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The Isolation and Characterization of a Putative p-Aminobenzoate Biosynthetic Gene in

Neurospora crassa

by

Jason B. Edwards

A thesis submitted to

the Faculty of Graduate Studies and Research

in partial fulfilment of

the requirements of the degree

Master of Science

Ottawa-Carleton Institute of Biology

Carleton University

Ottawa, Ontario

September, 1998

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acceptance of the thesis

“The Isolation and Characterization of a Putative p-Aminobenzoate Biosynthetic Gene in
Neurospora crassa”

submitted by Jason B. Edwards
in partial fulfilment of the requirements
for the degree Master of Science.

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Date

Abstract

p-Aminobenzoic acid (PABA) is a precursor to tetrahydrofolate, an essential vitamin required to synthesize nucleic acids. The biosynthetic pathway for this precursor, however, is poorly characterized in both prokaryotes and eukaryotes. The subject of the current study is the *pab-2* locus of *Neurospora crassa*, the mutation of which results in auxotrophy. The *pab-2*⁺ coding sequences were isolated from genomic and cDNA libraries and sequenced. The gene contains a single intron which is differentially spliced. PCR screening of several λ cDNA libraries showed that the longer message, which codes for a 276 aa protein, represents the dominant transcript. Primary and secondary structure analysis of PAB-2 suggest homology with PabC of *E. coli* and *B. subtilis*. This putatively identifies PAB-2 as a 4-amino-4-deoxychorismate lyase; a PABA biosynthetic enzyme. The *pab-2* mutant allele was found to have a premature stop codon which significantly truncated the carboxy-terminus of the gene product. No evidence was found for feedback inhibition of *pab-2* transcription. High levels of PABA in the growth media were not toxic.

Dedication

I dedicate this thesis both to my parents, Ron and Irene, whose support during my academic years has been unwavering, and to my sister Christy, the personification of persistence.

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First and foremost I would like to thank Dr. Vierula, for donating his time, expertise and lab equipment to aid me in my quest for some extra initials after my name. I would also like to thank NSERC for their generous funding, C. Micali for the constant floggings, and the entire Smith/Lambert consortium for their moral and technical support. Finally, I would like to express my sincere gratitude to K. Campsall (lab buddy) for the transfer of great wisdom, and for preventing me from becoming a gibbering, basket case. No cute, fuzzy animals were harmed in the making of this thesis.

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Abbreviations

ADC	4-amino-4-deoxychorismate
Amp	ampicillin
ATP	adenosine triphosphate
cDNA	complementary DNA
C:I	Choroform:Isoamyl alcohol (24:1)
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EtOH	ethanol
FGSC	Fungal Genetics Stock Centre
Hg ^r	Hygromycin B resistance
IPTG	isopropyl- β -D-thiogalactoside
Kb	kilobase
LBA	LB media + 100 μ g/ml ampicillin
mRNA	messenger RNA
NaOAc	Sodium acetate
O/N	Overnight
³² P	radioisotope of phosphorous
PA	Polyalamar
PABA	p-aminobenzoic acid
pBSKS+	pBluescript II KS+
P:C:I	phenol:chloroform:isoamyl alcohol (25:24:1)
PP	Polypropylene
ppt	precipitate
RNase	ribonuclease
RT	Room Temperature
rRNA	ribosomal RNA
³⁵ S	radioisotope of sulphur
sdH ₂ O	sterile, distilled water
sol'n	solution
SDS	sodium dodecyl sulphate
SSC	(for 1x conc) 150 mM NaCl, 15 mM sodium citrate (pH 7.0)
SSPE	(for 1x conc) 150 mM NaCl, 10 mM NaH ₂ PO ₄ , 1 mM EDTA (pH 7.4)
STE	100 mM NaCl, 10 mM Tris.Cl (pH 8.0), 1mM EDTA (pH 8.0)
sssDNA	sheared salmon sperm DNA
T ₁₀ E ₁	10 mM Tris-Hcl; 1 mM EDTA
tRNA	transfer RNA
μ tube	1.5 ml Eppendorf Tube
VMM	Vogel's Minimal Media + 15 g/L sucrose
X-Gal	5-Bromo-4-chloro-3-indolyl- β -D-galactoside
'	minutes

I. INTRODUCTION

***Neurospora crassa*: Background**

Neurospora crassa was first identified as a serious contaminant in bakeries, and thus was initially known as "pink bread mold". *N. crassa* is classified as an ascomycete, which has traditionally related it to yeasts like *Saccharomyces cerevisiae* and, more distantly, to basidiomycetes (mushrooms) (Davis and deSerres, 1970). Recent RNA based phylogenies, however, have related *Neurospora* most closely to *Sordaria* (Eriksson, 1995). In its native environment, *N. crassa* is typically found in tropical and subtropical areas feeding on burnt cellulosic plant remains (Davis and deSerres, 1970; Alexopoulos *et al.*, 1996).

