Cloning of the gene for glutamate decarboxylase and its expression during conidiation in *Neurospora crassa*

Ruyi HAO* and Joseph C. SCHMIT†

Medical Biochemistry, Southern Illinois University at Carbondale, Carbondale, IL 62901, U.S.A.

Neurospora crassa glutamate decarboxylase (GAD) is produced during conidiation and stored in dormant conidia. Polyclonal antibody was generated to GAD that had been purified to homogeneity. The anti-GAD antibody was specific for *N. crassa* GAD and inhibited GAD activity. The level of GAD protein decreased during conidial germination, indicating that GAD was degraded during this phase of development. The anti-GAD antibody was used to isolate a cDNA clone of GAD from a λ_{ZAP} cDNA expression library. *Escherichia coli* containing a plasmid

with the cDNA insert produced GAD activity. The cDNA clone contained a 2.6 kbp insert and hybridized to a 2.6 kb mRNA species from conidiating cultures of *N. crassa*. GAD mRNA was not present in vegetative hyphae. In conidiating cultures, GAD mRNA was first detected when conidia began to appear. The level of GAD mRNA increased as conidiation progressed. This is the first example of the cloning of an enzyme that is regulated at the level of mRNA during the asexual developmental cycle of *N. crassa*.

INTRODUCTION

N. crassa is an eukaryotic organism with a simple asexual developmental cycle. During asexual development *N. crassa* produces three distinct cellular types, aerial hyphae, conidia and vegetative hyphae [1,2]. Conidiation occurs when vegetative hyphae grow on the surface of the growth medium. Under these growth conditions, specialized aerial hyphae develop, and conidia bud from the tips of the aerial hyphae. Conidia are dormant cells that germinate and grow into vegetative hyphae when incubated in medium containing inorganic salts and a suitable carbon source [1]. Because of its simple asexual developmental cycle, *N. crassa* should be a convenient organism in which to study cellular differentiation and development. However, very few metabolic pathways or enzymes are known that are unique to conidiation or germination phases of the asexual cycle [1].

A metabolic pathway that is apparently unique to conidial germination involves the conversion of glutamic acid into γ -aminobutyric acid [3]. Dormant conidia contain a large pool of free glutamic acid [4]. This amino acid alone can account for up to 2.5% of the conidial dry weight. During conidial germination 75% of the pool of free glutamic acid is metabolized. The enzyme glutamate decarboxylase (GAD; EC 4.1.1.15) appears to be responsible for the first step in the metabolism of glutamic acid during germination [3–5]. GAD has been purified to homogeneity from dormant conidia [6]. The active form of GAD is a monomeric protein of 30 500 Da. GAD is a relatively abundant protein in dormant conidia, representing 0.5–1% of the extractable protein [6]. GAD activity is not present in vegetative hyphae [3]. As conidial germination progresses, GAD activity disappears and does not reappear until the next round of conidiation [3].

In this report we describe the generation and characterization of polyclonal antibody to *N. crassa* GAD. The polyclonal antibody was used to isolate a cDNA clone of GAD. An mRNA species that hybridized to the GAD cDNA clone was found in

cells from conidiating cultures, but not in vegetative hyphae. This is the first example of the cloning of a gene encoding a known enzyme that is expressed during conidiation in *N. crassa*.

MATERIALS AND METHODS

Strains and materials

The wild-type strain of *N. crassa*, FGSC 988, was obtained from the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS, U.S.A. The λ_{ZAP} cDNA expression library, which had been prepared with mRNA from a conidiating culture of *N. crassa*, was a gift from Dr. Matthew Sachs (Stanford University, CA, U.S.A.). *Escherichia coli* strains BB4 and XL1-Blue and the ϕ 1 bacteriophage R408 were from Dr. Ramesh Gupta (Southern Illinois University at Carbondale). Complete and incomplete Freund's adjuvant, horseradish-peroxidase-conjugated goat anti-rabbit IgG, 3,3'-diaminobenzidine, Ponceau S, isopropyl thio- β -D-galactoside, oligo(dT)-cellulose and CNBr-activated LB4 Sepharose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[1- 14 C]Glutamic acid (46 mCi/mmol) was purchased from NEN Research Products (Boston, MA, U.S.A.). Nitrocellulose sheets for Western blotting were from Bio-Rad Laboratories (Melville, NY, U.S.A.). Restriction enzymes were purchased from Bethesda Research Laboratories Life Technologies (Gaithersburg, MD, U.S.A.). Nitrocellulose filters (BA85) for plaque lifts were from Schleicher and Schuell (Keene, NH, U.S.A.).

