Regulation of the NADP-glutamate dehydrogenase gene \textit{gdhA} in \textit{Aspergillus nidulans} by the Zn(II)2Cys6 transcription factor LeuB

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NADP-dependent glutamate dehydrogenase (NADP-GDH) is a key enzyme in the assimilation of alternative nitrogen nutrient sources through ammonium in fungi. In \textit{Aspergillus nidulans}, NADP-GDH is encoded by \textit{gdhA}. Several transcription factors are known to regulate \textit{gdhA} expression, including \textit{AreA}, the major transcription activator of nitrogen metabolic genes, and \textit{TamA}, a co-activator of \textit{AreA}. \textit{TamA} also interacts with LeuB, the regulator of leucine biosynthesis. We have investigated the effects of leucine biosynthesis on \textit{gdhA} regulation, and found that leucine regulates the levels of NADP-GDH activity and \textit{gdhA} expression. We show, using mutants with perturbed levels of \textit{\alpha}-isopropylmalate (\textit{\alpha}-IPM), that this leucine biosynthesis intermediate affects \textit{gdhA} regulation. Leucine regulation of \textit{gdhA} requires a functional LeuB with an intact Zn(II)2Cys6 DNA-binding domain. By analysing the prevalence of putative LeuB DNA-binding sites in promoters of \textit{gdhA} orthologues we predict broad conservation of leucine regulation of NADP-GDH expression within ascomycetes except in the fusaria and fission yeasts. Using promoter mutations in \textit{gdhA}–\textit{lacZ} reporter genes we identified two sites of action for LeuB within the \textit{A. nidulans} \textit{gdhA} promoter. These two sites lack sequence identity, with one site conforming to the predicted LeuB DNA-binding site consensus motif, whereas the second site is a novel regulatory sequence element conserved in \textit{Aspergillus gdhA} promoters. These data suggest that LeuB regulates NADP-GDH expression in response to leucine levels, which may act as an important sensor of nitrogen availability.

INTRODUCTION

Ammonium is the preferred nitrogen source for many micro-organisms and its assimilation requires the activity of NADP-dependent glutamate dehydrogenase (NADP-GDH). NADP-GDH catalyses the synthesis of glutamate from 2-oxoglutarate, an intermediate of the Krebs cycle, and ammonium sourced from either the external environment or internally via metabolism. In \textit{Aspergillus nidulans} and Neurospora crassa, NADP-GDH is encoded by a single gene, \textit{gdhA} and \textit{am}, respectively (Arst & MacDonald, 1973; Gurr \textit{et al.}, 1986; Hawkins \textit{et al.}, 1989; Kinghorn & Pateman, 1973, 1975; Kinnaird & Fincham, 1983). In contrast, \textit{Saccharomyces cerevisiae} has two NADP-GDH genes, \textit{GDH1} and \textit{GDH3}, which are active under different growth conditions (Avendano \textit{et al.}, 1997; DeLuna \textit{et al.}, 2001).

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Abbreviations: \textit{\alpha}-IPM, \textit{\alpha}-isopropylmalate; \beta-IPM, \beta-isopropylmalate; NADP-GDH, NADP-dependent glutamate dehydrogenase.

Mutants of \textit{gdhA}, \textit{am} or \textit{GDH1/GDH3} lacking NADP-GDH function have reduced growth on ammonium compared with WT strains (Arst & MacDonald, 1973; Avendaño \textit{et al.}, 1997; Fincham, 1988; Fincham & Baron, 1977; Fincham \textit{et al.}, 2000; Kinghorn & Pateman, 1973). In \textit{A. nidulans}, \textit{gdhA} mutants are leaky as the assimilation of nitrogen through ammonium occurs by the non-preferred route of glutamine synthetase and glutamate synthetase (Arst \textit{et al.}, 1975; Kinghorn & Pateman, 1976; Macheda \textit{et al.}, 1999; Margelis \textit{et al.}, 2001). \textit{gdhA} mutants also show derepression of several alternative nitrogen assimilation pathways (Hynes, 1974; Kinghorn & Pateman, 1973; Pateman \textit{et al.}, 1973). As NADP-GDH is the primary assimilation route for nitrogen sources via ammonium, draws substrates from the tricarboxylic acid cycle and is required for correct regulation of alternative nitrogen assimilation genes, its synthesis is highly regulated, particularly in response to different nitrogen sources.

