Regulation of the *acuF* Gene, Encoding Phosphoenolpyruvate Carboxykinase in the Filamentous Fungus *Aspergillus nidulans*

Michael J. Hynes,* Oliver W. Draht,† and Meryl A. Davis

Department of Genetics, University of Melbourne, Parkville, Victoria 3010, Australia

Received 25 July 2001/Accepted 11 October 2001

Phosphoenolpyruvate carboxykinase (PEPCK) is a key enzyme required for gluconeogenesis when microorganisms grow on carbon sources metabolized via the tricarboxylic acid (TCA) cycle. *Aspergillus nidulans* *acuF* mutants isolated by their inability to use acetate as a carbon source specifically lack PEPCK. The *acuF* gene has been cloned and shown to encode a protein with high similarity to PEPCK from bacteria, plants, and fungi. The regulation of *acuF* expression has been studied by Northern blotting and by the construction of *lacZ* fusion reporters. Induction by acetate is abolished in mutants unable to metabolize acetate via the TCA cycle, and induction by amino acids metabolized via 2-oxoglutarate is lost in mutants unable to form 2-oxoglutarate. Induction by acetate and proline is not additive, consistent with a single mechanism of induction. Malate and succinate result in induction, and it is proposed that PEPCK is controlled by a novel mechanism of induction by a TCA cycle intermediate or derivative, thereby allowing gluconeogenesis to occur during growth on any carbon source metabolized via the TCA cycle. It has been shown that the *facB* gene, which mediates acetate induction of enzymes specifically required for acetate utilization, is not directly involved in PEPCK induction. This is in contrast to *Saccharomyces cerevisiae*, where Cat8p and Sip4p, homologs of FacB, regulate PEPCK as well as the expression of other genes necessary for growth on nonfermentable carbon sources in response to the carbon source present. This difference in the control of gluconeogenesis reflects the ability of *A. nidulans* and other filamentous fungi to use a wide variety of carbon sources in comparison with *S. cerevisiae*. The *acuF* gene was also found to be subject to activation by the CCAAT binding protein AnCF, a protein homologous to the *S. cerevisiae* Hap complex and the mammalian NFY complex.

Microorganisms capable of growth on two-carbon compounds, fatty acids, or other sources of tricarboxylic acid (TCA) cycle intermediates as sole carbon sources require the production of hexose sugars via gluconeogenesis. Regulation of the enzymes involved is of great importance in avoiding futile cycling between the degradation and biosynthesis of sugars by glycolysis opposed by gluconeogenesis. There are two key enzymes unique to gluconeogenesis. Fructose-1,6-bisphosphatase (EC 3.1.3.11) is necessary for growth on all carbon sources that require gluconeogenesis. Phosphoenolpyruvate carboxykinase (PEPCK [EC 4.1.1.32]) converts oxaloacetate to phosphoenolpyruvate, which is then converted to hexose phosphates and is necessary for growth on compounds that are metabolized via TCA cycle intermediates.

A single gene (*PCK1*) encodes PEPCK in *Saccharomyces cerevisiae* (14). PEPCK activity is strictly controlled by glucose-induced inactivation of enzyme activity (8, 23) and increased mRNA instability (49, 50). In addition, *PCK* transcription is strongly repressed by even very low concentrations of glucose (49). *PCK1* transcription is dependent on the *CAT8* gene, which encodes a protein with a Zn(II) Cys6 DNA binding domain and, together with the related Sip4p, is necessary for the activation of a wide variety of genes for enzymes required for growth on nonfermentable carbon sources, such as acetate

and ethanol (21, 24, 25, 26, 46). Each of these genes contains one or more *cis*-acting elements termed carbon source response elements (CSRE) in their 5' regions, and Cat8p and Sip4p binding in vitro to these elements has been found (21, 26, 35, 40, 46). Glucose repression of *CAT8*-dependent genes, including *PCK1*, is likely to result from the activity of the Mig1p repressor (39) as well as an unknown low-glucose sensing mechanism (49), and derepression is dependent on the Snf1p serine/threonine protein kinase (21, 37). In the yeast *Kluyveromyces lactis*, however, *Kl CAT8*, a homolog of *CAT8*, is not involved in the regulation of PEPCK. Deletion of *Kl CAT8* was found not to affect transcription of the *Kl PCK1* gene, while at the same time, derepression of the glyoxalate cycle enzymes, isocitrate lyase and malate synthase, was impaired (22).