N. crassa growth conditions

Large quantities of *N. crassa* conidia were obtained from baking-dish cultures containing minimal medium {2% (w/v) glucose, 2% (w/v) agar and Vogel's salts [7]} as described previously [6]. The conidia were freeze-dried and stored at -70°C . Germinating conidia were obtained from liquid cultures containing minimal

Abbreviation used: GAD, glutamate decarboxylase.

* Present address: Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198, U.S.A.

† To whom correspondence should be addressed.

medium that were incubated at 20 °C on a rotatory shaker at 150 rev./min. The cells were harvested by filtration on to glass-fibre filters [6]. Conidiating cultures were grown in minimal liquid medium without shaking at room temperature. Mycelial mats formed on the surface of liquid medium after 3 days, and the cultures began to conidiate after 4 days.

Preparation of protein extracts and GAD assays

Protein extracts of dormant conidia, germinating conidia and vegetative hyphae were obtained by homogenizing the cells with glass beads in a Bead Beater (Biospec Products, Bartlesville, OK, U.S.A.) [6]. GAD activity was assayed by the release of $^{14}\text{CO}_2$ from L-[1- ^{14}C]glutamic acid as described previously [6].

Western blotting

The proteins were separated on 12.5%-polyacrylamide slab gels containing 0.1% SDS [8] and were electrophoretically transferred from the SDS gels to nitrocellulose sheets [9]. In some cases, the proteins that had been transferred to the nitrocellulose sheets were stained with Ponceau S to locate the positions of the transferred proteins before treating the filters with anti-GAD antibody [9,10]. The unoccupied binding sites on the nitrocellulose sheets were blocked by incubating the filters in 1% dry milk in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.4) for 1 h at room temperature [10]. After three washes with PBS, the nitrocellulose sheets were incubated with agitation with a 1:1000 dilution of anti-GAD serum for 1 h at room temperature. The sheets were rinsed three times with PBS containing 0.05% Tween 20 and incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) for 1 h. After washing, the nitrocellulose sheets were incubated with the peroxidase substrate 3,3'-diaminobenzidine for 1–2 min [10]. Control experiments were done with pre-immune serum instead of anti-GAD serum.

Screening the λ_{ZAP} cDNA library

The *E. coli* antibodies in the polyclonal anti-GAD serum were removed by incubating the serum with *E. coli* proteins bound to CNBr-activated LB4 Sepharose [10]. The purified antiserum was used to screen a λ_{ZAP} cDNA expression library for phage that produced GAD [10]. λ phages were isolated from plaques that showed positive reactions with anti-GAD antibody and re-screened until all of the plaques on the plate gave a positive reaction. Bluescript plasmids containing cDNA inserts were excised *in vivo* from the λ_{ZAP} vector by co-infecting XL-1 Blue cells with the helper phage R408 [11]. Plasmid DNA was isolated by alkaline lysis and digested with *Eco*R1 to release the cDNA insert [10].

Preparation of poly(A)⁺ RNA

The freeze-dried cells were ground to a fine powder with an RNAase-free mortar and pestle that was chilled with liquid N_2 . The ground cells were suspended in 5 ml of 6% (w/v) sodium *p*-aminosalicylate, 1% (w/v) sodium tri-isopropyl-naphthalene-sulphonate, 20 mM EDTA and 50 mM Tris/HCl, pH 7.5 [12]. The RNA solution was extracted twice with equal volumes of phenol and chloroform.