In \textit{A. nidulans}, \textit{gdhA} mRNA is highly expressed during growth on ammonium and glutamine, whereas strains
grown on glutamate have lower levels of gdhA mRNA expression (Hawkins et al., 1989). This pattern of regulation by these nitrogen sources is also observed for NADP-GDH activity (Pateman, 1969), and is conserved in Aspergillus awamori and Botrytis cinerea (Cardoza et al., 1998; Santos et al., 2001). Additionally, Caddick et al. (2006) found that different nitrogen regimes do not regulate stability of the gdhA mRNA transcript. Therefore, differences in gdhA expression are likely due to transcriptional regulation. Several transcription factors are known to regulate NADP-GDH expression. In N. crassa, am is regulated by the CCAAT-binding factor AAB (Chen & Kinsey, 1994; Chen et al., 1998; Frederick & Kinsey, 1990a, b), as well as the global nitrogen utilization GATA transcription factor NIT2 (Dantzig et al., 1979). Likewise, S. cerevisiae requires the homologues of these transcription factors, HAP2/3/4/5 and Gln3p, respectively, for full expression of GDH1 and GDH3 (Dang et al., 1996; Daugherty et al., 1993; Hernández et al., 2011; Riego et al., 2002). GDH1 and GDH3 encode isoforms that arise during whole-genome duplication and they are differentially expressed dependent upon the carbon sources available in a mechanism involving chromatin remodelling by Swi/Snf and Gcn5p (Avendaño et al., 2005; DeLuna et al., 2001). GDH1 is also regulated by the amino acid starvation pathway transcription regulator Gcn4p (Hinnebusch, 1988; Riego et al., 2002) and the leucine biosynthesis pathway transcription regulator Leu3p (Hu et al., 1995). A. nidulans gdhA is regulated by the CCAAT-binding complex AnCF (Papagiannopoulos et al., 1996; M. A. Davis unpublished data) and the global nitrogen regulator AreA (Christensen et al., 1998). AreA is itself highly regulated by multiple mechanisms, including autogenous regulation and differential transcript stability (Caddick et al., 2006; Langdon et al., 1995; Morozov et al., 2000, 2001, 2010; Platt et al., 1996), regulated nuclear export (Todd et al., 2005), and interactions with the corepressor NmrA (Andrianopoulos et al., 1998; Kotaka et al., 2008; Lamb et al., 2004) and a co-activator TamA (Davis et al., 1996; Small et al., 1999, 2001). These mechanisms combine to minimize AreA activation of most nitrogen catabolic target genes during growth on ammonium (reviewed by Wong et al., 2008). However, the regulation of certain genes for ammonium uptake and assimilation, such as menA (Monahan et al., 2006) and gdhA, is markedly different than the regulation of AreA-regulated catabolic genes. High-level expression of gdhA requires AreA for activation during growth on ammonium (Christensen et al., 1998; Polotnianka et al., 2004). It was hypothesized that additional gdhA promoter-specific proteins may assist activation. Investigation of the co-activator TamA, which interacts with the extreme C-terminus of AreA, revealed that tamAA has a more severe effect than areAA on NADP-GDH levels, suggesting that TamA has a role in gdhA regulation in addition to its role as a co-activator of AreA (Polotnianka et al., 2004; Small et al., 1999). A yeast two-hybrid screen for proteins that interact with TamA identified LeuB, the homologue of S. cerevisiae Leu3p, which regulates the synthesis of leucine (Polotnianka et al., 2004).

S. cerevisiae Leu3p acts as a dual activator/repressor and has DNA-binding sites in the promoters of several genes in the branched-chain amino acid biosynthesis pathway, including BAT1, LEU1, LEU2, LEU4, ILV2 and ILV5 (Friden & Schimmel, 1988; Hellauer et al., 1996; Kohlhaw, 2003). Leu3p transcriptional activation function requires z-isopropylmalate (z-IPM), the product of z-IPM synthetase encoded by LEU4 and LEU9 (Baichwal et al., 1983; Casalone et al., 2000; Chang et al., 1984, 1985). High cellular leucine levels inhibit z-IPM synthetase activity, reducing z-IPM abundance and converting Leu3p to a repressor (Sze et al., 1992). LeuB and Leu3p are required for WT levels of expression of NADP-GDH from gdhA and GDH1, respectively (Hu et al., 1995; Polotnianka et al., 2004). In this paper, we characterize the effects of loss of z-IPM synthetase or z-IPM isomerase activity on gdhA expression in A. nidulans, and provide evidence that changes in z-IPM levels regulate NADP-GDH gene expression. We identify conserved putative LeuB/Leu3p sites in gdhA orthologues in certain phylogenetic groups within the Ascomycota. We also determine that the activity of LeuB as a transcriptional regulator requires a functional Zn(II)2Cys6 DNA-binding domain for activation of gdhA expression through two promoter elements, one of which conforms to the known Leu3p consensus DNA-binding motif.

**METHODS**

**A. nidulans strains, media and growth conditions.** *A. nidulans* strains used in this study are listed in Table 1 using annotation described by Clutterbuck (1974). *A. nidulans* growth conditions and media were as described by Cove (1966), with pH adjusted to 6.5. *Aspergillus* nitrogen-free minimal medium (ANM), appropriately supplemented for auxotrophs, contained 1% (w/v) glucose as the carbon source and nitrogen sources at a final concentration of 10 mM. Genetic analysis was performed as described by Clutterbuck (1974) and Todd et al. (2007).

**Transformation and enzyme assays.** *A. nidulans* protoplasts were prepared according to Yelton et al. (1984), with omission of β-glucuronidase and replacement of Novozyme 234 by Lysing Enzymes (Sigma), and transformation was carried out as described by Andrianopoulos & Hynes (1988), except with 10–30 min PEG treatments and omission of the wash to remove PEG prior to plating. β-Galactosidase and NADP-GDH assays were performed as described by Davis et al. (1988) and Pateman (1969), respectively, using soluble protein extracts from mycelia grown for 16 h at 37°C. β-Galactosidase specific activity is expressed as A420 nm × 10−6 per minute per milligram of soluble protein. One unit of NADP-GDH specific activity is defined as 1 nmol NADP reduced per minute per milligram of soluble protein. Protein concentrations were calculated according to Bradford (1976) using the Bio-Rad Protein Assay reagent (Bio-Rad) following the manufacturer’s instructions.

**Molecular techniques.** *Escherichia coli* strains used in this study were Top 10 (Invitrogen) and NM522 (Promega). Standard DNA manipulation methods were as described by Sambrook & Russell (2001). Ex Taq (TaKaRa) or Phusion (Finzymes) DNA polymerases

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Table 1. A. nidulans strains used in this study

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| MH12106 | A.f.pyroA–gdhA (–487 to –368)–gpdA

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were used for PCR. Restriction enzymes (Promega) were used following the manufacturer’s instructions. Plasmid DNA was prepared using the Wizard Plus SV Miniprep DNA Purification System (Promega) and genomic DNA was isolated according to Lee & Taylor (1990). Applied Genetic Diagnostics (University of Melbourne, Australia) or Kansas State University DNA Sequencing and Genotyping Facility (Kansas, USA) carried out DNA sequencing. For Southern analysis, DNA was transferred from gels to Hybond-N+ membranes (GE Healthcare) using 0.4 M NaOH. Probes were made either by random hexamers priming with [α-32P]dATP (Bresatec) and the Klenow fragment of DNA polymerase I (Promega) or the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) following the manufacturer’s instructions.