In the filamentous fungus *Aspergillus nidulans*, the *facB* gene has been shown to encode a protein with similarity to Cat8p, Sip4p, and KICat8p in the DNA binding domain (22, 44). Loss-of-function mutations in *facB* result in an inability to grow on two-carbon compounds metabolized via acetyl coenzyme A (acetyl-CoA), and it has been found that FacB activates acetate-inducible expression of the *facA* (acetyl-CoA synthase), *acuD* (isocitrate lyase), *acuE* (malate synthase), and *facC* (cytoplasmic acetyl carnitine transferase) genes, which are required for growth on acetate as a sole source of carbon (2, 3, 28, 30, 42, 43). In addition, FacB activates expression of the *amdS* gene encoding acetamidase (28, 43). Two dissimilar binding sites for FacB have been identified in the 5' region of acetate-induced genes (43).

A. nidulans *acuF* mutants have been isolated by virtue of an

* Corresponding author. Mailing address: Department of Genetics, University of Melbourne, Victoria 3010, Australia. Phone: 61 3 83446239. Fax: 61 3 8344 5139. E-mail: mjhynes@unimelb.edu.au.

† Present address: Molecular Microbiology, University of Goettingen, Goettingen 37077, Germany.

inability to grow on acetate and found to specifically lack PEPCK activity (3). They are also unable to grow on glutamate or butyrate as sole carbon sources, consistent with a role for PEPCK in gluconeogenesis. Unlike *S. cerevisiae* (47), *A. nidulans* is capable of using amino acids such as glutamate and proline as sole carbon sources, and *facB* mutants are unaffected in the utilization of these compounds (3). Previous studies have shown that PEPCK activity is induced not only by acetate but also by glutamate, proline, and other sources of TCA cycle intermediates (31). It is therefore likely that transcriptional regulation of *acuF* differs significantly from that of the *PCK1* gene. We now report the cloning of the *acuF* gene and show that it encodes a protein with high similarity to the ATP-dependent PEPCK enzyme of other organisms. Analysis of regulation of the gene via Northern blotting and *lacZ* fusions confirms that expression is induced by sources of TCA cycle intermediates and the pattern of regulation is not consistent with direct regulation by FacB. Furthermore, deletion analysis of the 5' region of *acuF* shows that the region responsible for induction lacks FacB binding sites. In addition, we have demonstrated that the CCAAT binding complex, AnCF (7), activates *acuF* expression but does not affect induction.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *A. nidulans* media and growth conditions used were as described by Cove (11). Growth of mycelium was in 250-ml Erlenmeyer flasks shaken at 37°C for all experiments. The strains used for RNA isolation for the wild type (MH 0001), *facB101* (MH 0764) *facA303* (MH0058), *facC::argB* (MH 8361), and *acuJ211* (MH 8352) have been described (42). The *acuH253*-containing strain (A653) was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City. The strain containing the *amdS-lacZ* gene (MH3408) has been described (13). The plasmid constructs containing the *acuF-lacZ* fusion (see below) were transformed into the *argB1* mutant strain (MH3018 [42]), and single-copy *ArgB*⁺ integrants were obtained by the method described by Punt et al. (36). Standard crosses were used to generate the appropriate double mutants containing *creA204* (29), *facA303* (2), *facB101* (2), *acuD306* (3), *acuF205* (3), *acuG223* (3), *prn-309* (18), *gdhB101* (31), *hapC::riboB* (34), and an *acuJ* disruptant allele, *acuJ::pyrG* (M. J. Hynes, unpublished data).

General methods. *A. nidulans* transformation was as previously described (1). Genetic manipulations of *A. nidulans* were carried out as described by Clutterbuck (9, 10). Plasmid and genomic DNA and RNA were isolated as previously described (42). Northern and Southern blot analyses were as previously described (42). DNA sequencing of subcloned fragments in pBluescript SK(+) (Stratagene) was performed by the Australian Genome Research Facility using ABI377 automatic DNA sequencing.

