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Transcriptional Control of Gluconeogenesis in *Aspergillus nidulans*

Michael J. Hynes,*,[1] Edyta Szewczyk,*,[2] Sandra L. Murray,* Yumi Suzuki,* Meryl A. Davis* and Heather M. Sealy-Lewis†

**Department of Genetics, University of Melbourne, Victoria, 3010 Australia and* †*Department of Biological Sciences, University of Hull, Hull HU6 7RX, England*

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ABSTRACT

Aspergillus nidulans can utilize carbon sources that result in the production of TCA cycle intermediates, thereby requiring gluconeogenesis. We have cloned the *acuG* gene encoding fructose-1,6 bisphosphatase and found that expression of this gene is regulated by carbon catabolite repression as well as by induction by a TCA cycle intermediate similar to the induction of the previously studied *acuF* gene encoding phosphoenolpyruvate carboxykinase. The *acuN356* mutation results in loss of growth on gluconeogenic carbon sources. Cloning of *acuN* has shown that it encodes enolase, an enzyme involved in both glycolysis and gluconeogenesis. The *acuN356* mutation is a translocation with a breakpoint in the 5′ untranslated region resulting in loss of expression in response to gluconeogenic but not glycolytic carbon sources. Mutations in the *acuK* and *acuM* genes affect growth on carbon sources requiring gluconeogenesis and result in loss of induction of the *acuF*, *acuN*, and *acuG* genes by sources of TCA cycle intermediates. Isolation and sequencing of these genes has shown that they encode proteins with similar but distinct Zn(2) Cys(6) DNA-binding domains, suggesting a direct role in transcriptional control of gluconeogenic genes. These genes are conserved in other filamentous ascomycetes, indicating their significance for the regulation of carbon source utilization.

An important feature of filamentous fungi in their role in the environment and as pathogens is their ability to metabolize a diverse range of organic molecules as carbon sources. Most filamentous fungi are saprophytes that grow on environmental compounds as nutrients. Growth on stored carbon sources is also important during various developmental stages and for the provision of substrates in the synthesis of secondary metabolites. The utilization of carbon sources during infection by fungal pathogens may have profound effects on pathogenicity (*e.g.*, Thines *et al.* 2000; Lorenz and Fink 2002; Wang *et al.* 2003; Barelle *et al.* 2006).

For a substrate serving as the sole carbon source, all cellular carbon components must be derived from this compound. Organisms rearrange the expression of genes encoding enzymes catalyzing the appropriate steps in metabolic pathways according to the substrates available usually by induction of enymes specific to the breakdown of the particular compound. It is also necessary to alter the central carbon metabolic pathways to enable the generation of essential carbon-containing biosynthetic intermediates and energy and reducing power to deal with metabolic stresses. This is clearly shown by comparing growth on sugars that feed into glycolysis with growth on compounds that result in tricarboxylic acid (TCA) cycle intermediates where there is a requirement for the net formation of sugars—*i.e.*, gluconeogenesis, a reversal of glycolysis with oxaloacetate being converted to hexose sugars. Futile cycling between degradation and biosynthesis of sugars by glycolysis opposed by gluconeogenesis is avoided by appropriate regulation of the synthesis and activity of the relevant enzymes. The key enzymes specific for gluconeogenesis are phosphoenolpyruvate carboxykinase (PEPCK; E.C.4.1.1.32), which converts oxaloacetate to phosphoenolpyruvate, and fructose-1,6-bisphosphatase (FBP; E.C.3.1.3.11), which catalyzes the final step in hexose monophosphate formation—the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and phosphate.

Saccharomyces cerevisiae has a strong preference for growth on fermentable monosaccharides, resulting in the production of ethanol. When glucose is exhausted, metabolism switches to a respiratory mode in which the ethanol is consumed by rearrangement of gene expression (DeRisi *et al.* 1997; Schuller 2003; Daran-Lapujade *et al.* 2004). In contrast to filamentous fungi, *S. cerevisiae* can use only a limited number of gluconeogenic substrates as the sole sources of carbon and

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[1]*Corresponding author:* Department of Genetics, University of Melbourne, Victoria, 3010 Australia. E-mail: mjhynes@unimelb.edu.au

[2]*Present address:* Department of Molecular Genetics, Ohio State University, Columbus, OH 43210.

energy. These include ethanol, acetate, and fatty acids, all of which result in the production of acetyl-CoA. The enzymes isocitrate lyase (ICL; E.C.4.1.3.1) and malate sythase (MAS; E.C.4.1.3.2), constituting the glyoxalate cycle, are necessary for the net conversion of acetyl-CoA to oxaloacetate, which is then used for gluconeogenesis. The Zn(2) Cys(6) binuclear (Schjerling and Holmberg 1996; Todd and Andrianopoulos 1997) proteins, Cat8 and Sip4 (Rahner *et al.* 1996; Haurie *et al.* 2001; Schuller 2003; Roth *et al.* 2004), control the expression of the genes for the synthesis of acetyl-CoA and its metabolism by the glyoxalate bypass as well as gluconeogenesis. Some of the genes are also regulated by the Cys2His2 zinc-finger protein Adr1 (Young *et al.* 2003). Therefore, growth on ethanol or acetate as sole carbon sources is dependent on the Cat8, Sip4, and Adr1 activators as well as on the Snf1 kinase (Vincent and Carlson 1998; Young *et al.* 2003). In the presence of glucose, the Cys2His2 zinc-finger Mig1 protein represses the expression of gluconeogenic genes both directly and by repressing the expression of Cat8 (Gancedo 1998; Zaragoza *et al.* 2001; reviewed in Schuller 2003). Single genes encode PEPCK (*PCK1*) and FBP (*FBP1*) and their transcription is dependent on Cat8/Sip4 activation. The levels of these enzymes are also regulated post-transcriptionally in response to glucose. The 5′ regions of genes regulated by Cat8/Sip4 contain *cis*-acting elements (carbon source response elements) to which Cat8p and Sip4p bind (Zaragoza *et al.* 2001; Schuller 2003; Roth *et al.* 2004). Therefore, in *S. cerevisiae* the expression of genes for both the glyoxalate cycle and gluconeogenesis is controlled by a single circuit that responds to a lack of the fermentable carbon source glucose. However, in *Kluyveromyces lactis*, regulation of both gluconeogenic genes KlFBP1 and KlPCK1 is independent of KlCat8p (Georis *et al.* 2000), indicating that the roles of Cat8/Sip4 as general regulators of gluconeogenesis are not conserved.

Filamentous fungi can grow at the expense of a great diversity of compounds that feed into the TCA cycle and therefore require gluconeogenesis (Hondmann and Visser 1994). These include not only sources of acetyl-CoA (ethanol, acetate, fatty acids) but also sources of 2-oxoglutarate (amino acids) and sources of both succinate and acetyl-CoA (aromatic acids and fatty acids containing odd numbers of carbon), which are not dependent on the glyoxalate cycle (Arst *et al.* 1975; Kinghorn and Pateman 1976; Kuswandi and Roberts 1992; Brock *et al.* 2000; Brock 2005).

In *Aspergillus nidulans*, mutations in the *facB* gene result in an inability to grow on two-carbon compounds metabolized via acetyl-CoA (Armitt *et al.* 1976; Hynes 1977; Katz and Hynes 1989). FacB is a Zn(2) Cys(6) binuclear cluster protein with similarity to Cat8 and Sip4 of *S. cerevisiae*, and binding sites for this activator have been found in the 5′ region of the acetate-induced genes *acuD* (ICL) and *acuE* (MAS) as well as *facA* (acetyl-CoA synthase) and *facC* (cytoplasmic acetyl carnitine transferase) genes, which are required for growth on acetate as a sole source of carbon (Todd *et al.* 1997, 1998; Stemple et al. 1998). *facB* mutants are unaffected in the utilization of other gluconeogenic carbon sources, clearly suggesting that FacB is a specific regulator of two-carbon metabolism and does not control gluconeogenesis (Armitt *et al.* 1976). This contrasts with *S. cerevisiae* where both the glyoxalate cycle and gluconeogenesis are controlled by a single circuit.