The cells of *N. crassa* are bounded by a double-layered plasma membrane. There is a strong cell wall composed of glucose polymers, a peptide-polysaccharide complex, chitin, and polygalactosamine (Mahadevan and Tatum, 1965). The hyphae of this fungus are divided by porous septa, and each "cell" compartment can contain multiple nuclei (Davis and deSerres, 1970). It is the hyphae, large numbers of which are referred to as mycelia, that make *Neurospora* a "filamentous" fungus.

When subject to carbon source deprivation or desiccation, *N. crassa* undergoes the asexual sporulation process of macroconidiation (a.k.a. conidiation); a process which involves the growth of aerial hyphae. These specialized hyphae subsequently bud to

form chains of multinucleate macroconidia which break apart to form wind-borne conidiospores. Nitrogen limitation induces *Neurospora* to enter its sexual cycle. This involves the formation of a "female" reproductive structure known as a protoperithecia; any *Neurospora* colony can produce these structures since *Neurospora* is functionally hermaphroditic. The protoperithecia then sprout trichogynes, specialized hyphae which seek out "male" cells of the opposite mating type, fuse with them, and obtain a fertilizing nucleus. The male and female nuclei fuse into a heterokaryon and genetic recombination occurs. The eight meiotically-derived, haploid nuclei are encapsulated in ascospores within a sac known as an ascus. The asci are assembled into structures called perithecia. Fully developed perithecia then burst open and release their ascospores. Ascospores, and conidiospores, both give rise to haploid, vegetative mycelia when they germinate (Springer, 1993).

N. crassa has several characteristics, aside from being a different model than *S. cerevisiae*, which make it a useful experimental system. It is a eukaryote that can be handled as easily as bacteria (Davis and deSerres, 1970). This microorganism has a relatively small, haploid genome (approximately 27 000 Kb) and genes typically contain only one or two short introns (Edelmann and Staben, 1994); these qualities greatly simplify genetic manipulations and analysis (Vollmer and Yanofsky, 1986). This fungus has also been well characterized with respect to its metabolism and nutritional requirements. Extensive genetic and cytogenetic information is also available (Vollmer and Yanofsky, 1986). This type of information is valuable in the context of this research due to parallels between the enzymic system under investigation (p-aminobenzoate

biosynthesis) and the previously characterized tryptophan biosynthetic pathway (discussed below). Finally, newly germinated conidiospores can be converted into spheroplasts, which in turn facilitates DNA transformations (Case *et al.*, 1979). Such transformations invariably result in ectopic (non-homologous) DNA integration in *Neurospora* (Timberlake, 1991). An easy system of transformation is of particular use in the current set of experiments where complementation of a mutation is key to the isolation of a functional gene.

PABA: a Precursor of Folate

Neurospora crassa, as discussed above, is a useful model system for both genetic and metabolic studies. It is therefore useful to further develop *Neurospora's* potential as an experimental system. One way to accomplish this goal is to characterize the biosynthetic pathways of this fungus. Such research not only serves to elucidate the metabolic process, thereby satisfying scientific curiosity, but also aids in the development of selection/marker systems which can be applied to a broad range of further studies.

The biosynthesis of the vitamin tetrahydrofolate meets the above criteria. Although this pathway has not been characterized in *Neurospora*, it is known that folate plays an important role in the generation of nucleotides used in the construction of DNA (Figure 1). The essential nature of this vitamin is evident when one considers that there is a class of antibiotics (sulfonamides) that function by interrupting its biosynthesis (Mills

Figure 1 - A generic scheme for the synthesis of the vitamin folate, from PABA, in microorganisms. Humans must ingest, rather, than synthesize folate, making the biosynthetic portion of this pathway an attractive drug target since there are minimal side effects on human metabolism (Mills and Masur, 1990). Sulfonamides, like sulfamethoxazole, are potent inhibitors of DHFS and therefore useful in the treatment of certain human pathogens (e.g. *P. carinii*). This figure was adapted from Mills and Masur (1990).

and Masur, 1990). Specifically, sulfonamides prevent the incorporation of the folate precursor p-aminobenzoic acid (PABA). Given the pivotal nature of this intermediate, and the fact that there are already PABA auxotrophic mutants of *N. crassa* available for study, the biosynthetic genes of PABA hold considerable potential for use as selectable markers.