Cloning of *acuF* gene. A BLAST search of the *A. nidulans* expressed sequence tag (EST) database (<http://www.genome.ou.edu/asper.html>) using the *S. cerevisiae* *PCK1* sequence identified the following ESTs as encoding a potential PEPCK: 13a02a1, n1D07a1, v1a06a1, s8 h08a1, l8g09a1, r4f12a1, s3a02a1, u4a06a1, z1f08a1, and w5a09a1. Two primers based on these sequences, ACUF3 (5'-ACTGGTCGCTCTCTTCA3') and ACUF4 (5'-CTGGACAACGGGACC GGC3'), were used in a PCR on *A. nidulans* genomic DNA using 30 cycles at 95°C for 1 min, 52°C for 30 s, and 72°C for 2 min, with a final cycle of 72°C for 4 min. The 1.8-kb product was cloned into pGEMT Easy (Promega), and sequencing confirmed the presence of a PEPCK-encoding sequence. The PCR product was labeled and used to probe an *A. nidulans* genomic bacterial artificial chromosome (BAC) library (provided by Ralph Dean, Clemson University). One of three positive clones was used for subcloning into pBluescript SK(+) (Stratagene) for further analysis.

Construction of *acuF-lacZ* fusion. A *Bgl*II fragment (coordinates -2364 to +1718) of the *acuF* gene was cloned into the unique *Bam*HI site of the plasmid pAN923-43B (45), generating an in-frame fusion of the predicted first 440 amino acids of *acuF* with the *lacZ* gene. The *argB*⁺ gene of this plasmid was mutated by filling in the unique *Bgl*II site with Klenow DNA polymerase and religating, thereby allowing the selection of *argB*⁺ transformants generated by crossing over with the *argB1* mutation (36). Crossing of the resulting *acuF-lacZ* strain to an

acuF205 mutant showed that the encoded fusion protein lacked activity. 5' Deletions of *acuF* in this plasmid were generated by cutting with *Nru*I and either *Bam*HI or *Not*I, end filling with Klenow polymerase, and religating, thereby generating deletions to -1012 and -486, respectively.

Nucleotide sequence accession number. The GenBank accession number of the *acuF* gene is AY049067.

RESULTS

Cloning and sequencing of *acuF* gene. A BLAST search of the *A. nidulans* EST database (<http://www.genome.ou.edu/asper.html>) revealed numerous sequences predicted to encode proteins with high similarity to the PEPCK enzymes of various yeast, plant, and bacterial species. Primers based on these sequences were used to amplify genomic DNA via the PCR and yielded a product of approximately 1.8 kb. This was cloned and partially sequenced to confirm that the predicted encoded product was similar to PEPCK. The PCR product was used to probe an *A. nidulans* BAC library, and three positive clones were identified. One of these was used to generate plasmid subclones for sequencing. A DNA segment of 4.71 kb was sequenced and predicted to encode a polypeptide of 556 amino acids with three introns, which were confirmed by comparison with homologous sequences in the *A. nidulans* EST database. The most 5' EST sequence began at position -52 (relative to the ATG start codon), and the sequenced 5' untranslated region was 2,314 bp in length.

Confirmation that the clone contained the *acuF* gene was obtained by complementation of the *acuF205* mutation (3). A subclone containing 2 kb of 5' sequence and extending to a *Cla*I site approximately 1.5 kb 3' of the sequenced region was cotransformed with the *riboB*⁺-containing plasmid pL3 (33) into a *riboB2 acuF205* strain, and *Ribo*⁺ transformants were selected. Of 25 transformants, 15 were fully complemented for the *AcuF*⁻ phenotype in that they showed restoration of growth on the carbon sources, acetate, proline, and glutamate.

The predicted amino acid sequence of *AcuF* showed throughout a high degree of similarity to bacterial, plant, and *S. cerevisiae* ATP-dependent PEPCK enzymes (Fig. 1). There was no similarity to the GTP-dependent class (EC 4.1.1.32) found in most animals and the anaerobic rumen fungus *Neocallimastix frontalis* (38). *AcuF* was predicted to be cytoplasmic by application of the PSORT program and the Target P version 1.01 program (20). The Protein Sequence Analysis program (<http://www.softberry.com/protein.html>) predicted a PEPCK (ATP) signature sequence between amino acids 275 and 290.

Regulation of *acuF* transcription. Northern blot analysis revealed a single RNA hybridizing to an *acuF*-specific probe (Fig. 2). In the presence of glucose, the RNA was present at low levels and induced by proline, glutamate, and gamma aminobutyrate (GABA) when present as sole nitrogen sources but was not induced by acetate in the presence of glucose. The *acuF* message was induced by acetate, proline, glutamate, and GABA as sole carbon sources. Mutations that result in an inability to grow on acetate as the sole carbon source all resulted in a loss of induction (Fig. 2B). These data were consistent with acetate induction being dependent on metabolism of acetate via the glyoxalate cycle.