Mutations in the *acuF* gene have been isolated by virtue of their leading to an inability to grow on acetate and found to specifically lack PEPCK activity (Armitt *et al.* 1976). *acuF* mutants are also unable to grow on carbon sources requiring gluconeogenesis (Figure 1). PEPCK activity is induced not only by acetate but also by glutamate, proline, and other sources of TCA cycle intermediates, but is not strongly repressed by glucose (Kelly and Hynes 1981). Analysis of regulation of the *acuF* gene confirmed that expression is induced by sources of TCA cycle intermediates, and mutations preventing the metabolism of inducers to TCA cycle intermediates prevented induction. The pattern of regulation is not consistent wih direct regulation by FacB. Furthermore, deletion analysis of the 5′ region of *acuF* showed that the region responsible for induction lacks FacB-binding sites (Hynes *et al.* 2002).

A. nidulans acuG mutants are unable to grow on acetate and specifically lack FBP activity (Armitt *et al.* 1976) and, consistent with this, they are unable to utilize any gluconeogenic carbon sources, or glycerol (Figure 1). Here we report the isolation of the *acuG* gene and the study of its regulation. As for *acuF*, there is increased expression under conditions where TCA cycle intermediates accumulate, suggesting an induction mechanism, and there is no evidence for direct induction by FacB.

The *acuN356* mutation (Armitt *et al.* 1976) results in loss of growth on gluconeogenic carbon sources (Figure 1). Surprisingly, we have found that *acuN* encodes enolase (E.C.4.2.1.11), one of the reversible enzymes that are essential for both glycolysis and gluconeogenesis. The *acuN356* mutation is due to a translocation with a breakpoint in the 5′ region, resulting in the loss of expression in response to gluconeogenic carbon sources but not glycolytic carbon sources.

The *acuK* and *acuM* genes were identified in the original screen for acetate mutants (Armitt *et al.* 1976). Mutations in these genes do not just affect growth on acetate but also on all carbon sources requiring gluconeogenesis (Figure 1). We have found that the *acuK248* and *acuM301* mutations each result in the loss of induction of the *acuF*, *acuN*, and *acuG* genes by sources of TCA cycle intermediates. A direct role for the products of *acuK* and *acuM* in transcriptional activation of gluconeogenic genes is indicated by the finding that these genes encode proteins with similar but distinct

Zn(2) Cys(6) DNA-binding domains. Furthermore, these genes are conserved in other filamentous ascomycetes, indicating that this gluconeogenic control circuit is of broad significance for fungal biology.

MATERIALS AND METHODS

***A. nidulans* strains, media, enzyme assays, and transformation:** Media and conditions for growth of *A. nidulans* were as described by Cove (1966). Carbon and nitrogen sources were added as appropriate to minimal salts. The pH of these was adjusted to 6.5 where necessary. Mycelia for enzyme assays and RNA and DNA preparations were grown in 100 ml of medium in 250-ml Ehlenmeyer flasks at 37°. β-Galactosidase assays were carried out by the method of Davis *et al.* (1988). All strains were derived from the original Glasgow strain and contained the *velA1* mutation to promote uniform flat conidiation, and standard genetic manipulations were as previously described (Clutterbuck 1974, 1994). Preparation of protoplasts and transformation were as described (Tilburn *et al.* 1983; Andrianopoulos and Hynes 1988).

Molecular techniques: Standard methods for DNA manipulations, RNA isolation, nucleic acid blotting, and hybridization have been described (Sambrook *et al.* 1989; Todd *et al.* 2005; Hynes *et al* 2006).

Cloning and analysis of the *acuN* gene: A strain containing the *acuN356* mutation was crossed to a strain containing the *pyrG89* mutation to generate an *acuN356;pyrG89* double mutant. This strain was transformed with DNA of the genomic library in the autonomously replicating vector pRG3AMA1 (Osherov and May 2000) selecting for pyrimidine prototrophy. Plates containing transformants were velvet replicated to medium with 50 mm acetate as the sole carbon source, and complementing colonies growing on acetate were purified and genomic DNA prepared. Plasmid DNA was recovered by transforming this DNA into *Escherichia coli* selecting for ampicillin resistance. Extensive subcloning and further transformation experiments identified plasmid subclones capable of complementing the *acuN356* mutation. DNA sequencing identified the complementing sequences as corresponding to the annotated gene AN5746.3 (http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/Home.html). Southern blot analysis confirmed that this was a unique gene.

Sequences flanking the proposed translocation breakpoint in the *acuN356* mutant were cloned by inverse PCR. Genomic DNA of an *acuN356* strain (G520, *wA3;pyroA4;acuN356*) was digested with *Hin*dIII, ligated, and used for the PCR with the primers inverse PCR-1 (CGTGGATCTTGGAGATAGGC) and inverse PCR-2 (CCCGCTCAGTCTACGACTCT). The resulting product was cloned into the *Eco*RV site of pBluescriptSK+ (Stratagene, La Jolla, CA) and sequenced. Comparison of this sequence with the *A. nidulans* genome sequence allowed the identification of the translocation breakpoint (see results).

To disrupt the *acuN* gene, a *Hin*dIII–*Sma*I fragment containing the *riboB* gene from plasmid pPL1 (Oakley *et al* 1987) was used to replace a *Hin*dIII–*Eco*RV fragment of *acuN* (coordinates −567 to +833) to generate the plasmid pSM5644. A linear *Not*I–*Xho*I fragment from this plasmid was gel purified and used to transform a diploid strain homozygous for the *riboB2* mutation with selection for Ribo$^+$ transformants. A transformant heterozygous for the predicted *acuN::riboB* gene replacement (*acuN*Δ) was detected by Southern blot analysis.

Promoter sequences of *acuN* used to construct *lacZ* fusions were generated by the PCR using the primers AcuNlacZ-2 (CTAGATCTTGAGAGGCATCAGCGAACTA, coordinates −721 to −701) and AcuNlacZ-3 (CTAGATCTGTCTCGATCCATCAGCGATT, coordinates −208 to −189), together with AcuNlacZ-1 (GAAGATCTGCGTGGATCTTGGAGATAGG, coordinates +4 to +23), incorporating *Bgl*II sites to allow insertion into the *Bam*HI site of pAN923-42B with the *argB*$^+$ gene mutated by end filling the unique *Bgl*II site with Klenow DNA polymerase and religating (van Gorcom *et al.* 1986; Punt *et al.* 1995) The constructs were checked by sequencing and plasmids transformed into a strain of genotype *yA2 pabaA1; argB1* selecting for ArgB$^+$. Single-copy insertions at the *argB* locus were detected by Southern blotting.

Cloning and analysis of the *acuG* gene: A BLAST search performed on the University of Oklahoma *A. nidulans* EST database (http://www.genome.ou.edu/fungal.html) revealed sequences (x8e05a1.f1, x8e05a1.r1, and g7g01a1.r1) with a high level of similarity to FBP-encoding genes from yeast species. The EST x8e05a1 clone was obtained from the Fungal Genetics Stock Center (http://www.fgsc.net/) and used to probe an *A. nidulans* BAC library (kindly provided by Ralph Dean, Clemson University), and three hybridizing clones were identified. Clones 4P22, 12H22, and 16D24 all showed an identical restriction pattern in the hybridizing region in Southern blots. Derivative *acuG* subclones from 16D24 in pBluescript SK(+) were used in *acuG223* mutation complementation experiments and for further manipulations. Sequencing (accession no. AF525021) showed that the gene corresponded to AN5604.3 in the *A. nidulans* database.