Deletion of AN0840 (leuC). A 3.3 kb genomic DNA fragment containing AN0840 (leuC) (–975 to +2302) was amplified from MH1 using primers leuCFwd (5′–CCACCTTTCCATAAGTTGAA–3′) and leuCrev (5′–ATTATATGGCGGTGAGCA–3′), and cloned into pGEM-T-easy (Promega) to create pBT7168. The BamHI–NruI fragment of pSM6358 containing the A. fumigatus pyroA (A.f.pyroA) selectable marker was cloned into the BglII and StuI sites of leuC in pBT7168 replacing bases –92 to +1986 and creating plasmid pDD7357. The deletion cassette was amplified with leuCFwd and leuCrev, and transformed into MH11036 (pyroA4 nkuA:: Bar riboB2). Transformants were selected for pyridoxine prototrophy, tested for leucine auxotrophy and single-copy gene replacement of leuC confirmed by Southern analysis. Segregation was confirmed by crossing to MH11879 (wA1 pyroA4). Complementation of the leuCA mutant was performed by gene-targeting WT leuC (pBT7168) to leuCA 5′ flanking sequences by single crossover in RT219 (pyroA4 nkuA:: Bar riboB2). Leucine prototrophs were directly selected. Integration of leuC+ was confirmed by Southern blot.

Construction of a leuCB69A mutant. pRP5390 (Polotnianka et al., 2004) was digested with Stul/EcoRV and self-ligated to produce pBT6962. Inverse PCR of pBT6962 using leuBfinv1 (5′–Phos-GAC-GGACGCTTCCCGCTG–3′; mutation underlined) and leuBzinv2 (5′–Phos–CATGGATCTCGACAAACATC–3′) and subsequent ligation mutated TG to GC in pBT7164. The Smal–Spel fragment was ligated into SmaI–Spel-cut pSM636 containing A.f.pyroA to create pDD7356. pDD7356 was transformed into MH12609 (yA1 pabaA1 leuΔ pyroA4 nkuA:: Bar niiA4) and transformants were selected for pyridoxine prototrophy. Single-copy integrants targeted to the 5′ flank of the leuBΔ were confirmed by Southern analysis.

Construction of gdhA reporter fusions. gdhA promoter fragments were amplified from PCR by MH1 genomic DNA using the gdhA5xho (5′–CCGCTTCGAGCGCATATACGACGCGACG–3′) forward primer with the gdhA2BglII (5′–GGAGATCTGGAGGTTAGACAGTTTGGCG–3′) reverse primer. PCR products digested with Xhol and BglII were ligated into Xhol/BamHI-cut pYS225, a plasmid containing a 3′ truncated gpdA min–lacZ fusion from pAN5-mini.
lacking the 3’ 370 codons of lacZ (Punt et al., 1995) and A.f.pyroA from pSM6363 (M. J. Hynes, M. A. Davis and S. Murray unpublished). Ligation into Xhol/BamHI sites replaced the gda sequence creating translational fusion of gdhA–lacZ in pSL6976. The −487 to −368 bp gdhA promoter fragment was amplified with gdhAfwXho2 (5’-CCGCTCGAGGCTTCGGCCAGAATAA-3’) and gdhArevPat1 (5’-AATCGCAGAGGACCATAGGATCGGAA-3’). The Xhol/Pat1-digested PCR product was cloned into pYS7225 creating pSL6973, containing the gdhA promoter fragment in the gdpApmin–lacZ reporter. These plasmids were transformed into MH11094 (anrd±lacZ pyroA nkuA mlaA) and targeted to anrd±lacZ to generate full-length gdhA–lacZ fusions by homologous recombination (Nayak et al., 2006). Transforms were selected for pyridoxine prototrophy and single-copy integrants were confirmed by Southern analysis.

**Mutation of the gdhA promoter.** Inverse PCR mutagenesis of pDD149 containing the Xhol–HindIII gdhA promoter fragment from pSL6976 in pBluescriptSK+ (Stratagene) was performed by amplification with leu1delfwd (5’-Phos-GTGCGGTCTCAGGCGCGAATGATAA-3’) and leu1delrev (5’-Phos-CAGTATAC-TCTTCCGGGCCG-3’) to delete bases −501 to −492, leu2delfwd (5’-Phos-CAGTATAC-TCTTCCGGGCCG-3’) and leu2delrev (5’-Phos-AATCCGGGAGATCTTGCTGAC-3’) to delete bases −250 to −241, condelfwd (5’-Phos-CTCTTGCTGATTATGCTTTGC-3’) and condelrev (5’-Phos-GGTTGTGATTGCGAAGGTTA-3’) to delete bases −431 to −422, leu2delfwd and leu1delrev to delete bases −501 to −421, and leu2delfwd and sk + shover (5’-Phos-CTCTAGGGGGGCGGCCC-3’) to create a 5’ truncation at −240 bp. Self-ligation of PCR products produced pDD134, pDD135, pDD130, pDD205 and pDD208, respectively. pDD134 was further amplified with leu2delfwd and leu2delrev, and ligated to create pDD136; subsequent amplification with condelfwd and condelrev produced pDD204. The mutated Xhol–HindIII promoter fragments were cloned into Xhol/HindIII-cut pSL6976, replacing the WT gdhA promoter, to make pDD137 (Δ−501 to −492), pDD138 (Δ−250 to −241), pDD131 (Δ−431 to −422), pDD207 (Δ−501 to −241), pDD210 (−240 bp), pDD139 (Δ−501 to −429, Δ−250 to −241) and pDD206 (Δ−501 to −492, Δ−431 to −422, Δ−250 to −241). These plasmids were gene-targeted in single copy in MH11094 as above.