Regulation of *acuF-lacZ* fusion. A fusion of the *lacZ* gene to *acuF* was constructed to encode a fusion protein with the first 440 amino acids of *AcuF* fused to *LacZ*. This was inserted in

An_PEPCK	MAATSVRRGALHFGGVQEG.....	19
At_PEPCK	JSAAGGNAANNDGGSFSGKGPVMPKLTGAAGKRSQVCHDSGPTVNAATIDELSLQKK	60
Ec_PEPCK	AVYNG.....	6
Sc_PEPCK	NSPKMNAVQSTS.....	14
An_PEPCK	KGTSTFEELHEHAHVDYRVAIA.....	44
At_PEPCK	RSATPTPIQMAAAFAAVSSEBRKIQLOSASASLASTRESGPKVVRGDPAAKKTDS	120
Ec_PEPCK	HTPELEATGSDVDIYN.....	26
Sc_PEPCK	EVEOKIRQELALSVDYTHR.....	35
An_PEPCK	NFSVAALYEDALVY.....EYGAFTSS	67
At_PEPCK	TPAYAGQHSIFSPATGAVSDSLKFTLVLYLSPALYEQAKY.....BKSGFTSN	176
Ec_PEPCK	PSVDLYQEEQPSLTGYERGVDNL	52
Sc_PEPCK	NAPALVYEDGK.....ENKTVISSS	57
An_PEPCK	GALTAYSCAKTGRSPDKRIYKESSEDFHWG.....PVNKPMPDVWIRNRERAVDY	121
At_PEPCK	GALTATSGAKTGRDPKRRVVRDATTEDPFWWG.....SPNIEDEHETMVRNRERAVDY	232
Ec_PEPCK	GAVADTGITFGASPKDRIYVDDATROTMMADKGGKNDKPKSPBTWOHLKGLVTRQ	112
Sc_PEPCK	GALTAYSGVKTGRSPDKRIYKESSEDFHWG.....PVNKPMPDVWIRNRERAVDY	111
An_PEPCK	INTERRIYVDFAGWDERYRISVRVVCARVYHALFMNMLIRPSEELAHFEPDYVY	180
At_PEPCK	INSLEKVFNDQTLNMDPNRIKRVIVSARAYSLFMNMLIRPSEELAHFEPDYVY	292
Ec_PEPCK	ISG.KRFLPVVDACGAPEDTRESVETIEVAQOEYVNMIRPSEELAHFEPDYVY	170
Sc_PEPCK	INTERRIYVDFAGWDERYRISVRVVCARVYHALFMNMLIRPSEELAHFEPDYVY	171
An_PEPCK	NAGSPFANRFT.....GHTMSTSVAINPDEHENVILCTEYAGEMKKGVFLIFVYVPMKHMV	239
At_PEPCK	NAGQPPCNRYT.....HYMTSGTSDVILNARRENVILCTEYAGEMKKGVFLIFVYVPMKHMV	350
Ec_PEPCK	NKAKTNPQKCGENENFYVATNTERMOLGSGTYGEMKKGVFLIFVYVPMKHMV	229
Sc_PEPCK	NAGQPPANRFT.....GHTMSTSVAINPDEHENVILCTEYAGEMKKGVFLIFVYVPMKHMV	230
An_PEPCK	TLHSSANEGQNGDVVFVFLSGTGKTTLSADPKRALIGDDEHCWTDGVPNIEGGCYAK	298
At_PEPCK	TLHSSANEGQNGDVVFVFLSGTGKTTLSADPKRALIGDDEHCWTDGVPNIEGGCYAK	409
Ec_PEPCK	TLHSSANEGQNGDVVFVFLSGTGKTTLSADPKRALIGDDEHCWTDGVPNIEGGCYAK	288
Sc_PEPCK	TLHSSANEGQNGDVVFVFLSGTGKTTLSADPKRALIGDDEHCWTDGVPNIEGGCYAK	290
An_PEPCK	CTGLSAEKPEPDIHNAIFGSLVENVVDFPSRVVDYDSSHTENTRCAYPIETENAKVF	358
At_PEPCK	CTGLSAEKPEPDIHNAIFGSLVENVVDFPSRVVDYDSSHTENTRCAYPIETENAKVF	469
Ec_PEPCK	CTGLSAEKPEPDIHNAIFGSLVENVVDFPSRVVDYDSSHTENTRCAYPIETENAKVF	347
Sc_PEPCK	CTGLSAEKPEPDIHNAIFGSLVENVVDFPSRVVDYDSSHTENTRCAYPIETENAKVF	350
An_PEPCK	CTGLSHSNNILLTCDASGVLPVSKLTPEQWYHFIISGYTSKMGTEGQVTEPPFFSS	418
At_PEPCK	CTGLSHSNNILLTCDASGVLPVSKLTPEQWYHFIISGYTSKMGTEGQVTEPPFFSS	528
Ec_PEPCK	CTGLSHSNNILLTCDASGVLPVSKLTPEQWYHFIISGYTSKMGTEGQVTEPPFFSS	407
Sc_PEPCK	CTGLSHSNNILLTCDASGVLPVSKLTPEQWYHFIISGYTSKMGTEGQVTEPPFFSS	410
An_PEPCK	CFAPQFLALHPHYRAHLADRSQHRANAHLNTGVCVCGATGGRCELYKTRATIDAI	478
At_PEPCK	CFAPQFLALHPHYRAHLADRSQHRANAHLNTGVCVCGATGGRCELYKTRATIDAI	587
Ec_PEPCK	CFAPQFLALHPHYRAHLADRSQHRANAHLNTGVCVCGATGGRCELYKTRATIDAI	462
Sc_PEPCK	CFAPQFLALHPHYRAHLADRSQHRANAHLNTGVCVCGATGGRCELYKTRATIDAI	470
An_PEPCK	ISGSLANAEYTYDVENLHVSCEGVDFDSENNNSWLAATTS.....NSDWNKLAKIFENEF	537
At_PEPCK	ISGSLANAEYTYDVENLHVSCEGVDFDSENNNSWLAATTS.....NSDWNKLAKIFENEF	646
Ec_PEPCK	ISGSLANAEYTYDVENLHVSCEGVDFDSENNNSWLAATTS.....NSDWNKLAKIFENEF	521
Sc_PEPCK	ISGSLANAEYTYDVENLHVSCEGVDFDSENNNSWLAATTS.....NSDWNKLAKIFENEF	530
An_PEPCK	OKYA.....DQATKEVHLAGPVPVQ	556
At_PEPCK	EVVAHKKIGVDGKLTPEELAGPFG	671
Ec_PEPCK	DKYT.....DTPGALVLAGPVL	540
Sc_PEPCK	KIQ.....DQATKEVHLAGPVPVQ	549