A Review of the PABA Pathway in Bacteria

E. coli mutants requiring PABA for growth were initially created by Lampen *et al.* (1949) using X-ray mutagenesis. Some of the first genetic characterization of PABA mutants, generated by chemical mutagenesis, was later undertaken by Huang and Pittard (1967). The results of this initial research suggested that two genes at distinct loci, dubbed *pabA* and *pabB*, were responsible for the synthesis of p-aminobenzoate. The *pabA* and *pabB* components together were referred as PABA synthase; this was later discovered to be a misnomer (see below). Gibson *et al.* (1964) found that PABA and anthranilate, an intermediate of the tryptophan biosynthetic pathway, were both synthesized from chorismate and glutamine in *Aerobacter aerogenes*. This was also determined to be true for *E. coli* (Huang and Gibson, 1970). The link between the mechanisms for PABA synthesis and anthranilate synthesis (Figure 2) provided the foundation for much further research.

Zalkin and Murphy (1975) investigated whether a mutation in the *E. coli* *trp(G)D* gene (a glutamine aminotransferase required for synthesis of anthranilate) could

Figure 2 – The biosynthesis of PABA (p-aminobenzoate) and anthranilate (o-aminobenzoate) from chorismate in *Escherichia coli*. PABA formation begins when an amidotransferase (❶) removes the amido group from glutamine and donates it to an aminase (❷) which substitutes it for the C-4 hydroxyl group of chorismate. A 4-amino-4-deoxychorismate lyase (❸) then cleaves off the pyruvate to form PABA. Anthranilate is formed in a similar fashion; a second amidotransferase (❶) supplies another aminase (❷) with an amido group from glutamine. Anthranilate is produced through the liberation of pyruvate by a lyase (❸). Note that the activities of ❷ and ❸ are performed by the same enzyme. ❶- PabA; ❷- PabB; ❸- PabC; ❶-TrpG portion of Trp(G)D; ❷-TrpE (as aminase); ❸-TrpE (as lyase)

be complemented by other glutamine aminotransferases. They found they could not; indicating that the enzyme responsible for glutaminase activity in the PABA pathway was not interchangeable with that of anthranilate. Despite this, Reiners *et al.* (1978) discovered that antibodies raised against anthranilate synthase cross reacted with PABA synthase components in crude extracts from *E. coli*. The authors suggested that the two proteins were therefore structurally related, and therefore mechanistically related, since they shared common epitopes.

The analogy relating p-aminobenzoate synthase and anthranilate synthase was better supported at the physiological level in other microorganisms. Sawula and Crawford (1972) and Buvinger *et al.* (1981) found that *trpG* mutants of *Acinetobacter calcoaceticus* and *Pseudomonas acidovorans* were blocked in both anthranilate and p-aminobenzoate synthesis. This indicated that TrpG and PabA glutaminase function were encoded by the same enzyme. It was also determined that the *trpG* of *Bacillus subtilis* was amphibolic (Kane *et al.*, 1972; Slock *et al.*, 1990). Slock *et al.* (1990) later revealed, however, that TrpE and Pab (PabB equivalent) were coded by separate genes in *B. subtilis*, thus demonstrating the two pathways were only functionally interlocked at one enzyme.

Kaplan and Nichols (1983) were the first to sequence *E. coli pabA* and establish a $M_r = 21,700$; Goncharoff and Nichols (1984) subsequently sequenced *pabB* and determined a $M_r = 53,400$. Since that time Kaplan *et al.* (1985) have also sequenced the *pabA* genes of *Salmonella typhimurium*, *Klebsiella aerogenes* and *Serratia marcescens*.

Goncharoff and Nichols (1988) characterized *pabB* in *S. typhimurium* and *K. aerogenes*. Arhin and Vining (1993) found PabA and PabB equivalents in *Streptomyces lividans*. *Streptomyces griseus* and *Streptomyces venezuelae* are unusual in that they each possess a bifunctional gene encoding PabA and PabB at the N- and C- terminal domains respectively (Criado *et al.*, 1993; Brown *et al.*, 1996). PabA and PabB from all these organisms share significant homology with each other.

Comparison of the predicted amino acid sequences of *E. coli* anthranilate synthase and p-aminobenzoate synthase showed a high degree of structural similarity between these two enzymes (Kaplan *et al.*, 1985; Goncharoff and Nichols, 1988; Nichols *et al.*, 1980; Nichols *et al.*, 1981). PabA was found to share 44% sequence identity with Trp(G)D (Kaplan and Nichols, 1983) as well as similarity to other glutamine amidotransferase enzymes in *E. coli* (Nyunoya and Lusty, 1984; Zalkin *et al.*, 1985). Given the similarities, Kaplan and Nichols (1983) suggested that *pabA* and *trp(G)D* likely arose from a gene duplication event. Goncharoff and Nichols (1984) believed that 26% amino acid homology between PabB and TrpE showed that both enzymes must be the chorismate-utilizing components in their respective pathways.