**DNA-binding site alignments.** The 1.0 kb gdhA upstream sequences from Aspergillus clavatus, A. nidulans, Aspergillus niger, Aspergillus oryzae, Aspergillus flavus, Aspergillus fumigatus, Aspergillus terreus and Neurospora crassa (also known as Aspergillus fischeri) were obtained from the Broad Institute database (http://www.broadinstitute.org/annotation/genome/aspergillus_group). The 1.0 kb upstream sequences of luA, leuC, ANO912, ANO4956 and AN2525 were sourced from the Aspergillus Genome Database, AspGD (www.aspgd.org) (Arnaud et al., 2012). Putative DNA-binding sites and flanking nucleotides were identified through manual searches for CCGN4CGG consensus sites and manually aligned. Consensus motifs of aligned sequences were generated using WebLogo (www.weblogo.berkeley.edu) (Crooks et al., 2004; Schneider & Stephens, 1990).

**DNA and protein sequences and phylogenetic analyses.** Gene identification and primary analysis were performed using AspGD and SGD (Arnaud et al., 2012; Cherry et al., 2012). In silico manipulation of DNA and protein sequences was carried out in Geneious version 5.3.5 created by Biomatters (http://www.geneious.com). Alignments, unless otherwise stated, were performed using CLUSTALW2 (Larkin et al., 2007) and shaded using MacBoxshade 2.15E (M. D. Baron) with default settings. For phylogenetic analysis of ascomycete NADP-GDH promoters, genes were identified by BLASTP using full-length A. nidulans GdhA and S. cerevisiae Gdh1p, and the Broad Institute Fungal Genomes Initiative FGI: Fungal Genomes Protein database (http://www.broadinstitute.org/annotation/genome/FGI_Blast/blast. html, 15 April 2013). The NADP-GDH protein sequences identified were used to construct an unrooted neighbour-joining phylogenetic tree in Geneious using the default settings.

**RESULTS**

**Leucine inhibits gdhA expression.** In S. cerevisiae, the LeuB orthologue, Leu3p, is activated by α-IPM, a leucine biosynthesis intermediate produced when cellular levels of leucine are low. High cellular leucine levels deplete α-IPM and cause Leu3p to function as a repressor (reviewed by Kohlhaw, 2003). To determine the effects of leucine availability on gdhA regulation in A. nidulans we examined the effects of exogenous leucine on levels of the native NADP-GDH enzyme and a gdhA–lacZ reporter gene. We gene-targeted in single copy a reporter construct containing a translational fusion of −753 bp of the gdhA promoter to lacZ. Like the native gdhA transcript and NADP-GDH enzyme (Hawkins et al., 1989; Pateman, 1969), the gdhA–lacZ reporter gene was highly expressed during growth in the presence of ammonium or glutamine and had reduced levels of expression during growth on glutamate (Fig. 1a). Consistent with previous reports of NADP-GDH enzyme activity (Pateman, 1969), over the 5–20 mM range the effect of nitrogen source rather than the effect of nitrogen concentration was the main determinant of gdhA–lacZ levels. Both the gdhA–lacZ reporter (Fig. 1b) and NADP-GDH encoded by the WT gene (Table 2) showed lower levels of activity in the presence of 2 mM leucine than in its absence, consistent with conservation of the mechanism of leucine metabolic control of NADP-GDH expression between A. nidulans and yeast. No negative effect on levels of the gdhA–lacZ reporter resulted from addition of the other two branched-chain amino acids, isoleucine and valine, or by tyrosine (Fig. 1b).