An *acuG::lacZ* construct (pES4979) was created using a *Bgl*II–*Eco*RV fragment (coordinates −1546 to +61) of the *acuG* gene cloned into the unique *Bam*HI–*Sma*I sites of the plasmid pAN923-43B (van Gorcom *et al.* 1986), generating an in-frame fusion of the predicted first 20 amino acids of *acuG* with the *E. coli lacZ* gene. The *argB*$^+$ gene of the pAN923-43B plasmid was mutated by end filling the unique *Bgl*II site with Klenow DNA polymerase and religating, allowing the selection of *argB*$^+$ transformants generated by crossing over with the *argB1* mutation to produce single-copy insertions at the *argB* locus.

To disrupt the *acuG* gene, a 2.2-kb *Sma*I–*Cla*I fragment containing the *A. nidulans pyrG* cassette of pAB4342 (Borneman *et al.* 2001) was cloned into the *Eco*RV–*Cla*I sites of pES5431. Insertion of the 1-kb *Cla*I fragment of pES4723 into the *Cla*I site of the latter plasmid created pES5397. A 7-kb *Not*I–*Apa*I fragment was transformed into a *pyrG89* strain, and transformants were selected for PyrG$^+$. One transformant (*acuG*Δ) with an AcuG$^-$ phenotype was shown by Southern blotting to have the predicted restriction map for relacement of the wild-type gene with the disruption construct.

Cloning of *acuK*: Pools of cosmids from the chromosome 4-specific pWE15 and pLORIST2 libraries (Brody *et al.* 1991) were used together with a plasmid (pPL5) containing the *riboB* gene (Oakley *et al.* 1987) to cotransform strain H1579: *yA2biA;acuK248;riboB2* and Ribo$^+$ transformants were selected. The transformants were grown on glucose minimal medium and then replicated onto acetate medium. In this way, a positive pool was identified and individual cosmids were eventually identified (SW22F05 and SW04205) as clones containing a sequence that could complement *acuK258*. The cosmid SW04205 was completely digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sal*I, *Not*I, and *Pst*I and the individual digests were used in cotransformations with pPL5 of H1579. *Hin*dIII- and *Sal*I-digested cosmid DNA gave transformants that were able to grow on acetate medium. The *Hin*dIII digest gave eight bands on digestion and DNA from each of the bands was used in cotransformations. One band of ~9 kb was identified as the *acuK* complementing sequence. This band was digested with *Sal*I, and a *Hin*dIII–*Sal*I fragment derived from the original band of 4.8 kb complemented the *acuK* containing strain. This fragment was sequenced (accession no. AY255811) and corresponds to AN7468.3 in the *A. nidulans* database.

Cloning of *acuM*: Initially, a strategy similar to that described for the cloning of *acuK* was adopted using the chromosome 1-specific cosmid library but using strain H1576*: biA1, acuM301;wA3; riboB2* in the cotransformations. No positive transformants were identified. The *acuJ* gene is located 6 cM from *acuM* and a clone of *acuJ* was obtained by cotransformation of strain H1572: *yA2pabaA1acuJ217;riboB2* with pools derived from 29 cosmid clones from the left arm of chromosome 1. A cosmid clone, L7G03, was identified as complementing the lack of growth on acetate due to the *acuJ217* mutation (Armitt *et al.* 1976). Individual cosmids in close proximity to this clone were screened for a clone that could complement *acuM*, but none was found. The *acuJ* clone was used to probe a BAC library (kindly provided by Ralph Dean, Clemson University) and clones that hybridized to the *acuJ* cosmid clone were identified. Four of these were used to cotransform H1576 and one, 4D7, gave acetate$^+$ transformants. The BAC clone was completely digested with *Eco*RI, *Hin*dIII, and *Bam*HI and, when individual digests were transformed into the *acuM* recipient, all the digests gave positive colonies on acetate medium. Of seven *Hin*dIII bands, one fragment of ~4 kb complemented the *acuM* mutation in cotransformations. This fragment was entirely sequenced (accession no. AY256961) and corresponds to AN6293.3 in the *A. nidulans* database.

Sequencing of the *acuK* and *acuM* mutations: DNA from the respective mutant strains (G0306: *pabaA1yA2;acuK248* or G1101: *acuM301;wA3;pyroA4*) was made and PCR primers were designed to amplify overlapping sections of the entire coding region of the genes. The PCR products were then sequenced using the same primers for sequencing as those used for the original amplification. Additional primers were designed to fill any gaps, and the coding region of both genes was sequenced on both strands.

RESULTS

Analysis of the *acuN* gene: The *acuN* gene was originally defined by a single mutant isolated as unable to use acetate (Armitt *et al.* 1976). The *acuN356* mutation was found to result in loss of growth on a wide range of carbon sources requiring gluconeogenesis but was able to utilize glycerol (Figure 1). In this respect, the phenotype was identical to that of *acuF205* strains lacking PEPCK (Armitt *et al.* 1976; Hynes *et al.* 2002). The *acuN* gene was cloned by complementation of the *acuN356* mutation (see materials and methods). Sequencing of insert DNA in the recovered plasmids showed that the *acuN* gene corresponded to AN5746.3 in the genome sequence (Figure 2A) and encoded enolase (E.C.4.2.1.11). This was a surprising result as this enzyme is regarded as reversible and essential for both glycolysis and gluconeogenesis.

Crosses with *acuN356*-containing strains yielded abnormal slow-growing colonies, a phenomenon commonly associated with duplication–deficiency segregants arising from chromosomal rearrangements. Therefore, a diploid between an *acuN356* strain and the strain MSF with each linkage group genetically marked was constructed and subjected to haploidization analysis (McCully and Forbes 1965). This revealed that the *acuN356* strain contained a translocation event involving

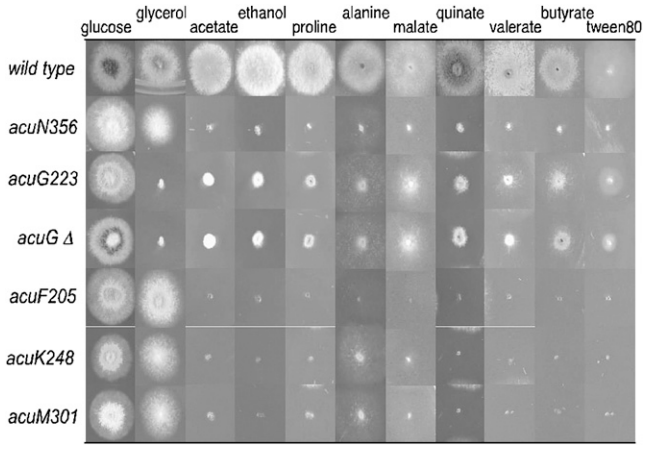

Figure 1.—Growth of relevant mutants on carbon sources. The following carbon sources were added to minimal medium with 10 mm ammonium chloride as the nitrogen source: glucose (1%), glycerol (0.5%), acetate (50 mm), ethanol (0.5%), L-proline (50 mm), L-alanine (50 mm), malate (10 mm), quinate (0.2%), valerate (10 mm), butyrate (20 mm), and Tween 80 (0.2%). All acids were adjusted to a pH of ~6.5 with sodium hydroxide where necessary. Tween 80 is a source of the long-chain fatty acid oleate. Growth was for 2–3 days at 37° except for malate (6 days).

linkage groups I and V. Southern blot analysis of *Hin*dIII- and *Hae*III-digested *acuN356* DNA, using cloned *acuN* DNA as a probe, localized a translocation breakpoint to the 5′ region of *acuN* ~150–300 bp upstream of the predicted enolase ATG (Figure 1 and data not shown). The breakpoint was precisely identified by designing primers for inverse PCR with circularized *Hin*dIII-digested DNA as a template, followed by cloning and sequencing (Figure 2A). Comparison with the genome sequence showed that the breakpoint was precise and resulted in truncation of the *acuN* promoter at −220 and fusion to the coding region of the annotated gene AN10770.3 on linkage group I (Figure 2B).