FIG. 1. Comparison of predicted amino acid sequence of product of *acuF* gene with PEPCK enzymes from other species. Identical residues are indicated by dark shading, and similar residues are indicated by light shading. The alignment was generated using Pileup (GCG software package [16]) and viewed with boxshade (bioinformatics group; ISREC). The proteins in the alignment were from *A. nidulans* (An [this paper]), *Arabidopsis thaliana* (At [GenBank accession no. T06034]), *Escherichia coli* (Ec [P22259]), and *S. cerevisiae* (Sc [P10963]).

single copy at the *argB* locus and used to investigate *AcuF* regulation under various growth conditions and in various genetic backgrounds (Fig. 3 and 4).

Induction by proline was observed in the presence and absence of glucose but was greater in the absence of glucose (Fig. 3A). Acetate induction was observed only in the absence of glucose. Acetate and proline induction was not additive (Fig. 3A and 4A), and the kinetics of induction by proline and acetate was similar (Fig. 4A).

The *creA* gene encodes a repressor protein with two C2H2 zinc fingers similar to those of *S. cerevisiae* Mig1p and Mig2p and is required for carbon catabolite repression (17, 39). The *creA204* mutation results in relief of repression by glucose of genes controlled by carbon catabolite repression (29). In a *creA204* background, expression of *AcuF-LacZ* was elevated

less than twofold in the presence of glucose and was unaffected in its absence (Fig. 3A). Notably, *creA204* did not relieve the effects of glucose on acetate induction.

Consistent with the Northern blotting results, loss-of-function mutations in the *facA*, *facB*, *acuD*, and *acuJ* genes, all of which prevent acetate utilization, resulted in greatly reduced acetate induction but did not affect proline induction (Fig. 3B and 4B). The catabolism of proline requires activities encoded by the *prm* gene cluster (5). A deletion of the *prm* cluster resulted in a loss of proline but not acetate induction, showing that proline catabolism is required for induction (Fig. 3C). The *gdhB* gene encodes NAD-dependent glutamate dehydrogenase, which acts to convert glutamate to 2-oxoglutarate and ammonia and consequently is required for both proline and glutamate utilization (6). The *gdhB101* mutation resulted in a loss of induction by both proline and glutamate but not acetate (Fig. 3D). There was a residual level of induction by glutamate and proline, possibly because the *gdhB101* mutation is not a complete loss-of-function mutation. These results indicated that the conversion of proline, glutamate, and acetate into TCA cycle intermediates was required for induction.