**α-IPM levels modulate gdhA expression.** During leucine biosynthesis, α-IPM produced by α-IPM synthetase is converted by α-IPM isomerase to β-isopropylmalate (β-IPM). In A. nidulans, α-IPM isomerase is encoded by lnuA and the loss-of-function mutant lnuA1 is a leucine auxotroph (Polotnianka et al., 2004). However, the gene for α-IPM synthetase has not been characterized. S. cerevisiae α-IPM synthetase is encoded by two genes, LEU4 and LEU9 (Casalone et al., 2000; Chang et al., 1984, 1985). BLASTP searches of the A. nidulans non-redundant protein sequence database with Leu4p and Leu9p identified a single protein encoded by AN0840, which we named leuC. BLASTN and TBLASTN searches of the A. nidulans genome sequence indicated that AN0840 is unique. Alignment of Leu4p, Leu9p and LeuC protein sequences revealed strong conservation throughout the protein, including the leucine feedback regulatory R-region (Cavalieri et al., 1999) (Fig. 1c), consistent with the three α-IPM synthetases sharing the
Leucine biosynthesis regulates \( \text{gdhA–lacZ} \). (a) LacZ enzyme activity was assayed for the \( \text{gdhA–lacZ} \) reporter strain, with 753 bp of the \( \text{gdhA} \) promoter fused to \( \text{lacZ} \), grown on 1 % glucose-minimal media (ANM) supplemented with 5, 10 or 20 mM ammonium tartrate (NH\(_4\)), glutamate (GLU) or glutamine (GLN), and (b) grown on ANM with 10 mM ammonium tartrate in the presence or absence of 2 mM leucine (LEU), isoleucine (ILE), valine (VAL) or tyrosine (TYR) for 16 h at 37 °C. (c) Alignment of \( \alpha\)-IPM synthetases from \( \text{S. cerevisiae} \) and \( \text{A. nidulans} \). The protein sequences for \( \text{S. cerevisiae} \) Leu4p (YNL104C), Leu9p (YOR108W) and \( \text{A. nidulans} \) LeuC (AN0840) were aligned using CLUSTALW and shaded using MacBoxshade. The R-region in Leu4p, which is required for leucine feedback regulation (Cavalieri et al., 1999), is underlined. (d) \( \text{leuC} \) (AN0840) was deleted by gene replacement with an \( \text{A.f.pyroA} \) cassette. Black boxes, \( \text{leuC} \) exons; horizontal lines, \( \text{leuC} \) flanking DNA or introns; grey box, selectable marker. The direction of \( \text{leuC} \) transcription is indicated by an arrow. (e) \( \text{luA1} \), \( \text{leuB} \), \( \text{leuBC^{C69A}} \) and \( \text{leuC} \) leucine biosynthesis mutants as well as complemented \( \text{leuCA} \) (\( \text{leuCD, leuC}^{+} \)) and WT strains were grown for 2 days on 10 mM ammonium tartrate in the presence of 0, 2, 5 and 10 mM leucine. (f) A WT strain and leucine biosynthesis mutants \( \text{luA1} \) and \( \text{leuC} \) were assayed for expression of the \( \text{gdhA–lacZ} \) reporter after 16 h growth in ANM supplemented with 10 mM ammonium tartrate and 2 mM leucine at 37 °C. For assays in (a), (b) and (f) soluble protein extracts were prepared from mycelia and measured for \( \beta\)-galactosidase specific activity. Error bars, SEM (\( n \geq 3 \)).
Table 2. Leucine biosynthesis regulates NADP-GDH activity

| Genotype  | NADP-GDH enzyme activity* |  |  |
|-----------|---------------------------|  |  |
|           | NH₄  | NH₄ + 2 mM leucine |  |  |
| WT        | 118.4 (±7.88)  | 62.35 (±8.48) |  |  |
| leuΔ      | 42.84 (±3.38)  | ND  |  |  |
| leuC<sup>C69A</sup> | 66.83 (±4.71)  | ND  |  |  |
| luA1      | ND  | 142.1 (±16.43) |  |  |

*NADP-GDH enzyme specific activity was assayed for soluble protein extracts of mycelia grown in 1 % glucose-minimal media supplemented with 10 mM ammonium tartrate (NH₄) for 16 h at 37 °C in the presence or absence of 2 mM leucine. SEM is given in parentheses (n≥3). ND, Not determined.

The same common ancestor (Larson & Idnurm, 2010). LeuC has a C-terminal extension absent in both Leu4p and Leu9p. We cloned the leuC gene using PCR primers designed to the genome sequence and deleted leuC by homologous gene replacement with the A. f. pyroA selectable marker (Fig. 1d). The leuCΔ mutant is a strict leucine auxotroph, indicating that leuC is the only significant z-IPM synthetase-encoding gene in A. nidulans (Fig. 1e). In an outcross, leucine auxotrophy segregated as a single gene with reduced viability of leuCΔ progeny and co-segregated with the A. f. pyroA selectable marker (data not shown). Complementation of leuCΔ with WT leuC restored leucine prototrophy (Fig. 1e).

The luA1 z-IPM isomerase mutant, predicted to have higher levels of z-IPM due to inability to convert z-IPM to β-IPM, showed higher levels of gdhA–lacZ expression (Fig. 1f) and NADP-GDH enzyme specific activity (Table 2) than the WT strain. Conversely, the leuCΔ z-IPM synthetase mutant, unable to synthesize z-IPM, had lower levels of gdhA–lacZ expression than WT (Fig. 1f). Overall, the genetic data indicate that the levels of z-IPM influence the levels of activation of gdhA expression.

**LeuB DNA-binding domain is required for function**

To determine the contribution of LeuB to gdhA expression we measured NADP-GDH enzyme activity and gdhA–lacZ reporter levels in a leuBΔ mutant. NADP-GDH enzyme activity (Table 2) and gdhA–lacZ reporter levels (Fig. 2a) were reduced in a leuBΔ mutant compared with WT during growth on ammonium. Unlike in WT, there was no negative effect of leucine addition in a leuBΔ mutant. Therefore, inhibition of gdhA–lacZ expression by leucine is LeuB dependent. To avoid any potential complications due to effects of leuBΔ on general protein synthesis and amino acid starvation, leuB mutant strains were supplemented with 2 mM leucine for subsequent gdhA–lacZ experiments.

To determine whether LeuB contributes to gdhA expression as a DNA-binding protein, we assessed the effect of specifically altering the LeuB zinc cluster DNA-binding motif while retaining the remainder of the protein. Mutation of the third cysteine codon of the Leu3p zinc cluster confers loss of function (Bai & Kohlhaw, 1991). We mutated the equivalent codon to an alanine codon to generate leuB<sup>C69A</sup> (Fig. 2b). The mutant gene was targeted in single copy at leuB in a leuBΔ mutant (Fig. 2c). The
leuB<sup>C69A</sup> transformant, like leuB<sub>Δ</sub>, is a leaky leucine auxotroph (Fig. 1e). Furthermore, the level of reduction of gdhA–lac<sub>Z</sub> activity was equivalent for leuB<sub>Δ</sub> and leuB<sup>C69A</sup> strains (Fig. 2a). Mutation of the LeuB DNA-binding domain also reduced NADP-GDH enzyme activity expressed from the WT promoter (Table 2). Therefore, although currently it is not possible to preclude other effects of this mutation such as those on protein stability or protein folding, the most likely explanation is that the DNA-binding domain of LeuB is required for WT regulation of both leucine biosynthesis and gdhA expression.