These results suggested that the *acuN356* mutation resulted in abnormal regulation of enolase expression rather than a complete loss of function. Therefore, the *acuN* gene was inactivated by insertion of the *riboB*$^+$ selectable marker (Figure 2C) and selection for Ribo$^+$ transformants of a diploid strain homozygous for *riboB2*. Southern blot analysis confirmed the successful isolation of a diploid strain heterozygous for the *acuN* deletion (data not shown). Haploidization of this diploid yielded no Ribo$^+$ haploids—only haploids containing the linkage group V *facA*$^+$ marker in coupling with *acuN*$^+$ (Figure 2C). In this experiment, haploids were isolated using glucose-containing media. A less extensive haploidization experiment using media with proline as the sole carbon source also failed to yield Ribo$^+$ haploids. This confirmed that the *acuN* gene is essential for growth on either glycolytic or gluconeogenic carbon sources, consistent with a single enolase enzyme being essential for both pathways.

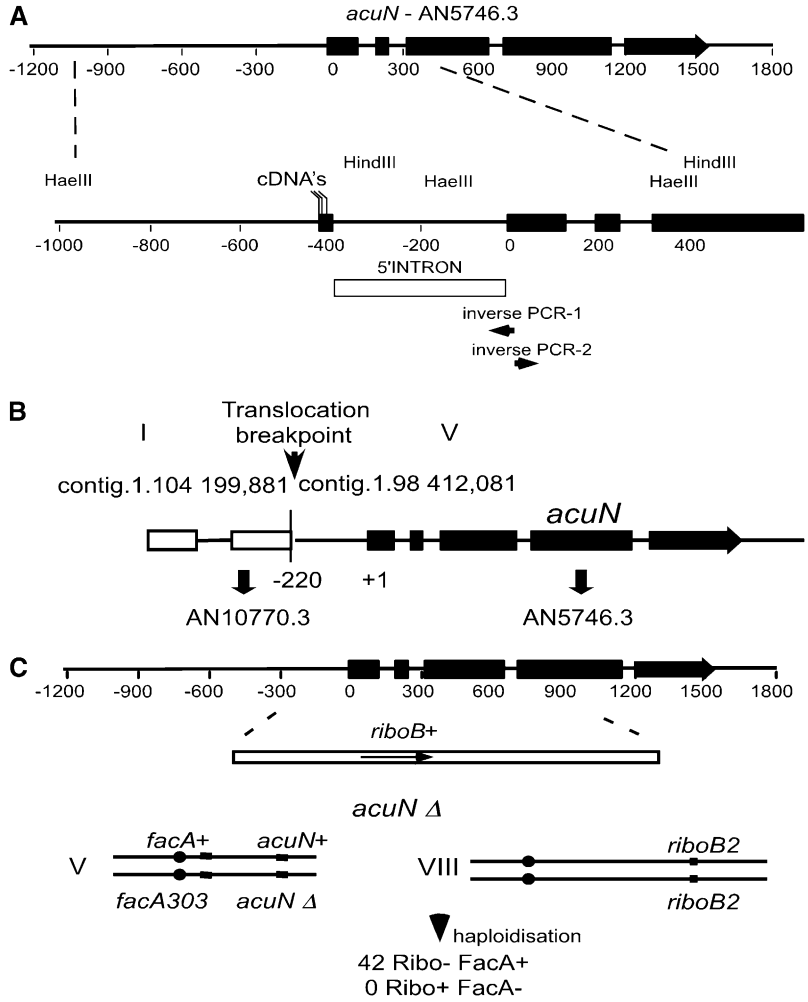

FIGURE 2.—Characterization of the *acuN356* mutation. (A) Schematic of the *acuN* gene corresponding to AN5746.3 in the *A. nidulans* database. The positions of exons are shown as thick lines and the coordinates are relative to the position of the ATG start codon. An expanded view of the 5′-end of the gene shows the position of the *Hin*dIII and *Hae*III restriction sites used to map the *acuN356* translocation breakpoint by Southern blotting of genomic DNA. The position of the intron in the 5′ untranslated region is shown (open box) and the position of 5′ endpoints of sequenced cDNAs is indicated, as are the positions of primers (inverse PCR-1 and -2) used for cloning the *acuN356* translocation region. (B) Schematic of the *acuN356* translocation breakpoint showing the contig coordinates on chromosomes 1 and 5 and the position of the breakpoint relative to the genes AN10770.3 and AN5746.3 (*acuN*). (C) Deletion of the *acuN* gene by replacement with the *A. nidulans riboB* gene in a *riboB2* homozygous diploid strain. The relevant genotypes on linkage groups V and VIII of the resulting diploid are shown and phenotypes of haploids are recovered by haploidizing this diploid. All haploids have the *riboB2* mutation (linkage group VIII) but none were phenotypically Ribo$^+$ and all were FacA$^+$, indicating that no haploids with the *acuN*Δ were recovered.

Expression of the *acuN* gene was studied by Northern blotting (Figure 3A). No transcript was detected after transfer to medium lacking a carbon source while similar levels were found with glucose or the gluconeogenic carbon sources, acetate, glutamate, or γ-aminobutyric acid. The presence of both glucose and the gluconeogenic carbon sources did not result in additive expression. Under these circumstances, uptake and metabolism of these gluconeogenic substrates would be repressed by glucose. Fusions of *acuN* 5′ sequences to the *lacZ* reporter gene were constructed and integrated in single copy at the *argB* locus. When the 5′ sequence extended to −721, high-level expression of β-galactosidase was observed in the presence of glucose and glycerol (glycolytic) or acetate, proline, and quinate (gluconeogenic). Expression was greatly reduced on the gluconeogenic carbon sources when the 5′ sequence was shortened to −206 while expression on glucose and glycerol was affected by less than twofold (Figure 3B). Consistent with these data, the *acuN* transcript was present at low levels in mycelium transferred to acetate or proline-containing media in the *acuN356* mutant (Figure 3C). This was strong evidence for separate controls of *acuN* expression. During glycolysis, sequences downstream of −207 (with perhaps some contribution from upstream sequences) are used for activation of transcription while during gluconeogenesis sequences upstream of −207 are required. In the *acuN356* mutant, a reciprocal translocation between chromosomes 1 and 5 has resulted in loss of gluconeogenic regulation without eliminating glycolytic regulation, consistent with the phenotype of the *acuN356* mutant. On the basis of EST sequences, a large intron is predicted to occur in the *acuN* 5′ untranslated region and cDNA 5′-ends have been found at −426 and at −4 (http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/Home.html). Comparison of the *A. nidulans* sequence with the 5′ sequences of the corresponding genes in *Aspergillus oryzae* and *Aspergillus fumigatus* (accession nos. BAE57291 and AAK49451) indicate conservation of splice sites for the 5′ intron. Splicing of the 5′ intron from RNA synthesized under gluconeogenic conditions is predicted to result in similarly sized transcripts under both glycolytic and gluconeogenic growth conditions as observed in Northern blot analysis.