It was demonstrated that the conversion of chorismate to anthranilate occurs via the intermediate 2-amino-4-deoxychorismate, which remains bound to anthranilate synthase during the reaction (Teng and Ganem, 1984; Policastro *et al.*, 1984; Viswanathan *et al.*, 1995). Experiments by Nichols *et al.* (1989) showed that, as with anthranilate synthesis, PabA (or Trp(G)D), could be omitted if large amounts of NH_3

were present at high pH. This confirmed that PabA was indeed an amidotransferase. Interestingly, the PabA component did not enhance the NH_3 dependant reaction catalyzed by PabB, as occurs for the anthranilate synthase complex (Trp(G)D + Trp E) (Ito and Yanofsky, 1969; Zalkin, 1973). To utilize glutamine as the amino donor, PabA must be present and must be able to physically interact with PabB; the same holds true for the TrpG subunit of anthranilate synthase (Nichols *et al.*, 1989).

Nichols *et al.* (1989) made the significant discovery that PABA was synthesized by three enzymes, rather than two as had previously been believed (and is the case for anthranilate synthesis). They showed that extracts from *E. coli*, genetically modified to overproduce PabA and PabB, could not synthesize PABA until the soluble extract from a *pabA pabB* double mutant was added. This study revealed that a previously unknown enzyme, which had no apparent homologue in the anthranilate pathway, was involved. It was considered likely that the substrate of this third enzyme, dubbed PabC, was 4-amino-4-deoxychorismate (ADC), since this compound could be converted to PABA *in vitro* in the absence of NH_3 (Teng *et al.*, 1985). The TrpE protein catalyzes NH_3 -dependant chorismate amination to 2-amino-4-deoxyisochorismate and its subsequent aromatization by *syn* elimination of the elements of pyruvate (C2-H, C3-enolpyruvyl loss) (Policastro *et al.*, 1984; Teng and Ganem, 1984). Slock *et al.* (1990) point out that there are three genes coding for PABA synthesis as opposed to just two for the generation of anthranilate; therefore, there is no specific counterpart of PabC in *E. coli* anthranilate synthase to perform the final aromatization reaction. One might expect, however, that PabC would share some homology with the region of TrpE responsible for a similar enzymic (i.e.

lyase) activity. Goncharoff and Nichols (1984) explain some of the differences between the two biosynthetic systems by remarking that although TrpE responds to feedback inhibition by tryptophan, PabB does not.

The reason this latter component of PABA biosynthesis remained in obscurity for so long was likely because partial activity by other enzymes masked its mutation, and therefore made it impossible to isolate mutant strains (Green and Nichols, 1991). Nichols and Green (1992), for instance, showed that overproduction of chorismate lyase (UbiC) in *E. coli* could complement the *pabC* mutation. UbiC is involved in the formation of p-hydroxybenzoate for ubiquinone biogenesis. Interestingly, PabC and UbiC share no amino acid homology (Nichols and Green, 1992). Alternatively, *pabC* interruptions could have been inherently leaky, requiring several generations of growth to fully deplete endogenous PABA and thereby affect growth (Green *et al.*, 1992).

There have also been considerable difficulties purifying the PABA biosynthetic enzymes from wild-type *E. coli*, which have in turn led to difficulties in characterizing enzymatic steps (Ye *et al.*, 1990). It is also evident that the experimental preparations from *pabA* and *pabB* mutant strains would also harbor PabC (unknown to researchers of the time). This undoubtedly affected assay results in the past and perpetuated the erroneous conclusion that PABA biosynthesis required only two enzymes. The precedent set by the multi-functional TrpE could not have helped but exacerbate matters.

Ye *et al.* (1990) engineered a strain of *E. coli* which produced ~25% of its total soluble cell protein as PabB. These researchers were then able to successfully purify and characterize PabB. It was confirmed that PabB produces a diffuseable aminated intermediate from chorismate, and that PabB does not disturb the enolpyruvyl group. The aminated intermediate was identified as distinct from PABA, chorismate and *p*-hydroxybenzoate and subsequently isolated via HPLC. This chemical was shown to be converted to PABA by PabC. The authors reasoned that the newly isolated compound was likely 4-amino-4-deoxychorismate identified by Teng *et al.* (1985), and given that PabC removes the enolpyruvyl group, named this protein 4-amino-4-deoxychorismate lyase (ADC lyase). Ye *et al.* (1990) also explained the low similarity between PabB and TrpE, since only part of TrpE function is to act as an aminase, and PabB lacks the lyase function of TrpE. It follows that TrpE should share some homology to PabC in light of shared lyase function.