FacB is required for acetate induction of *AcuF-LacZ* (Fig. 4B). However, this is likely to be due to an indirect effect on the conversion of acetate into TCA cycle intermediates. Consistent with this, induction by acetate of the *amdS-lacZ* fusion, where it has been shown that acetate metabolism is not required, was much more rapid than for the *acuF-lacZ* fusion (compare Fig. 4A and E). Furthermore, the TCA intermediates malate and succinate were found to result in the induction of *acuF-lacZ* (Fig. 4C), although induction was slower than for acetate or proline. Both succinate and malate are poor carbon sources for *A. nidulans* and uptake may be limiting. Induction by malate and succinate were also observed in the *facB101* mutant background (Fig. 4D). Weak induction by the poor carbon source, 2-oxoglutarate, was observed (results not shown) while oxaloacetate, which is not a sole source of carbon for *A. nidulans*, was not found to act as an inducer.

PEPCK was previously found to be elevated in an *acuG* mutant lacking fructose-1,6-bisphosphatase (3). Expression of *acuF-lacZ* was increased in an *acuG223* mutant background in the absence of glucose and in the presence and absence of the addition of proline, acetate, and malate as sources of induction (Fig. 3F). Similarly, the presence of the *acuF205* mutation resulted in elevated expression (Fig. 3F).

The 5' region of the *acuF* gene was found to contain a number of CCAAT sequences, the closest to the start of the gene being at -283 relative to the ATG start codon. CCAAT sequences have been found to be a binding site for the AnCF transcription factor (comprising the HapB, HapC, and HapE subunits) which affects, both negatively and positively, the expression of a diverse range of genes in *A. nidulans* (7, 34, 41). The expression of the *acuF-lacZ* fusion was found to be reduced by approximately 60% in a *hapC* deletion mutant background, but induction by proline or acetate was not affected (Fig. 3E).

Effects of 5' deletions on *acuF-lacZ* expression. Two deletion derivatives of the *acuF-lacZ* construct were made and integrated in single copy at the *argB* locus. Deletion to -1012 was found to lead to reduced levels of expression without affecting induction. However, the response to carbon starvation was

FIG. 2. Analysis of *acuF* expression by Northern blot analysis. Total RNA was isolated from the wild-type strain (A) and strains with relevant genotypes (B). Mycelia were grown for 16 h in 1% glucose–10 mM ammonium tartrate and then were transferred to medium containing glucose (1%) or no added carbon source (C-free) with the following additions as indicated: NH₄ (10 mM ammonium chloride), pro (10 mM L-proline), Ac (50 mM sodium acetate, pH 6.5), glu (10 mM L-glutamate), and gaba (10 mM gamma-amino butyric acid). *acuF* RNA was detected by using a 2.32-kb *Pst*I-*Xho*I fragment of the *acuF* gene (coordinates –961 to +1359) as a probe. The blots were also hybridized with the *Eco*RI fragment of the histone H3 gene (19) as a loading control.

eliminated. In contrast, deletion to –486 resulted in very high constitutive levels of expression, but again, induction by proline and acetate was observed (Fig. 5). These data indicate that there may be one or more positive elements upstream of –1012 that are involved in determining the level of expression, particularly in response to carbon starvation and that there may be one or more negative elements between –486 and –1012 that act to repress expression. However, it cannot be excluded that these effects result from adventitious juxtaposition of vector sequences affecting expression. Nevertheless, the results show that the element or elements responsible for induction lie downstream of –486. The *hapC* deletion mutation was found to reduce expression of the –486 deletion construct (results not shown) consistent with a CCAAT element at –283 being functional.

DISCUSSION

The *acuF* gene is defined by mutations resulting in the loss of the ability to grow on acetate, butyrate, and glutamate and other sources of TCA cycle intermediates as sole carbon sources (3). We have cloned a gene capable of complementing an *acuF* mutation, and the sequence shows high conservation with PEPCK genes from other organisms, consistent with specific loss of PEPCK activity in *acuF* mutants (3).