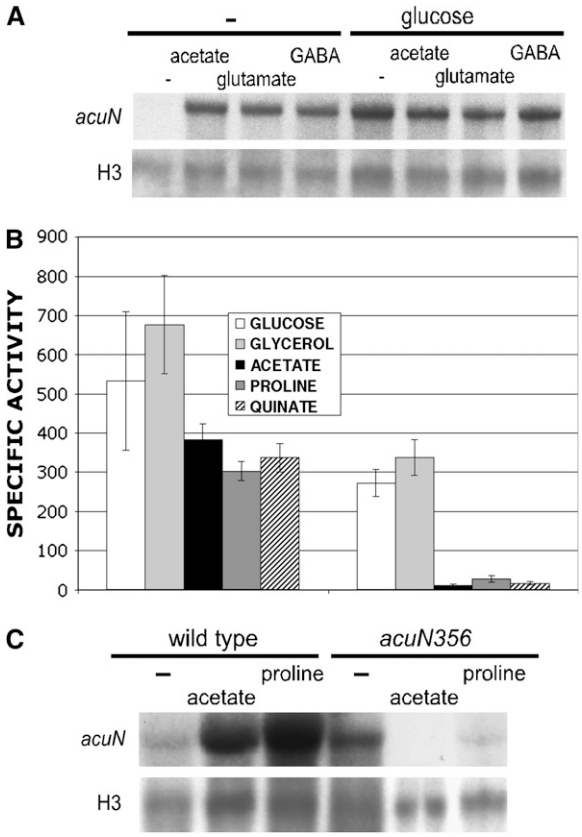

FIGURE 3.—Analysis of expression of *acuN* and the effects of the *acuN356* mutation. (A) Northern blot analysis of expression in a wild-type strain. RNA was extracted from mycelium grown for 16 hr in 1% glucose–10 mM ammonium tartrate medium and then transferred to medium with or without glucose (1%) with ammonium chloride (10 mM) as the nitrogen source with the following additions as indicated: acetate (50 mM); L-glutamate (10 mM); and γ-aminobutyric acid–GABA (10 mM) for 4 hr. *acuN* RNA was detected by hybridization with a 3.8-kb *Eco*RI fragment (coordinates −461 to +3376). Hybridization with the *Eco*RI fragment of the histone H3 gene (EHINGER *et al.* 1990) was used as a loading control. (B) Effects of *acuN* 5′ sequences on expression of a *lacZ* reporter. Mycelium from strains with single copies of plasmids with the indicated 5′ sequences driving *lacZ* expression were grown for 20–24 hr in medium containing one of the carbon sources: glucose (1%), glycerol (0.5%), acetate (50 mM), proline (50 mM), and quinate (0.5%) together with ammonium chloride (10 mM) as the nitrogen source before harvesting, extraction, and determination of β-galactosidase activities. The specific activity is expressed as Miller units per minute per milligram of protein (DAVIS *et al.* 1988) and represents the average of three independent experiments. The bars show standard errors. (C) Effects of the *acuN356* mutation on *acuN* expression. RNA was extracted from wild type and an *acuN356* strain grown under the same conditions as described in A and the Northern blot hybridized as in A.

Analysis of the *acuG* gene: Mutations in the *acuG* gene specifically lack FBP (ARMITT *et al* 1976). These mutations result in loss of growth on gluconeogenic carbon sources as well as on glycerol where FBP but not enolase or PEPCK are required (see Figure 1). Somewhat leaky growth on gluconeogenic carbon sources is thought to be due to the presence of a nonspecific phosphatase activity (ARMITT *et al* 1976).

A BLAST search performed on the *A. nidulans* EST database revealed sequences with a high level of similarity to FBP-encoding genes of other species. An EST clone was used to probe an *A. nidulans* BAC library (see MATERIALS AND METHODS). A DNA segment of 3.4 kb was sequenced and predicted to encode a polypeptide of 356 amino acids with extensive similarity to FBP enzymes from other fungi. Subsequently, this sequence was found to correspond to AN5603.3 in the *A. nidulans* database. Subclones were cotransformed with the *riboB*$^+$-containing plasmid pPL3 (OAKLEY *et al* 1987) into a *riboB2 acuG223* strain, and Ribo$^+$ transformants were selected. A total of 44–72% of transformants with any of the subclones containing the full *acuG* coding region and at least 135 bp of 5′ sequence were fully complemented for the AcuG$^-$ phenotype in that they showed restoration of growth on acetate, proline, glutamate, or glycerol, confirming that the cloned DNA fragment contained the *acuG* gene. An *acuG* deletion mutation was made with the *pyrG*$^+$ selectable marker, replacing the coding region corresponding to amino acids 21–348 (see MATERIALS AND METHODS). This resulted in a phenotype identical to that of the *acuG223* mutant (Figure 1).

An *acuG::lacZ* fusion gene was constructed that encoded a fusion protein with the first 20 amino acids of AcuG fused to *E. coli* LacZ (see MATERIALS AND METHODS). This was inserted in single copy at the *argB* locus and used to investigate *acuG* regulation under various growth conditions and in various genetic backgrounds.

Expression of AcuG-LacZ was significantly higher with acetate and slightly higher with proline or glycerol in comparison to glucose-grown conditions (Figure 4A). Levels of expression were slightly higher on xylose or fructose in comparison to glucose. It should be noted that a significant level of expression of AcuG-LacZ (~50 units) was observed even under the most repressed conditions (glucose and sucrose). This might reflect a level of constitutive expression. An alternative explanation is that this is due to an artifact of insertion of the fusion gene at an ectopic location. Growth on decreasing concentrations of glucose for 20 hr resulted in increased expression (Figure 4B). The *creA* gene in *A. nidulans* encodes a repressor protein with two C2H2 zinc fingers similar to those of *S. cerevisiae* Mig1p and is required for carbon catabolite repression (DOWZER and KELLY 1991). The *creA204* mutation in the DNA-binding domain results in loss of glucose repression (SHROFF *et al.* 1996). In a *creA204* background, AcuG-LacZ expression was elevated about twofold in the presence of glucose and sucrose (Figure 4A). The effects of the nonmetabolizable analogs of glucose, 2-deoxy-D-glucose (2-DOG) and 3-*O*-methyl-D-glucose (3-MeGlc), able to bring about carbon catabolite repression in *S. cerevisiae* (GANCEDO and GANCEDO 1985), were also tested (Figure