**Identification of predicted LeuB DNA-binding sites in the gdhA promoter**

Zn(II)2Cys6 proteins usually bind DNA as dimers. The amino acid sequence between the final cysteine residue of the zinc binuclear cluster and the first heptad repeat of the coiled-coils together with the DNA sequence of the target site are thought to determine DNA-binding site specificity (Liang et al., 1996; Marmorestein et al., 1992; Noël & Turcotte, 1998; Reece & Ptashne, 1993). Alignment of the linker–heptad regions of Leu3p and LeuB showed conservation of both the linker length and hydrophobic residues at the 1 and 4 positions of three heptads (Fig. 3a). Therefore, like Leu3p, LeuB is predicted to bind inverted CCG triplet repeats separated by a 4 nt spacer, CCGN<sub>4</sub>CGG (Hellauer et al., 1996; Marmorestein et al., 1992; Noël & Turcotte, 1998; Mamane et al., 1998). We compared the promoter sequences of five A. nidulans leucine biosynthesis pathway genes with S. cerevisiae homologues that are directly regulated by Leu3p binding. The 1.0 kb sequences upstream of the ATG start codons of luA (LEU1), AN0912 (LEU2), leuC (LEU4), AN4956 (ILV2) and AN2525 (ILV5) contain multiple direct, inverted and everted CCG/CGG triplet repeat pairs; however, the only spacing between triplets found in all five promoters was 4 bp (data not shown), consistent with LeuB recognizing DNA-binding sites conforming to a CCGN<sub>4</sub>CGG consensus. Interestingly, the N<sub>4</sub> spacer region of putative LeuB DNA-binding sites shows less conservation than that for Leu3p (Fig. 3b). In S. cerevisiae, Leu3p recognizes the same consensus motif in the GDH<sub>1</sub> promoter as that found in leucine biosynthesis gene promoters (Hellauer et al., 1996). We also identified two putative LeuB DNA-binding sites, CCGCAAGCGG and CCGACTCCGG at −501 to −492 and −250 to −241 bp, respectively, upstream of the gdhA start codon (Fig. 3c). We searched the promoters of gdhA genes from seven closely related Aspergillus species for CCGN<sub>4</sub>CGG sites. In all of these species, a proximal site was found within 300 bp of the gdhA start codon, and in three species (A. fumigatus, A. terreus and N. fischeri) a second distal site was also identified between 780 and 470 bp upstream of the start codon. Alignment of the distal sites and proximal sites from each species shows absolute conservation of the proximal binding site, whereas the distal binding site has conserved triplets but less conservation of the spacer region (Fig. 3d).

**Putative LeuB binding sites are present in NADP-GDH gene promoters in many ascomycetes**

Regulation of NADP-GDH expression by leucine biosynthesis has only been reported in A. nidulans (Polotnianka et al., 2004; this work) and S. cerevisiae (Hu et al., 1995). We were interested to what extent this mechanism may operate throughout the Ascomycota. By BLASTP analysis of sequenced genomes available from the Broad Institute searched with the protein sequences of Gdh1p and GdhA, we identified 44 NADP-GDH-encoding genes from ascomycetes. These 44 genes represent 21 unique genera and 38 unique species. Our analysis included multiple sequenced isolates of Candida albicans (two), Histoplasma capsulatum (three) and Paracoccidioides brasiliensis (three) for within-species comparison. S. cerevisiae was the only species with multiple NADP-GDH-encoding genes. No putative Leu3p/LeuB sites (CCGN<sub>4</sub>CGG) were found 1.0 kb upstream of 18 gdhA orthologues, 16 NADP-GDH gene promoters contained a single putative LeuB binding site and 10 of the promoters contained two predicted LeuB sites (Fig. 4). None of the promoters contained more than two putative LeuB/Leu3p DNA-binding sites. Interestingly, two of the P. brasiliensis isolates had one putative binding site, whereas the third isolate had two putative LeuB/Leu3p binding sites. Fungi that lacked putative LeuB/Leu3p DNA-binding sites upstream of the NADP-GDH gene were generally clustered into closely related genera (Fig. 4), suggesting those clades may have lost direct leucine regulation of gdhA orthologues. LeuB/Leu3p DNA-binding sites upstream of gdhA were found in all Aspergillus species analysed, in N. crassa and H. capsulatum as well as in some Candida species, but were not found in either the fission yeasts or the fusaria.

**LeuB activates gdhA expression via the proximal consensus LeuB DNA-binding site**

To determine whether LeuB acts via either or both of the two putative LeuB DNA-binding sites in the A. nidulans gdhA promoter we deleted both sites separately and in combination from the gdhA–lac<sub>Z</sub> reporter gene (Fig. 5a). Deletion of the highly conserved proximal site, −250 to −241, resulted in strongly reduced β-galactosidase levels. Therefore, the proximal site likely mediates LeuB activation. However, deletion of the distal site, −501 to −492, caused an increase in expression of gdhA–lac<sub>Z</sub> compared with the WT promoter. When both predicted LeuB DNA-binding sites were removed from the gdhA–lac<sub>Z</sub> promoter expression was higher than when the proximal site alone was deleted, indicating that the negative effect of deleting the proximal site was countered by removal of the distal site. We measured expression of the gdhA–lac<sub>Z</sub> reporter genes lacking either or both LeuB DNA-binding sites in the leuB<sub>Δ</sub> mutant. Two key observations inform our understanding of gdhA regulation. First, all three mutated reporter genes, including...
the reporter lacking both LeuB sites, showed leucine inhibition via WT LeuB and reduced expression in the leuB mutant, indicating that leuB and deletion of the LeuB DNA-binding sites are additive (Fig. 5a). Therefore, LeuB contributes to activation of gdhA expression independently of the consensus LeuB DNA-binding sites, either via regulation of another transcription factor that activates gdhA expression or by LeuB DNA binding at a non-consensus regulatory element. Second, gdhA–lacZ levels were lower in the leuB mutant in the absence of the proximal site than when it remained intact, suggesting that an unknown positive factor activates gdhA expression via the proximal site or an overlapping sequence in the absence of LeuB.

