl of anti-HapC antibody (Ab) were added to the binding assay as indicated

properties was observed between the DNA-protein complexes detected under inducing and non-inducing conditions.

To identify the regions responsible for protein binding, competition experiments with synthetic oligonucleotides (ckp0, ckp1, ckp2 and ckp3; for sequences, see Table 1) spanning different parts of the 137-bp fragment were carried out (Fig. 3A). Oligonucleotides ckp0, ckp1 and ckp2, but not ckp3, strongly competed with the 137-bp fragment for protein binding. Consistent results were obtained when ckp1, 2 and 3 were labeled and used as probes in EMSA (Fig. 3B). We therefore conclude that protein binding to the *nag1* promoter takes place in the area between positions –284 and –228.

In order to identify individual bases that are essential for protein binding within this 56-bp segment of the *nag1* promoter, we performed EMSAs with oligonucleotides carrying specific mutations (see Table 1; Fig. 3C). In the region covered by oligonucleotide ckp1, the mutation AG→CT at positions –281/–280 leads to a complete loss of binding, and a similar effect was observed when any change was introduced into the sequence CCACTG (–269 to–264). Mutations in the region between –279 and –270 only caused minor reductions in the strength of complex formation. This is seen particularly with oligonucleotides ckp12 and ckp16 (see Table 2), which carry mutations in the CCAAT box that have been reported to impair the binding of the Hap

Table 2 Oligonucleotides used for characterization of protein/DNA interaction

Name	Sequence (5′→3′)[a]	Complex formation[b]	Competition[b]
ckp1	acgtGCCAGGTAGCATTGGCCACTGGATC	+	+
ckp11	acgtGCCAGGT**C**GCATTGGCCACTGGATC	nd	+
ckp12	acgtGCCAGGTAGCA**AA**GGCCACTGGATC	+	nd
ckp13	acgtGCC**CT**GTAGCATTGGCCACTGGATC	–	–
ckp14	acgtGCCAG**TG**AGCATTGGCCACTGGATC	+/–	+/–
ckp15	acgtGCCAGGTA**AT**ATTGGCCACTGGATC	+	–
ckp16	acgtGCCAGGTAGCAT**CCC**CCACTGGATC	+	nd
ckp17	acgtGCCAGGTAGCATTGG**AAC**CTGGATC	–	nd
ckp18	acgtGCCAGGTAGCATTGGCCA**AGT**GATC	–	nd
ckp19	acgtGCCAGGTAGCATTGGCCACTG**TCG**C		
ckp2	acgtGTCGCGTGTCAGGGGGCCGTGCT	+	+
ckp21	acgtGTCGCGTGTC**C**GGGGGCCGTGCT	+/–	+/–
ckp22	acgtGTCGCGTGTCAG**T**GGGCCGTGCT	+/–	+/–
ckp23	acgtGTC**T**CGTGTCAGGGGGCCGTGCT	–	+/–
ckp24	acgtGTCGCGTGTCAG**TTT**GCCGTGCT	–	+/–
OamdS	CGTAAAATTCGGCGAAGCCG CCAATCACCAGCTAGGCACCA	nd	+

[a] Mutations are indicated in *bold*, bases added for labeling are shown in *lower case*
[b] The + sign indicates formation of a specific complex identical to that formed with the wild-type oligonucleotide, or that the oligonucleotide competes with the wild-type oligo; +/– indicates a minor reduction/weak competition; – indicates abolition of complex formation or no competition, respectively. nd, not determined

complex to the CCAAT motif in the *A. nidulans amdS* promoter (van Heeswijck and Hynes 1991), but also include A_{-277} which was found to be protected against methylation in vivo. Since mutations in CCAAT-boxes have recently been shown to lead only to incomplete competition (Zeilinger et al. 1998), an additional competition experiment with an oligonucleotide from the *A. nidulans amdS* promoter (Oamds), which carries a functional CCAAT-box (Papagiannopoulos et al. 1996), was performed; this sequence competed for the complex formed with ckp1 almost quantitatively (Fig. 3E). Furthermore, when an antibody against *A. nidulans* HapC (Kato et al. 1998) was added to the binding assay, a supershift was observed. These data suggest that, despite the marginal effects of mutations in the CCAAT box on binding in vitro, the protein complex that binds to ckp1 contains the Hap2/3/5 protein complex of *T. atroviride*.

With oligonucleotide ckp2 as a probe, two different DNA-protein complexes were observed. Oligonucleotides containing mutations in positions –247 or –238 to –236 were partially impaired in their ability to compete with ckp2 for binding (Fig. 3D), and did not form a specific complex with *T. atroviride* protein (Table 2). Both mutations led to the loss of both DNA-protein complexes. Hence, both the STRE element and an unknown motif including G_{247} are involved in binding to this area of the *nag1* promoter.