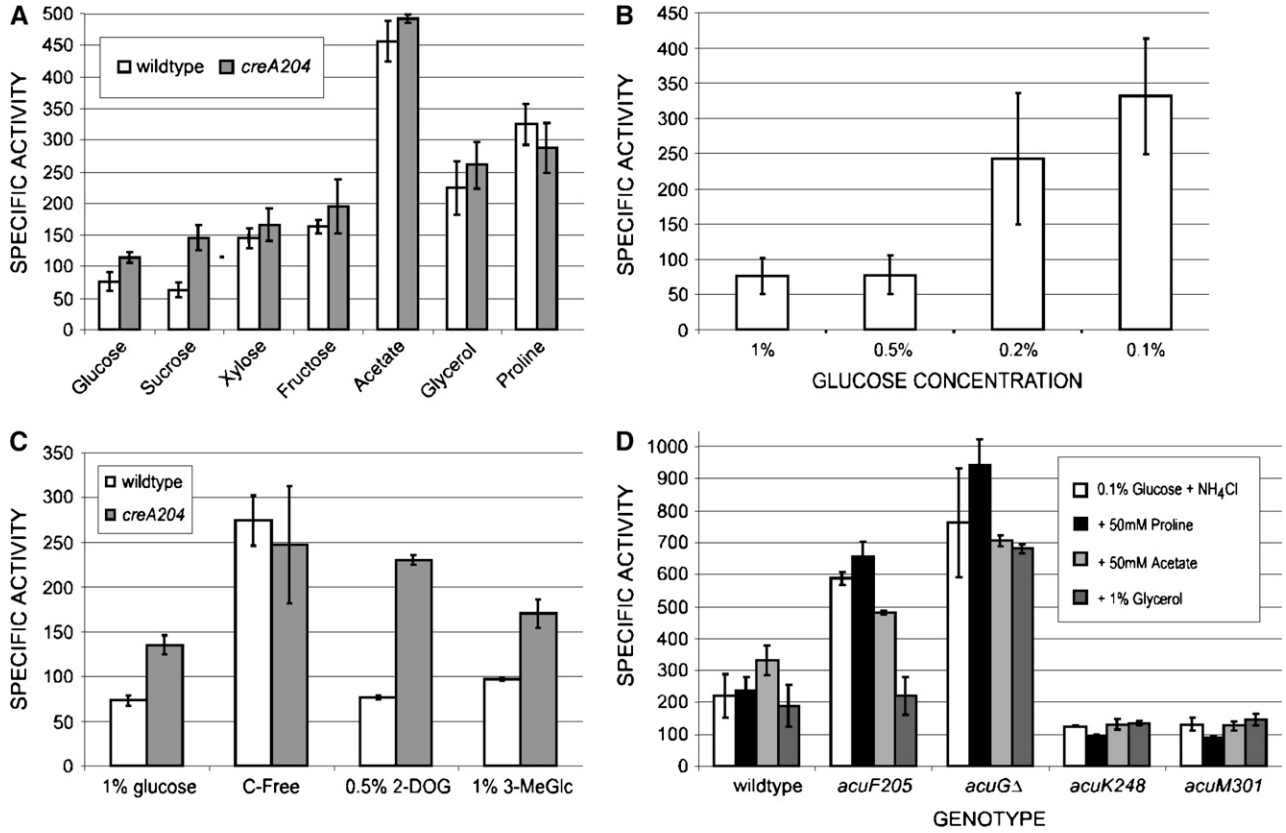

FIGURE 4.—Regulation of expression of the *acuG::lacZ* fusion gene. (A) Expression in the presence of various carbon sources and in the *creA204* mutant background. Mycelium was grown for 20–24 hr in minimal medium with 10 mM ammonium tartrate as the nitrogen source and the indicated carbon sources at 1% except for acetate and proline (50 mM). (B) Expression in mycelium grown for 20 hr in the indicated glucose concentrations with 10 mM ammonium tartrate as the nitrogen source. (C) Effects of nonmetabolizable glucose analogs on expression in the wild-type and *creA204* strains. 2-DOG and 3-MeGlc were present at the indicated concentrations. Mycelia were grown in 1% glucose–10 mM ammonium tartrate medium for 16 hr and then transferred to the indicated media with 10 mM ammonium chloride present as the nitrogen source for a further 4 hr before harvesting. (D) Expression in various mutant backgrounds. Mycelia of strains carrying the *acuG::lacZ* fusion gene in the indicated genetic background were grown in 0.1% glucose-minimal media for 16 hr with 10 mM ammonium chloride as the nitrogen source. The indicated carbon sources were added and growth was continued for 8 hr before harvesting. Harvested mycelium was extracted and assayed for β-galactosidase. The specific activity is expressed as for Figure 3.

4C). Both 2-DOG and 3-MeGlc resulted in levels of repression that were similar to glucose relative to incubation in media lacking a carbon source, and repression was relieved by the *creA204* mutation (Figure 4C). These results provided strong evidence for *acuG* being subject to CreA-mediated carbon catabolite repression.

The effects of carbon sources present during growth for 24 hr in a limiting concentration of glucose (0.1%) on expression were determined (Figure 4D). Glycerol, proline, or acetate addition had little effect in a wild-type background. The *acuF205* mutation resulted in elevated expression under all conditions except in the presence of glycerol. These results were interpreted as indicating a significant influence of endogenous induction occurring due to metabolic turnover under carbon-limiting conditions, with the *acuF205* mutant lacking PEPCK leading to inducer accumulation increasing this effect. A similar effect of the *acuF205* mutation on induction of an *acuF-lacZ* reporter has been described (HYNES *et al.* 2002 and see below). The *acuG*Δ mutation resulted in elevated expression under all conditions, including in the presence of glycerol. Again, this is similar to results with the *acuF-lacZ* reporter where the *acuG223* mutation was found to lead to increased expression (HYNES *et al.* 2002). In addition, metabolism of glycerol to hexose phosphates dependent on FBP might result in carbon catabolite repression both directly and indirectly by preventing inducer formation in the wild-type and *acuF205* backgrounds, but not in the *acuG*Δ strain. Overall, the results suggested that *acuG* is subject to CreA-mediated repression as well as to induction by a TCA cycle intermediate.

Characterization of the *acuK* and *acuM* genes: Mutations in the *acuK* and *acuM* genes result in a similar phenotype—loss of growth on gluconeogenic carbon sources. Growth on glycerol still occurs but is less than for wild-type strains (Figure 1). The *acuK* gene was cloned by complementation of the *acuK248* mutation for

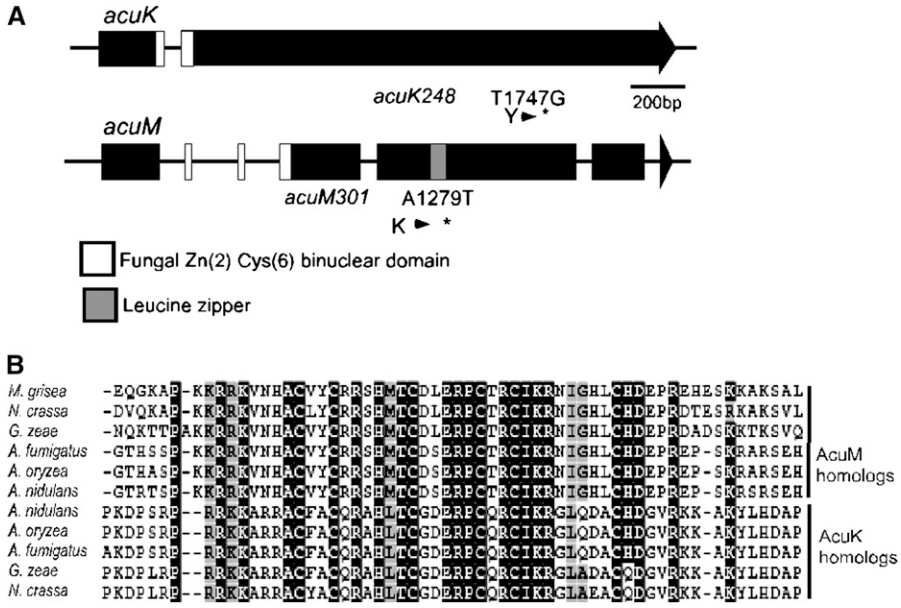

FIGURE 5.—The *acuK* and *acuM* genes. (A) The structure of the genes showing intron positions and the sequences coding for the indicated domains. The sequence changes and the resulting generation of stop codons in the *acuK248* and *acuM301* mutations are shown. (B) Comparison of the predicted Zn(2) Cys(6) domains of AcuK and AcuM homologs in filamentous ascomycetes. Sequences were aligned using Clustal W (THOMPSON *et al.* 1994) and visualized with Macboxshade (http://www.ch.embnet.org/software/BOX_form.html). Identical residues are indicated by a solid background and similar residues by a shaded background. Sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and from the Broad Institute Fungal Genome Initiative (http://www.broad.mit.edu/annotation/fungi/fgi/). Species and accession numbers were the following: AcuK—*A. nidulans* (AN7468.3), *A. oryzae* (BAE57193), *A. fumigatus* (XP_749712), *Neurospora crassa* (XP_957577), and *Gibberella zeae* (XP_388802); and AcuM—*A. nidulans* (AN6293.3), *A. oryzae* (BAE59871), *A. fumigatus* (XP_755565), *M. grisea* (XP_360718), *N. crassa* (CAD37032), and *G. zeae* (XP_381738).

growth on acetate using cosmid pools from chromosome 4 (see MATERIALS AND METHODS). This yielded a 4.8-kb complementing fragment, which was sequenced and found to correspond to AN7468.3 in the *A. nidulans* database. The *acuM* gene was isolated by first cloning the linked *acuJ* gene by complementation and using this sequence as a hybridization probe to isolate BAC clones. One of these complemented the *acuM301* mutation for growth on acetate (see MATERIALS AND METHODS). From this BAC clone, a complementing 4-kb fragment was identified, sequenced, and found to correspond to AN6293.3 in the *A. nidulans* database. The *acuK* and *acuM* genes were predicted to encode related proteins (identity, 30.5%; similarity, 22.4%) with highly similar Zn(2) Cys(6) DNA-binding motifs (Figure 5A). Predicted introns were confirmed by sequencing DNA generated by reverse-transcription PCR. Sequencing of the *acuK248* and *acuM301* mutations showed that each resulted from the generation of a stop codon, consistent with these mutations resulting in a loss of function (Figure 5A). This has been confirmed by the generation of deletion mutations that show identical phenotypes to these mutants (Y. SUZUKI, unpublished results). Genes encoding proteins with highly similar Zn(2) Cys(6) DNA-binding domains were identified in other filamentous ascomycetes, indicating a conserved function for these genes (Figure 5B). Interestingly, an AcuM, but not an AcuK, homolog was found in *Magnaporthe grisea*.