We have studied regulation of *acuF* expression by Northern blot analysis and *lacZ* fusions and found that regulation differs significantly from the regulation of the *PCK1* gene of *S. cerevisiae*. Even low concentrations of glucose result in strong repression of *PCK1* expression (50). In contrast, significant expression of *acuF* was observed in the presence of glucose. The *creA* gene, a homolog of the *MIG1* and *MIG2* genes of *S. cerevisiae*, is the major gene involved in carbon catabolite repression in *A. nidulans* (4, 17, 29). The partial loss-of-function *creA204* mutation was found to have only a small effect on *acuF* ex-

pression in the presence or absence of sources of induction and, although there are numerous potential CreA binding sites (consensus SYGGRG [12]) in the *acuF* 5' region, it is clear that CreA does not strongly regulate *acuF*. Carbon starvation in the absence of added inducer resulted in a twofold increase in activity. This was eliminated by deletion to –1012, indicating the possible presence of an upstream element responsible for this weak regulation.

Acetate results in strong induction of *acuF* expression. This is abolished by mutations in the genes *facA* (acetyl-CoA synthase [2, 3]), *acuD* (isocitrate lyase [3]), *facC* (cytoplasmic carnitine acetyltransferase [42]), *acuJ* (peroxisomal and mitochondrial carnitine acetyltransferase [42; M. J. Hynes, unpublished data]), and *acuH* (acetyl carnitine carrier protein [3, 15])—all of which result in loss of acetate metabolism via the TCA cycle. In addition, the loss-of-function *facB101* mutation affecting acetate induction of genes required for acetate utilization mediated by the *facB* activator (28, 30, 43, 44) results in the loss of acetate induction. However, a direct effect of FacB on *acuF* expression is unlikely. Mutations in the *acuH* and *acuJ* genes have been found to result in increased FacB-mediated acetate induction (42; M. J. Hynes, unpublished data), which was not observed for *acuF*. Furthermore, the expression of *facB* is subject to CreA regulation, resulting in FacB-mediated acetate induction being subject to glucose repression which is greatly reduced in a *creA204* background (30, 42). Glucose prevented acetate induction of *AcuF-lacZ* expression even in a *creA204* background, indicating that the effect of glucose is by affecting inducer formation rather than by repressing *facB* expression. In contrast, in *S. cerevisiae*, PEPCK is strongly regulated by glucose, with the major transcriptional control being via glucose-sensitive Cat8p activation (21, 25, 26, 35, 37).

acuF expression is also induced by compounds metabolized via glutamate, such as GABA and proline. Induction by proline

FIG. 3. Regulation of expression of *acuF-lacZ* gene. Mycelium was grown for 16 h in 1% glucose–10 mM ammonium tartrate medium and then was transferred to the indicated media (designations as for Fig. 2) for 4 h before harvesting. Mycelium was extracted and assayed for β -galactosidase as previously described (13). The specific activity is expressed as Miller units per minute per milligram of protein and presents the average of a minimum of three independent experiments. Standard errors were less than 25% of the average. The relevant genotypes of the *acuF-lacZ* strains are indicated.

and glutamate requires metabolism to 2-oxoglutarate since induction is reduced by the *gdhB101* mutation in the structural gene for NAD-dependent glutamate dehydrogenase (6). Glutamate, proline, and GABA are capable of acting as sole nitrogen sources for *A. nidulans* in the presence of glucose. Under these conditions, the genes involved are activated by AreA, the wide domain regulator involved in nitrogen metabolite repression of nitrogen catabolic genes (4, 48). Induction of *acuF* by these sources of 2-oxoglutarate was observed in the presence of glucose, although the level of induction was less than in its absence. This supports the conclusion that glucose does not strongly directly repress *acuF* expression but reduces the formation of the inducing metabolite(s).

Induction of *acuF* by acetate and sources of 2-oxoglutarate

appears to be via a single mechanism since proline and acetate induction is not additive and the kinetics of induction by each inducer are very similar. Therefore, it is proposed that *acuF* expression is induced by a single mechanism involving a metabolite that can be derived from either 2-oxoglutarate or acetate. Induction by succinate and malate, both of which can be derived from 2-oxoglutarate (via the TCA cycle) or from acetate (via the glyoxalate bypass), was observed—supporting the interpretation that a TCA cycle intermediate or derivative is the inducer. Possible candidates are malate or oxaloacetate, the substrate for PEPCK. Exogenous malate resulted in some induction, but oxaloacetate, which is not a carbon source, did not—probably because of a lack of uptake. The observations that *AcuF-LacZ* expression was elevated in an *acuG* mutant