Two sequence motifs in the *nag1* promoter are essential for induction by N-acetyl-glucosamine

In order to verify that the identified nucleotide motifs are relevant to the induction of *nag1* by N-acetyl-D-glucosamine in vivo, we transformed *T. atroviride* with reporter constructs bearing specific mutations in the *nag1* regulatory region, which had been shown by EMSA to eliminate protein binding, i.e. mutation #4 (AGGGG→AGTTT, oligonucleotide ckp24) and the combination of two mutations corresponding to oligonucleotides ckp13 and ckp18. No mutations in the CCAAT box were tested in vivo, as the respective mutations did not yield significant effects in vitro. The results are shown in Table 3: in both cases, the inducibility

Table 3 Effect of specific mutations in the *nag1* promoter on inducibility by N-acetyl-D-glucosamine *in vivo* 1

Construct[a]	Mutation	GOX activity (mU/ml)	
		Glycerol	N-acetyl-D-glucosamine
SJ3	–245 GTGTCAGGGGGCCGTGCTCC CGTC –220	12 ± 0.4	184 ± 21
N1M38	–285 TGCCC**CT**GTAGCATTGG CCA**AGT**GATC –260	16 ± 2.4	10.5 ± 2.0
N2M4	–245 GTGTCAG**TTT**GCCGTGCTCC CGTC –220	11 ± 0.2	19 ± 0.8

[a] Precultivation and induction was carried out as described in Materials and methods; experiments were performed with at least two strains for each mutated construct and repeated at least twice. Values are given in mU/ml (± standard deviation). Note that biomass density was the same in all experiments; strains carrying the mutated constructs as single-copy integrations were selected for the experiments. The mutations introduced are *highlighted in bold*

of *nag1* by N-acetyl-D-glucosamine was abolished; the low level of residual reporter activity observed under inducing conditions is comparable to the low level of constitutive expression obtained under non-inducing conditions (0.5% glycerol as carbon source).

Discussion

In this paper, we have used a combination of promoter deletion studies, in vivo footprinting, EMSA and in vivo mutagenesis to obtain an insight into the transcriptional regulation of a fungal chitinase gene; the results are summarized in Fig. 4. The data are consistent with a major role for the segment between –284 to –228 in the regulation of *nag1* induction by N-acetylglucosamine, and show that formation of protein contacts in at least two different positions in this area is essential for induction by N-acetyl-D-glucosamine. That not all of the relevant nucleotides are visualized by in vivo footprinting is not unexpected, because it is known that LM-PCR does not identify all binding sites (Wolschek et al. 1998; Muro-Pastor et al. 1999; M. Wolschek and C. P. Kubicek, unpublished data), probably due to differences in the stability of protein-DNA complexes. However, sites identified by this method have so far always been shown to be functional in vitro and in vivo (Zeilinger et al. 1998; Muro-Pastor et al. 1999).

The regions of *nag1* capable of binding protein are clustered in two areas. In the one further upstream, the bases identified as essential for binding in vitro and induction in vivo may be interpreted as part of a motif containing the sequence CCAG in dyad symmetry, separated by a 13-bp "spacer", in which a CCAAT motif is present (on the opposite strand). To the best of our knowledge, such a motif has not previously been identified as a target for a DNA-binding protein. Yet the organization of this binding site would be compatible with the binding of a $Zn(II)_2Cys_6$-type zinc-cluster protein (Todd and Andrianopoulos 1997). Although these proteins usually bind to a terminal nucleotide triplet (e.g. *S. cerevisiae* GAL4 and MAL63; or *Aspergillus nidulans* FacB; Vashee et al. 1993; Sirenko et al. 1995; Todd et al. 1998), the use of a terminal nucleotide quartet has been demonstrated in some cases (e.g. *A. nidulans* UaY; Suarez et al. 1995). The size of the spacer (13 bp) is also compatible with that (10–12 bp) in the target sites for GAL4 or FacB.