LeuB acts via a non-consensus site in the gdhA promoter

To determine the location of the additional non-consensus LeuB site of action within the gdhA promoter we created a −240 bp gdhA–lacZ 5’ truncated fusion containing the shortest gdhA promoter that retains the TATA-box. The −240 bp gdhA–lacZ reporter gene supported low basal levels of expression, but was not affected by the addition of leucine or by leuB (Fig. 5a). Therefore, LeuB acts within the −753 to −241 bp region upstream of gdhA. We next constructed a variant of the −753 bp gdhA–lacZ reporter lacking both LeuB consensus motifs and the intervening region (Δ−501 to −241 bp gdhA–lacZ). Like the −240 bp
reporter, neither addition of leucine nor leuΔ reduced expression of the Δ−501 to −241 bp gdhA–lacZ reporter (Fig. 5a), localizing the additional LeuB site of action between the two LeuB consensus sites. To confirm the additional site of action for LeuB we inserted gdhA promoter sequences from the region between the consensus sites, −487 to −368, into the minimal promoter of the gdpApmin–lacZ reporter gene (Punt et al., 1995) to create gdhAmin–lacZ. We assayed this reporter in WT and the leuΔ mutant, and found that either the addition of leucine or leuΔ conferred reduced reporter gene expression (Fig. 5b). Assays of a control gdpApmin–lacZ reporter containing a fragment of the acuJ promoter (Hynes et al., 2006) showed no negative effect of leucine or leuΔ (Fig. 5c). Therefore, LeuB activation of the gdhA promoter is mediated, in part, independently of LeuB consensus DNA-binding sites through the −487 to −368 bp region.

In a separate study, we have identified a positive-acting promoter element CCGAATTCGG located at −432 to −422 bp upstream of the gdhA ATG conserved within

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### Table: LeuB/Leu3p consensus sites

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Sequence</th>
<th>Location</th>
<th>Sequence</th>
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<tbody>
<tr>
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<td>−420</td>
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<td>−700</td>
<td>CCGTAATCGG</td>
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<td>Aspergillus niger</td>
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<td>−700</td>
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</tr>
<tr>
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<td>−700</td>
<td>CCGATTCGG</td>
</tr>
<tr>
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<td>−419</td>
<td>CCGTAATCGG</td>
<td>−700</td>
<td>CCGATTCGG</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>−419</td>
<td>CCGTAATCGG</td>
<td>−700</td>
<td>CCGATTCGG</td>
</tr>
<tr>
<td>Magnaportha oryzae</td>
<td>−419</td>
<td>CCGTAATCGG</td>
<td>−700</td>
<td>CCGATTCGG</td>
</tr>
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<td>Neurospora crassa</td>
<td>−419</td>
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</tr>
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<td>−700</td>
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**Fig. 4.** LeuB/Leu3p DNA-binding sites are present in NADP-GDH gene promoters within the Ascomycota. BLASTp was used to identify 44 NADP-GDH-encoding genes from ascomycetes. The 1.0 kb sequence upstream of the first ATG was searched for the presence of predicted LeuB/Leu3p CCGG DNA-binding sites. Promoter sequences were sourced from the Broad Institute Fungal Genome Initiative or for *F. fujikuroi* from GenBank. Cladogram showing evolutionary relationship of NADP-GDH orthologues constructed using protein sequences for each represented NADP-GDH gene. Branches represent relationships between orthologues, but not evolutionary distance. The CCGAATTCGG conserved element is only present in *Aspergillus* species *gdhA* promoters (†).
Aspergillus species gdhA promoters (D. J. Downes, M. A. Davis, K. H. Wong, S. D. Kreutzberger, M. J. Hynes & R. B. Todd, in preparation). This element contains a CCGN$_2$CGG motif similar to the LeuB consensus motif, varying only by 1 nt within the spacer. We deleted 10 nt of this element (Δ–431 to –422) from the –753 bp reporter

Fig. 5. LeuB regulates gdhA through two conserved sites. (a) gdhA–lacZ reporters containing deletions of putative LeuB regulatory elements, (b) the gpdA$_{min}$–lacZ reporter containing the –487 to –368 region of the gdhA promoter, and (c) the gpdA$_{min}$–lacZ reporter containing a fragment of the acuJ promoter were assayed in WT and leuBΔ strains grown in supplemented 1% glucose-minimal medium containing 10 mM ammonium tartrate for 16 h at 37 °C with or without 2 mM leucine (LEU). Soluble protein extracts were prepared from mycelia and assayed for β-galactosidase specific activity. Error bars, SEM (n ≥ 3).
lacking both predicted LeuB consensus sites. This triple-site deletion reporter was unaffected by either addition of leucine or leuBΔ (Fig. 5a), indicating that LeuB does not act at sites other than those deleted in this construct and that this conserved element is a LeuB site of action. A −753 bp gdhA-lacZ reporter lacking only the conserved element but retaining the proximal and distal LeuB consensus sites was reduced in a leuBΔ strain compared with WT (Fig. 5a), consistent with LeuB activation via the proximal LeuB consensus site. Therefore, the full LeuB activation of gdhA expression occurs via both the proximal consensus site and the non-consensus element. We searched the promoters of the 44 ascomycete NADP-GDH-encoding genes analysed above and found the CCGGACTTCGG element conserved only within Aspergillus species (Fig. 4).