Poly(A)⁺ RNA was prepared from oligo(dT)-cellulose columns [10]. The poly(A)⁺ RNA was separated by electrophoresis on 0.8% agarose gels containing formaldehyde [10]. The RNA was transferred to nitrocellulose filters by capillary transfer [10]. The plasmid p222 containing the cDNA clone of GAD was labelled with ^{32}P by nick translation using a kit from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). The filters were subjected to pre-hybridization and hybridization in 50% formamide, 5 × SSPE (20 × SSPE contained 3 M NaCl, 0.17 M NaH_2PO_4 , 0.02 M EDTA, pH 7.4), 2 × Denhardt's, 0.1% SDS and 10 μg of salmon sperm DNA/ml at 42 °C [10]. The filters were washed twice in 1 × SSC (150 mM NaCl, 17 mM sodium citrate, pH 7.0)/0.1% SDS and twice in 0.1 × SSC/0.1% SDS at room temperature [10] and then exposed to X-ray film (Kodak XAR-5) at –70 °C with an intensifying screen. After exposure to the X-ray film, the nitrocellulose filters were stained with Methylene Blue [10] to locate the positions of the rRNA bands which were used as size standards.

RESULTS AND DISCUSSION

Generation of polyclonal antibody to *N. crassa* GAD

GAD which had been purified to homogeneity [6] was used to immunize male New Zealand white rabbits. GAD (100 μg) was emulsified with complete Freund's adjuvant and injected subcutaneously [10]. The rabbits were given additional biweekly injections of 50 μg of GAD emulsified with incomplete Freund's adjuvant. Sera were collected, tested for the presence of GAD antibodies, and then stored at –70 °C. The anti-GAD serum gave a single precipitin band with purified GAD on Ouchterlony double-immunodiffusion plates. No precipitin band was observed with pre-immune serum. GAD activity was inhibited when the purified enzyme was incubated with increasing amounts of anti-GAD serum (Figure 1). GAD activity decreased 15% with a 1:2000 dilution of immune serum. At higher concentrations of immune serum, only 6–7% of the GAD activity remained. Pre-immune serum did not inhibit GAD activity.

Western blots of crude protein extracts of *N. crassa* conidia gave a single major band (Figure 2, lane A). Purified GAD gave a band at the same position on the gels (results not shown). In this experiment several faint higher-molecular-mass bands were seen (Figure 2, lane A). These bands corresponded to aggregates

Figure 1 Immunotitration of GAD

Samples containing 4 μg of purified GAD in a volume of 10 μl were incubated for 30 min at 25 °C with 10 μl of a series of dilutions of anti-GAD serum in PBS [10]. GAD assay buffer was added (40 μl), and the reaction was initiated by adding L-[1- ^{14}C]glutamate [6]. The reaction was stopped after 30 min at 37 °C, and the $^{14}\text{CO}_2$ released from glutamic acid was determined [6]. Controls were done with pre-immune serum and without serum.

Figure 2 Western blot of GAD from conidia, germinating conidia and vegetative hyphae

The germinating conidia and vegetative hyphae were obtained from shaken liquid cultures containing minimal medium that were incubated at 20 °C at 150 rev./min. Protein extracts were prepared as described in the Materials and methods section. Samples containing 10 µg of crude protein extracts from dormant conidia (lane A), germinating conidia after 1 h (lane B), germinating conidia after 2 h (lane C) and vegetative hyphae from a 24 h culture (lane D) were separated on an SDS/polyacrylamide slab gel. The proteins were transferred to a nitrocellulose membrane and treated with anti-GAD serum as described in the Materials and methods section.

of GAD. *N. crassa* GAD is known to form high-molecular-mass aggregates [6]. The only *N. crassa* protein that reacted with the rabbit antiserum was GAD.

Disappearance of GAD protein during conidial germination

GAD activity decreased by 65% within the 1 h of conidial germination, and there was a marked decrease in the amount of GAD protein (Figure 2, lane B). A further decrease in the amount of GAD protein was seen after 2 h of germination (Figure 2, lane C). No GAD activity and only a trace of GAD protein was detected in vegetative hyphae obtained from a 24 h culture (Figure 2, lane D). The trace amount of GAD protein that remained in these cultures could be due to a few conidia that failed to germinate. We conclude that the decrease in GAD activity during conidial germination was due to the degradation of GAD. Previous studies showed that cycloheximide inhibited the loss of GAD activity during germination [3]. Apparently some protein was synthesized during conidial germination that participated in the degradation of GAD. The nature of this protein is unknown. The degradation of GAD during the transition from dormant conidial to vegetative hyphae appears to be the result of a developmentally regulated process that leads to the proteolytic digestion of this enzyme.