Expression of an AcuF-LacZ reporter (HYNES *et al.* 2002) was greatly reduced by the *acuK248* and *acuM301* mutations (Figure 6A). As previously shown, the *acuF205* mutation resulted in high levels of expression, a result interpreted as due to inducer accumulation. Presence of the *acuK248* mutation eliminated the effects of *acuF205* (Figure 6A). Northern blot analysis supported these results with the *acuK248* and *acuM301* mutations showing low levels of *acuF* transcript (Figure 6C). These mutations also resulted in low levels of expression of the AcuG-LacZ reporter (Figure 4D and Figure 6B). A response to glucose repression was still observed in the mutants (Figure 6B). Expression of the *acuG* transcript was very low under carbon-starved conditions as well as in the presence of proline or acetate (Figure 6C). Expression of the *acuN* transcript in response to acetate or proline was also greatly reduced by the *acuK248* and *acuM301* mutations (Figure 6D). Therefore, these data were consistent with AcuK and AcuM being transcriptional activators of gluconeogenic genes.

DISCUSSION

In glycolysis, most enzymatic steps are reversible, thereby allowing gluconeogenesis to occur. In gluconeogenesis, two irreversible reactions of glycolysis are replaced by PEPCK and FBP. Consequently, regulation of the expression of the genes encoding these enzymes controls the utilization of carbon sources requiring gluconeogenesis. We have previously studied the regulation of PEPCK encoded by the *acuF* gene (KELLY and HYNES 1981; HYNES *et al.* 2002). Here we have extended this by studying the regulation of FBP, the product of the *acuG* gene. Unexpectedly, we have found that expression of the reversible glycolytic enzyme enolase is also subject to induction during growth on gluconeogenic carbon sources. This was discovered because of

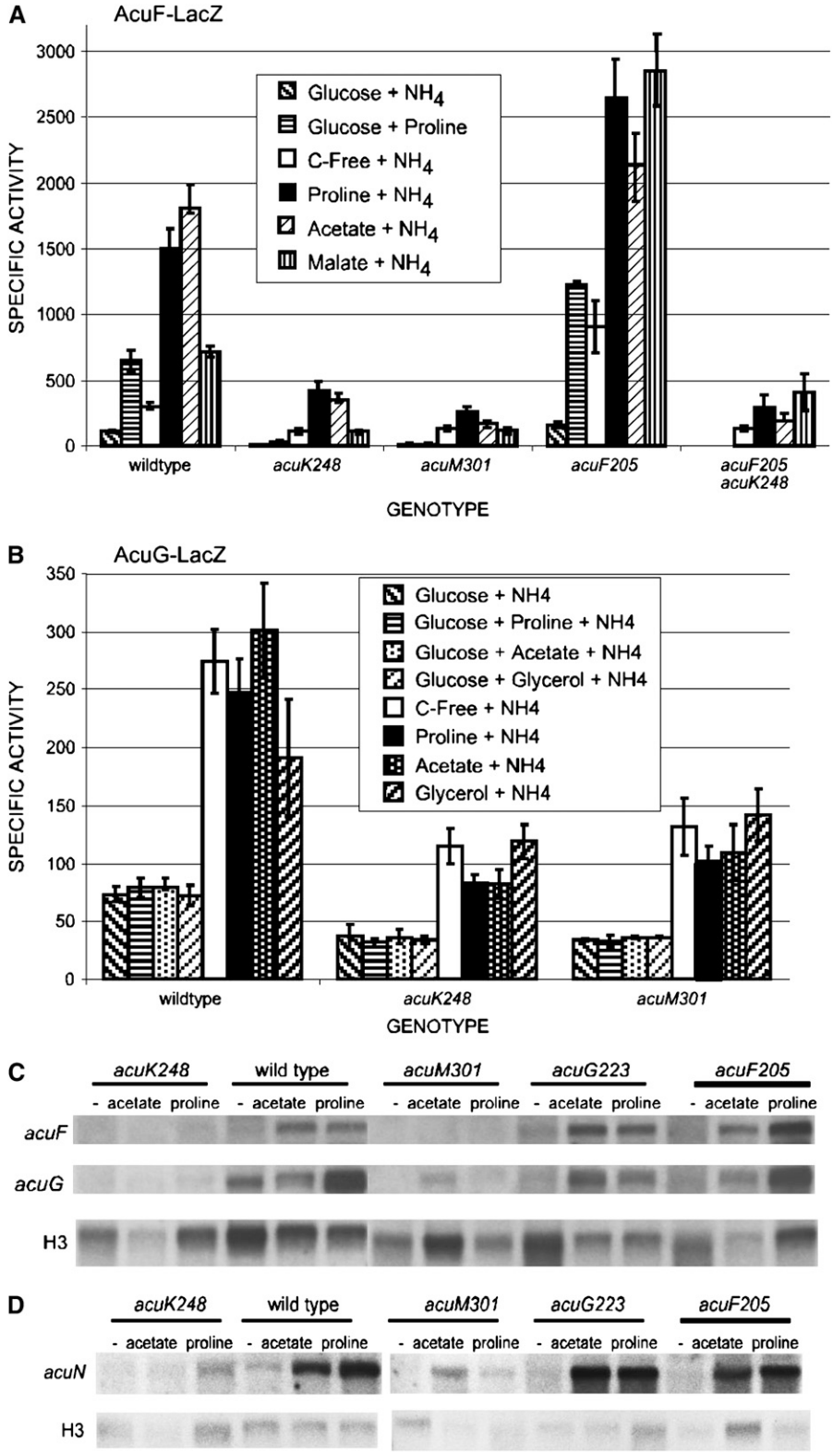

FIGURE 6.—Effects of the *acuK248* and *acuM301* mutations on gene expression. (A) Effects on AcuF-LacZ expression. Mycelia of *acuF-lacZ*-containing strains of the indicated relevant genotypes were grown for 16 hr in 1% glucose–10 mM ammonium tartrate and then transferred to the indicated media for 4 hr before harvesting, extraction, and assaying as described in the legends to Figure 3 and Figure 4. The nitrogen source was 10 mM ammonium chloride in all cases except for the glucose-plus-proline treatment where 10 mM proline was present as the sole nitrogen source. Carbon sources were glucose (1%), proline (50 mM), acetate (50 mM), and malate (10 mM). (B) Effect on AcuG-LacZ expression. Mycelia of strains carrying the *acuG::lacZ* fusion gene of the indicated relevant genotypes were grown for 16 hr in 1% glucose–10 mM ammonium tartrate and then transferred to the indicated media for 4 hr. Carbon sources were glucose (1%), glycerol (1%), proline (50 mM), and acetate (50 mM). Ammonium chloride (10 mM) was present as a nitrogen source. (C) Effects on *acuF* and *acuG* transcripts. RNA was extracted from the strains of the indicated relevant genotypes grown under the same conditions as indicated in A. The same Northern blot was hybridized sequentially to an *acuF* probe (a 2.3-kb *Pst*I–*Xho*I fragment with coordinates −961 to +1359), an *acuG* probe (a 1.7-kb *Eco*RV fragment beginning at coordinate +58 and containing the rest of the coding region), and the *Eco*RI fragment of the histone H3 gene. (D) Effects on *acuN* transcripts. RNA was as for C with hybridization to the same probes as for Figure 3A.

the unique nature of the single mutation defining the *acuN* gene. We have established that this gene encodes enolase and that the *acuN356* mutation results from a translocation breakpoint in the 5′ region separating glycolytic from gluconeogenic regulation, leading to loss of the ability to use gluconeogenic carbon sources while growth is unaffected on glycolytic carbon sources. Analysis of the 5′ region of the enolase gene from *A. oryzae* showed transcription start points at −17, −38, and −45, and deletion analysis of the promoter indicated that a 100-bp sequence between −121 and −228 is necessary for expression on glucose (MACHIDA *et al.* 1996;

Toda *et al.* 2001). This is consistent with our results indicating a downstream glycolytic control. A large intron in the 5′ untranslated region appears to be conserved in *Aspergillus* spp. and we propose that gluconeogenic regulation occurs upstream of this, consistent with a EST 5′-end at −426. The dual regulation of a single enolase gene in *A. nidulans* contrasts with the presence of two genes in *S. cerevisiae*. *ENO2* is 20-fold induced in the presence of glucose while *ENO1* is equally expressed on glucose or gluconeogenic carbon sources (Cohen *et al.* 1987).