FIG. 4. Kinetics of induction of expression of *acuF-lacZ* gene. Mycelium was grown for 16 h in 1% glucose–10 mM ammonium tartrate medium and then was transferred to medium lacking a carbon source with 10 mM ammonium chloride and the indicated additions (designations as for Fig. 2 and, in addition, 10 mM malate and 10 mM succinate as the sodium salts at pH 6.5). Mycelium was harvested at the indicated times and extracted and assayed as described for Fig. 3. (A and C) Results for *acuF-lacZ* in a wild-type background. (B and D) Results for *acuF-lacZ* in a *facB101* background. (E) Kinetics of induction by acetate of the *amdS-lacZ* gene integrated in single copy by replacement of the *amdS* gene (13).

and in an *acuF* mutant suggests that these mutations result in inducer accumulation. Increased expression in the absence of inducer may indicate that some endogenous induction occurs due to metabolic turnover under starvation conditions. The nature of the inducer cannot be determined unequivocally at present. In the *acuF* mutant, no phosphoenolpyruvate would be formed making it unlikely that this is an inducer. However, oxaloacetate would accumulate but might then result in the production of malate (via malate dehydrogenase) which would be converted to pyruvate via malic enzyme. In the *acuG* mutant, loss of metabolism of fructose-1,6-diphosphate could result in the accumulation of phosphoenolpyruvate by glycolytic

enzymes. Fructose-1,6-diphosphate has been found to activate pyruvate kinase, which, although present in low levels during growth on gluconeogenic carbon sources (reference 3 and reviewed in reference 27), could result in pyruvate formation. Accumulated pyruvate could also be converted via pyruvate carboxylase to oxaloacetate. Therefore, one (or more) of malate, oxaloacetate, or pyruvate could act as inducers. Further studies with other relevant mutants are required.

The effects of deletions of the *acuF* 5' region on expression of the *AcuF-LacZ* fusion showed that a possible weak positively acting *cis*-acting sequence is upstream of –1012 and a strong negatively acting sequence lies between –1012 and

	GROWTH CONDITIONS				
	1% glucose		No carbon source + NH ₄		
	NH ₄	Pro	—	Ac	Pro
-2364	110±10	560±80	280±60	1200±340	1200±340
-1012	80±20	230±50	80±5	670±60	580±40
-486	830±40	1900±110	1000±190	3100±600	3300±800

FIG. 5. Effects of 5' deletions on regulation of expression of *acuF-lacZ* gene. Mycelium was grown, extracted, and assayed as described for Fig. 3. Results are expressed as the average of at least three independent experiments, and standard errors are shown. Each *acuF-lacZ* construct was present as a single copy integrated at the *argB* locus (see Materials and Methods).

–486. It is clear that induction by acetate and by proline still occurs in the –486 deletion derivative. This region lacks sequences similar to the previously determined consensus FacB binding sites (43). Therefore, one or more novel *cis*-acting elements involved in induction are proposed to lie in this *acuF* 5' sequence.

In addition, the CCAAT binding complex, AnCF, acts via this same 5' region to affect the level of expression without affecting induction. The *A. nidulans* AnCF regulatory protein contains homologs of the Hap2p, Hap3p, and Hap5p subunits of the *S. cerevisiae* Hap complex as well as of the corresponding subunits of the NFY mammalian complex (7, 34, 41). AnCF acts at CCAAT sequences to affect the expression of a diverse array of genes with no obvious common role in metabolism (7). Binding of AnCF to a CCAAT sequence in the 5' region of the *amdS* gene has been shown to result in the formation of a nucleosome-free region (32). Here, we have shown that *AcuF-LacZ* expression is greatly reduced in a strain with a deletion in the gene encoding the HapC subunit of AnCF. However, induction was unaltered. For *S. cerevisiae*, it is not known whether the Hap complex influences *PCK1* expression.

It is clear that the regulation of PEPCK expression is fundamentally different in the filamentous fungus *A. nidulans* compared with *S. cerevisiae*. Induction of PEPCK allows gluconeogenesis to occur during growth on any carbon source metabolized via TCA cycle intermediates. In *S. cerevisiae* cells, PEPCK is 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 consequently, the regulation of gluconeogenesis is different in this group of organisms.

ACKNOWLEDGMENTS

The provision of the *A. nidulans* BAC library by Ralph Dean is gratefully acknowledged, as well as assistance by Sophie Delimitrou.

This work was supported by a grant from the Australian Research Council.

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