Table 1 *N. crassa* GAD activity produced in *E. coli*

E. coli (XL-1 Blue) containing the plasmid p222 with the GAD cDNA insert were grown in 200 ml of LB medium with ampicillin and isopropyl thiogalactoside [10]. The *E. coli* cells were isolated by centrifugation and resuspended in 40 mM potassium phosphate/2 mM EDTA/0.2 mM pyridoxal 5'-phosphate/2 mM 2-mercaptoethanol/1 mM phenylmethanesulphonyl fluoride/0.5 µM pepstatin A/10% (v/v) glycerol at pH 7.2. The cells were lysed by treating them with lysozyme and then with pancreatic DNAase and Triton X-100 [10]. Cell debris was removed by centrifugation, and the protein solution was assayed for 30 min for GAD activity [6].

Protein extract	¹⁴ CO ₂ released from L-[1- ¹⁴ C]glutamate (c.p.m.)
<i>E. coli</i> with the <i>N. crassa</i> cDNA plasmid p222	747.5 ± 30
<i>E. coli</i> control culture	46.3 ± 10
None	19.0 ± 3

Isolation of a cDNA clone of GAD

The λ_{ZAP} cDNA library was screened with anti-GAD antibody as described in the Materials and methods section. One plaque that reacted strongly with anti-GAD antibody through three rounds of antibody screening was isolated. The cDNA clone of GAD was subcloned in the plasmid vector pBluescript SK⁻ by an *in vivo* excision procedure [11]. When the excised plasmid, designated p222, was digested with the restriction enzyme *Eco*R1, the cDNA insert gave two restriction fragments of about 1200 and 1400 bp.

In order to get additional evidence that the cDNA clone was a clone of *N. crassa* GAD, a culture of *E. coli* with the plasmid p222 was incubated under conditions that allowed the expression of the β-gal-GAD fusion protein (Table 1). Protein extracts were prepared from *E. coli* containing the plasmid p222, and assayed for GAD activity. As shown in Table 1, GAD activity was produced in *E. coli* containing the cDNA plasmid, but not in the host strain without the *N. crassa* cDNA plasmid. The β-gal-GAD fusion protein was sufficiently complete to produce active GAD.

Appearance of GAD mRNA during conidiation

Poly(A)⁺ RNA was isolated from *N. crassa* cultures before and after they had begun to conidiate. Vegetative hyphae which had not yet begun to conidiate did not contain GAD mRNA (Figure 3, lane B). By day 4, conidia had begun to appear at the tips of the aerial hyphae, and an mRNA species that migrated between 18 S and 25 S rRNA was detected with the GAD cDNA probe (Figure 3, lane C). The length of this mRNA was about 2.6 kb, the same as the sum of the lengths of the two *Eco*R1 fragments in the cDNA insert. As conidiation progressed, the amount of GAD mRNA also increased (Figure 3, lanes D and E). mRNA extracted from dormant conidia (Figure 3, lane A) did not show any GAD mRNA. The variation in GAD mRNA levels as a function of development could be regulated by differentially controlling the rate of mRNA synthesis or degradation, or by a combination of these two processes. The relative contributions of synthesis and degradation in regulating transcript levels during asexual development in *N. crassa* for GAD is not known.

Several *N. crassa* conidiation-specific genes of unknown function have been cloned, and some of these clones have been characterized [13–16]. Two of these clones encode genes that hybridize to mRNA species about the same size as GAD mRNA

Figure 3 Appearance of GAD mRNA during conidiation

Each lane on the formaldehyde gel was loaded with 1.2 μ g of poly(A)⁺ RNA. Lane A contained RNA from dormant conidia. Lanes B, C, D and E contained RNA from conidiating cultures that had been grown for 3, 4, 7 and 8 days respectively. Conidia first appeared in these cultures on day 4. The probe was the plasmid p222, which contained the GAD cDNA insert. The positions of the rRNA bands were determined by staining the nitrocellulose membrane with Methylene Blue after autoradiography.

[16]. However, it is unlikely that the GAD clone is a duplicate of one of these genes, because the restriction-enzyme patterns are different.