The presence of a CCAAT motif within this putative spacer, on the opposite strand, is intriguing. Competition and antibody supershift experiments convincingly show that this CCAAT-motif binds a Hap-like protein complex of *T. atroviride*. This complex consists of at least three proteins – Hap2, Hap3 and Hap5 – which were originally described in *S. cerevisiae* but have more recently also been characterized from the filamentous fungi *A. nidulans* (Papagiannopoulos et al. 1996; Steidl et al. 1999) and *T. reesei* (Zeilinger et al. 2001). The fact that mutation of this motif did not result in complete loss of binding in vitro does not necessarily conflict with this conclusion, as similar findings have also been reported for other genes, such as the *cbh2* promoter of *T. reesei* (Zeilinger et al. 1998). In this case, a mutation in the CCAAT motif alone only marginally reduced binding in vitro and gene expression in vivo, but simultaneous mutation of the CCAAT box and an adjacent motif binding a second factor completely abolishes protein binding in vitro and gene expression in vivo (Zeilinger et al. 1998). Narendja et al. (1999) have recently demonstrated that the main function of the Hap proteins is the establishment of an open chromatin structure, and a similar role has recently been assigned by us to the CCAAT motif in the promoter of the *cbh2* gene (for cellobiohydrolase 2) of *T. reesei* (Zeilinger et al., manuscript submitted). We therefore believe that this element may play a similar role in the *T. atroviride nag1* promoter. Interestingly, results from EMSA suggest that the Hap complex interacts with the protein that binds to the palindromic motif, as mutations in the palindromic motif, but outside the CCAAT box, prevented binding of the Hap complex.

Fig. 4 Schematic representation of the 5′ regulatory region of *nag1* investigated in this study. The locations of synthetic oligonucleotides used in EMSAs are indicated. The CCAAT box (lower strand) and the STRE-like motif are *boxed*, the palindromic element is indicated by *bars above* the sequence; bases protected from methylation in vivo are indicated by *arrows*, bases that are essential for the formation of specific protein/DNA complexes (i.e., complex formation is abolished by mutation, as revealed by EMSA) are indicated by +

The motif at the 3′-end of the region required for induction, an AGGGG-box at –240, showed the strongest protection against methylation in vivo of all motifs in the *nag1* promoter, indicating that it is tightly bound to protein in vivo. The corresponding element on the template strand (CCCCT) has been identified as a binding site for the stress regulators Msn2p and Msn4p in *S. cerevisiae* (Marchler et al. 1993; Martinez-Pastor et al. 1996; Treger et al. 1998). According to the last group of authors, a single copy of this element is sufficient to confer responsiveness to certain environmental stress factors, independently of flanking sequences. If this is also valid for other fungi, the element found in *nag1* may be functional. A CCCCT element is also present in the promoters of the *Trichoderma* chitinase genes *ech42* and *chit33*. which are both upregulated by certain stress conditions (Mach et al. 1999; de la Dana et al. 2001), and the CCCCT-box in *ech42* has been shown to bind proteins from cell-free extracts of *T. atroviride* (M. Montero, P. Sposato and C. P. Kubicek, unpublished data). However, in contrast to *ech42*, *nag1* is not upregulated by stress (C. K. Peterbauer, unpublished data), and the presence of this motif in *nag1* must therefore serve another function. Interestingly, a similar motif has been identified in the cutinase promoter of *Haematonectria haematococca*, where it is involved in maintaining the basal expression level (Kamper et al. 1994). In *Yarrowia lipolytica*, the AGGGG motif is bound by the Mhy1p protein, the expression of which is dramatically increased during the yeast-to-hypha transition (Hurtado and Rachubinski 1999). It is thus not clear whether the AGGGG box fulfills the same function in other fungi as in *S. cerevisiae*.

In vivo mutagenesis confirmed that the motif that binds the putative $Zn(II)_2Cys_6$-type zinc-cluster protein and the AGGGG box are both essential for induction of *nag1* by N-acetyl-glucosamine, and that mutation of either of these motifs is sufficient to lead to complete loss of inducibility. This indicates that binding to both motifs is essential, which suggests that the proteins/protein complexes that bind to the two motifs interact with each other. In fact, results from EMSA showed that competition of the protein/DNA complex with an annealed pair of synthetic oligonucleotides spanning both regions of interaction (ckp0; Table 1) was almost quantitative, whereas competition with a mixture of the two oligonucleotide pairs ckp1 and ckp2 was much less effective (Fig. 3A), a result which supports this hypothesis.

While this study demonstrated the interaction of at least two segments in the region upstream of the *nag1* gene with proteins from cell-free extracts, it is interesting that we did not observe any change in the in vitro binding of the respective proteins under induced and non-induced conditions. This may indicate that other levels of regulation, such as nuclear transport or covalent modification, are involved in controlling the final steps in *nag1* induction.

In summary, we have identified the area in the *nag1* promoter which is responsible for the expression of the gene in response to N-acetyl-β-D-glucosamine. Cloning of the genes encoding the respective DNA-binding proteins by the one-hybrid system will facilitate further studies on the regulation of chitinase gene expression in *T. atroviride*.

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