It remains to be determined whether other genes required for both glycolysis and gluconeogenesis are subject to similar dual transcriptional controls. This may not be evident in comparisons of transcript levels on glycolytic and gluconeogenic carbon sources if both give similar expression levels, as indicated by our data for *acuN*. This is supported by recent microarray data comparing glucose-, ethanol-, and glycerol-grown cultures where the expression of enolase and other glycolytic genes was not affected by the carbon source (David *et al.* 2006). It will be necessary to investigate the promoters of glycolytic genes for different controlling sequences affecting gluconeogenic and glycolytic regulation. In addition, studies on gluconeogenic regulation of these genes in *acuK* and *acuM* mutants will be informative. The regulation of genes encoding glycolytic enzymes has been studied in the related species *A. oryzae* and it has been found that expression is generally high in the presence of glucose but low upon transfer to medium lacking a carbon source or to pyruvate, which was shown to be an extremely poor source of carbon (Nakajima *et al.* 2000). Studies were not carried out using strong gluconeogenic carbon sources.

We have cloned the single *acuG* gene encoding fructose-1,6-bisphosphatase. The cloned DNA fragment was shown to complement the *acuG223* mutation and the *acuG*Δ strain generated showed a phenotype similar to that of the *acuG223* mutant. Overall, the observed pattern of *acuG* regulation suggests the presence of a significant constitutive level of *acuG* expression and CreA-mediated carbon catabolite repression. Several putative CreA-binding sites (Cubero and Scazzocchio 1994) are detected in the promoter region with possible strong tandem sites at −805 and −435. In *A. oryzae*, the expression of the FBP-encoding gene has been shown to be glucose repressed (Nakajima *et al.* 2000). In addition, there is a significant response of *acuG-lacZ* to induction by sources of TCA cycle intermediates as well as endogenous induction due to metabolic turnover under starvation conditions. This is evident in mutants lacking PEPCK (*acuF*) and FBP (*acuG*), suggesting the accumulation of inducer(s) in these strains blocked in gluconeogenesis. Glycerol brought about an increase in expression only in the *acuG*Δ mutant and did not have this effect in the *acuF205* background. In this case, inducer accumulation might result from the accumulation of fructose-1,6-bisphosphate in the presence of acetate, proline, as well as glycerol with feedback inhibition resulting in accumulation of a TCA cycle intermediate. This pattern of regulation for *acuG* is similar to that previously described for *acuF* (Hynes *et al.* 2002) with the major difference being the presence of carbon catabolite repression for *acuG*. This likely reflects the fact that *acuG* but not *acuF* is required for glycerol utilization. The basal glucose-repressible level of expression would allow growth on glycerol without requiring induction. Recent microarray data generally support these results (David *et al.* 2006). Growth on the gluconeogenic carbon source, ethanol, results in high-level expression of both *acuG* and *acuF* in comparison with glucose; *acuF* expression, but not *acuG*, is elevated in ethanol compared to glycerol while, interestingly, both genes are moderately elevated in glycerol compared to glucose.

Identification of the inducing metabolite(s) poses some difficulties because of the central pathways involved. Nevertheless, the *acuF205* mutation leads to increased expression of both *acuF-lacZ* and *acuG-lacZ* reporters, indicating that the metabolite is before the PEPCK step. Malate, a weak inducer in wild type, is a much stronger inducer of *acuF-lacZ* in an *acuF205* background (Figure 6A). This indicates that accumulation of either or both malate and oxaloacetate may result in induction. We cannot distinguish between these because malate dehydrogenase interconverts malate and oxaloacetate. Three malate dehydrogenase genes are present in the *A. nidulans* genome. One is predicted to be mitochondrial while the other two genes lack obvious targeting signals and may be cytoplasmic with essential or contributing roles during growth on gluconeogenic carbon sources in converting malate to oxaloacetate in the cytoplasm.

Regulation of gluconeogenesis is significantly different from that observed in *S. cerevisiae* where the PEPCK and FBP enzymes are strongly regulated at the transcriptional level by glucose-sensitive Cat8p and Sip4p activation. This reflects the specialized growth of *S. cerevisiae*, which has a strong preference for the fermentation of glucose over growth on carbon sources requiring gluconeogenesis and is unable to use amino acids as sole carbon sources. In contrast, *A. nidulans* and other filamentous fungi are able to use a wide variety of carbon sources, and induction of FBP and PEPCK allows gluconeogenesis to occur during growth on any carbon source metabolized via TCA cycle intermediates. The FacB activator, which is similar to the Cat8/Sip4 proteins, is specific for the regulation of genes required for acetate utilization. The enzymes of the glyoxalate cycle that are required for growth on both acetate and fatty acids are additionally controlled by fatty acid induction independently of FacB, and regulatory genes involved in this have been identified (Hynes *et al.* 2006). In *S. cerevisiae*, the glyoxalate cycle genes are not regulated by

fatty acid induction but only by Cat8/Sip4 (Hiltunen *et al.* 2003).

We have evidence that the transcription factors controlling the induction of enzymes for gluconeogenesis are encoded by the *acuK* and *acuM* genes, which were identified in the original screen for acetate mutants (Armitt *et al.* 1976). However, in the initial limited testing and in our subsequent more extensive examination of carbon sources, it has been found that mutations in these genes affect not only growth on acetate but also growth on carbon sources metabolized via the TCA cycle. The *acuK248* and *acuM301* mutations each result in loss of induction of the PEPCK-encoding *acuF* gene by sources of TCA cycle intermediates. Similarly, activation of expression of the *acuG* and *acuN* genes has been found to be lost in these mutants. A direct role in transcriptional activation for the products of *acuK* and *acuM* is indicated by finding that the gene products contain Zn(2) Cys(6) binuclear cluster DNA-binding domains. It is clear that they encode related proteins with similar but distinct putative DNA-binding domains. Furthermore, database searches reveal that these genes are present in other filamentous ascomycetes, indicating that this control circuit is conserved. A previous suggestion (McCullough and Roberts 1974; Armitt *et al.* 1976) that AcuK and AcuM encode the NADP-malic enzyme (E.C. 1.1.1.40) is explained by these genes being required for expression of this enzyme. It has been been shown that gluconeogenic carbon sources can result in induction of this enzyme (Kelly and Hynes 1981). Furthermore, the gene encoding this enzyme has been identified (our unpublished data).

The phenotypes of *acuK* and *acuM* mutants are indistinguishable. Therefore, it is likely that they act together to regulate gene expression, possibly by forming an active heterodimer. An alternative possibility is that one of the genes is necessary for expression of the other. Pairs of related Zn(2) Cys(6) binuclear proteins acting together as transcriptional regulators are not uncommon in *S. cerevisiae* (Macpherson *et al.* 2006). It remains to be determined whether AcuK and AcuM act as direct activators of all of the gluconeogenic genes or indirectly by regulating the production of an inducing molecule. The conservation of these genes in other fungi indicates their importance for controlling carbon source utilization.

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