GAD is regulated as a function of cellular differentiation and development in a variety of organisms in addition to *N. crassa*. GAD has been associated with specific neurons in the central nervous system of animals [17–21], and γ -aminobutyric acid has been implicated in the regulation of neural growth during insect embryogenesis [22]. GAD has been found in many different plant tissues [23–26]. Changes in GAD activity have been reported in barley embryos [26] and barley aleurone [27] during seed germination. GAD has been shown to be involved in the germination of bacterial endospores [28–30]. This is the first report of the cloning of GAD from a fungal species. This is also the first report of the cloning of a gene for an intracellular enzyme of known activity that is specifically produced during conidiation in *N. crassa*.

We thank M. S. Sachs for the λ_{ZAP} cDNA library. The technical assistance of Scott Ross is gratefully acknowledged.

REFERENCES

- Schmit, J. C. and Brody, S. (1976) *Bacteriol. Rev.* **40**, 1–41
- Springer, M. L. and Yanofsky, C. (1989) *Genes Dev.* **3**, 559–571
- Christensen, R. L. and Schmit, J. C. (1980) *J. Bacteriol.* **144**, 983–990
- Schmit, J. C. and Brody, S. (1975) *J. Bacteriol.* **124**, 232–242
- Guignard, R. and Brody, S. (1983) *Exp. Mycol.* **7**, 133–140
- Hao, R. and Schmit, J. C. (1991) *J. Biol. Chem.* **266**, 5135–5139
- Vogel, H. J. (1964) *Am. Nat.* **98**, 435–446
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Short, J. M., Fernandez, J. M., Sorge, J. A. and Huse, W. D. (1988) *Nucleic Acids Res.* **16**, 7583–7600
- Kirby, K. S. (1965) *Biochem. J.* **96**, 266–269
- Roberts, A. N. and Yanofsky, C. (1989) *Nucleic Acids Res.* **17**, 197–214
- Roberts, A. N., Berlin, V., Hager, K. M. and Yanofsky, C. (1988) *Mol. Cell. Biol.* **8**, 2411–2418
- Sachs, M. S. and Yanofsky, C. (1991) *Dev. Biol.* **148**, 117–128
- Berlin, V. and Yanofsky, C. (1985) *Mol. Cell. Biol.* **5**, 849–855
- Denner, L. A., Wei, S. C., Lin, H. S., Lin, C.-T. and Wu, J.-Y. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 668–672
- Wu, J.-Y., Matsuda, T. and Roberts, E. (1973) *J. Biol. Chem.* **248**, 3029–3034
- Wu, J.-Y., Denner, L., Lin, C.-T. and Song, F. (1985) *Methods Enzymol.* **113**, 3–10
- Stapleton, A., Tyrer, N. M., Goosey, M. W. and Cooper, M. E. (1989) *J. Neurochem.* **53**, 1126–1133
- Spink, D. C., Wu, S. J. and Martin, D. L. (1983) *J. Neurochem.* **40**, 1113–1119
- Jackson, F. R., Newby, L. M. and Kulkarni, S. J. (1990) *J. Neurochem.* **54**, 1068–1078
- Matsumoto, T., Yamaura, I. and Funatsu, M. (1986) *Agric. Biol. Chem.* **50**, 1413–1417
- Inatomi, K. and Slaughter, J. C. (1971) *J. Exp. Bot.* **22**, 561–571
- Satyanarayan, V. and Nair, M. (1985) *Eur. J. Biochem.* **150**, 53–60
- Inatomi, K. and Slaughter, J. C. (1975) *Biochem. J.* **147**, 479–484
- Duffus, C. M., Duffus, J. H. and Slaughter, J. C. (1972) *Experientia* **28**, 635–636
- Foerster, H. F. (1971) *J. Bacteriol.* **108**, 817–823
- Foerster, H. F. (1972) *J. Bacteriol.* **111**, 437–442
- Aronson, J. N., Doerner, J. F., Akers, E. W., Borris, D. P. and Mani, M. (1975) in *Spores VI* (Gerhardt, P., Costilow, R. N. and Sadoff, H. L., ed.), pp. 404–410, American Society for Microbiology, Washington DC