DISCUSSION

NADP-GDH functions as a key enzyme in nitrogen assimilation and draws a substrate directly from the tricarboxylic acid cycle, intricately involving NADP-GDH in core carbon and nitrogen metabolism. gdhA expression therefore responds to metabolites sourced from the environment and through cellular processes via metabolic flux. Indeed, gdhA displays differential expression in response to ammonium, glutamate or glutamine and responds to nitrate (Pateman, 1969; Schinko et al., 2010). We have now shown that gdhA is regulated by leucine biosynthesis intermediates via the LeuB transcription factor. Therefore, regulation of gdhA expression is a potential response to metabolic flux. Leucine, despite comprising a significant proportion of the protein-incorporated amino acids in Aspergillus species, is one of the least-abundant free amino acids, representing <1% of the amino acid pool (Berger et al., 2008; Stokes & Gunnness, 1946). Inherently low levels of free leucine likely make it a sensitive sensor for general depletion of amino acids and may signal a need for greater levels of glutamate synthesis to replenish amino acid levels. Branched-chain amino acid biosynthesis requires glutamate as the amine donor in the final transamination step for the production of isoleucine, valine and leucine. In *S. cerevisiae*, leucine biosynthesis genes are regulated by Leu3p binding to a well-defined CCGN4CGG binding site (reviewed by Kohlhaw 2003). We have shown that the equivalent structural gene promoters in *A. nidulans* share a similar consensus binding site, as does the *A. nidulans* gdhA promoter. Furthermore, we have shown by perturbing α-IPM levels both physiologically with the addition of exogenous leucine and genetically by disrupting either α-IPM synthetase or α-IPM isomerase, that the activity of LeuB, like Leu3p, responds to α-IPM levels (Hu et al., 1995). Additionally, leucine inhibition of gdhA expression is directly mediated by LeuB, which requires a functional DNA-binding domain for gdhA regulation. Similarly, *S. cerevisiae* GDH1 is directly regulated by Leu3p via UAS binding sites in an α-IPM-dependent manner (Hu et al., 1995). Regulation of NADP-GDH by leucine biosynthesis is therefore conserved between *A. nidulans* and *S. cerevisiae*. We determined the extent to which leucine biosynthesis regulation of NADP-GDH may be conserved throughout the Ascomycota by analysis of 5′ gdhA homologues for LeuB/Leu3p consensus DNA-binding sites. Interestingly, numerous species have consensus CCGN4CGG binding site motifs in their NADP-GDH gene promoters, suggesting widespread conservation of LeuB/Leu3p regulation. Some species lack CCGN4CGG consensus sites as well as the non-canonical LeuB target site we found 5′ of *A. nidulans* gdhA, suggesting alternative regulatory mechanisms. For these species nitrogen may be preferentially incorporated via glutamine synthetase and glutamate synthetase, as is the case in some ectomycorrhizal fungi (Morel et al., 2006). In *F. fujikuroi*, a species lacking a LeuB/Leu3p site in the NADP-GDH promoter, GS plays both functional and regulatory roles in nitrogen metabolism (Teichert et al., 2004).

Our analysis of LeuB consensus sites in the *A. nidulans* gdhA promoter provided several new insights into the regulatory mechanisms underlying gdhA expression. Based on our finding that LeuB played a positive role in regulating gdhA expression and that the LeuB zinc finger was essential for this activation, we initially predicted that both sites would mediate this regulation. Deletion of the proximal site was consistent with this, resulting in significantly reduced gdhA expression and indicating that LeuB activation of gdhA occurs in part via the highly conserved proximal site. However, deletion of this site alone did not abolish the action of LeuB, suggesting that additional sequences were involved. Deletion of the distal site did not influence the contribution of LeuB, but rather led to an increase in expression. In addition to acting at the proximal LeuB site, we found that LeuB also acts via a novel site of action, which does not conform to the Leu3p DNA-binding site consensus. This novel element is conserved within *Aspergillus* species and, like the LeuB/Leu3p consensus motif, contains inverted CCG triplets, although the triplets are separated by a 5 bp spacer (CCGN4CGG). For Zn(II)2Cys6 transcription factors, spacer length is a key factor determining DNA-binding specificity, based in part on recognition by the linker region (MacPherson et al., 2006; Schjerling & Holmberg, 1996; Todd & Andrianopoulos, 1997). In vitro DNA binding of Leu3p is lost if the length of the spacer region is altered (Hellauer et al., 1996), thus it is unlikely that LeuB binds this site as a homodimer. One possibility is that LeuB may indirectly exert its effect by activating expression of another transcription factor, which binds this site. Leu3p is known to bind to the promoters of several other transcription factors (Tang et al., 2006). Alternatively, LeuB may bind this site directly as a heterodimer with another Zn(II)2Cys6 transcription factor, as seen for *A. nidulans* AcuK and AcuM (Suzuki et al., 2012), and *S. cerevisiae* Oaf1p and Pip2p (Rottensteiner et al., 1997; Trzcinska-Danielewicz et al., 2008) or Pdr1p and Pdr3p (Mannun et al., 2002; Wolfger et al., 1997). This novel site mediates a
significant contribution to gdhA expression. Therefore, the combined action of LeuB via the proximal consensus site and the conserved element, possibly by heterodimerization with or regulation of an unknown factor, may be a means by which cells utilize leucine levels to sense changes in nitrogen metabolic flux and respond by altering levels of the key nitrogen assimilation enzyme NADP-GDH.

ACKNOWLEDGEMENTS

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