Green *et al.* (1992) sequenced the *E. coli pabC* gene and determined it to encode a protein 29.7 kDa in size. Since PabC was found to behave as a 50-kDa protein when passed over gel filtration columns it is likely dimeric (Nichols *et al.*, 1989; Ye *et al.*, 1990). When compared on the basis of amino acid sequence, ADC lyase exhibits no similarity to TrpE, despite the fact that the two enzymes catalyze similar reactions (Green *et al.*, 1992). Green *et al.* (1992) did, however, find homology between the PabC of *E. coli* and *Bacillus subtilis*. They also found that *E. coli* ADC lyase was homologous with a number of amino acid transaminases. This similarity led to research which showed that PabC was capable of binding pyridoxal-phosphate, a cofactor used by the

transaminases. Green *et al.* (1992) went on to propose a mechanism by which the phosphate compound might act as a cofactor in PABA biosynthesis.

Anthranilate synthase, in *E. coli*, is a complex (TrpE & Trp(G)D) that is easily purified because its subunits are quite robust in their association (Ito and Yanofsky, 1969). Despite the long suspected subunit association of PabA and PabB, the enzymes have only recently been shown to be a $\alpha\beta$ dimer (Rayl *et al.*, 1996). This subunit association pattern again supports the similarity of these two pathways. PabC, which has no direct homologue in anthranilate synthesis, was not found to participate in an higher order structure with PabA and PabB.

PABA Synthesis in Fungi

The PABA pathway has remained poorly characterized in fungi. Edman *et al.*, (1993) isolated the gene for a bifunctional protein conferring sulfamethoxazole resistance in *Saccharomyces cerevisiae*. The protein was shown to possess sequences that were 29% and 24% identical to the PabA and PabB of *E. coli*. Increased levels of the PabAB enzyme caused an overproduction of PABA, which in turn outcompeted sulfamethoxazole for the active site of DHPS. No PabC equivalent has yet been found in this organism.

Tatum and Beadle (1942) were the first to characterize X-ray-induced PABA

mutants in *Neurospora crassa*. Drake (1956) established that X-ray induced mutations in *Neurospora* could be categorized into two separate loci, *pab-1* and *pab-2*, on linkage group V. Hendler and Srinivasan (1967) later showed, via cross-feeding experiments, that the *pab-1* gene product precedes that of *pab-2* in the PABA biosynthetic pathway. Beyond this, however, it remains unknown as to what constitutes the pathway in *N. crassa*. In *Neurospora*, it is not even known if chorismate is indeed the first step in the pathway (Perkins *et al.*, 1982). Given the extensive similarities between the anthranilate and PABA synthetic mechanisms in several microorganisms, it is interesting to speculate on whether these similarities will be evident in *N. crassa*. In *Neurospora*, anthranilate synthase is composed of two distinct subunits (TrpE (*trp-2*) & TrpGCF(*trp-1*)) and in an $\alpha_2\beta_2$ tetrameric structure (Hulett and DeMoss, 1975; Keesey *et al.*, 1981; Schechtman and Yanofsky, 1983).

Since there are only two genes (*pab-1* and *pab-2*) known to code for PABA biosynthesis in *N. crassa* (Perkins *et al.*, 1982), and the *pab-2* gene product has been putatively identified as ADC lyase (current work), it is possible that *pab-1* encodes a single, bifunctional enzyme with PabA and PabB activity, as in *S. cerevisiae* (Edman *et al.*, 1993). It remains of interest to determine if the PABA synthesis components will be separate as in *E. coli*, or arranged in a multimeric structure as suggested by similarities to the anthranilate synthase complex of *Neurospora*.

PABA Biosynthesis in *N. crassa*: a Model for *Pneumocystis carinii*

Pneumocystis carinii is an opportunistic fungal pathogen that causes a form of pneumonia in immuno-compromized individuals. It is currently the most common lethal infection afflicting AIDS patients (Edman *et al.*, 1989b). The combined use of antibiotics that target folate synthesis (e.g. trimethoprim and sulfamethoxazole) have proven a successful treatment for rare cases of this pneumonia in the past. The effectiveness of sulfamethoxazole is due, in part, to the fact that it selectively targets *P. carinii*. Selective targeting is possible because *P. carinii* must synthesize folate from the p-aminobenzoate precursor using the DHPS enzyme (Figure 1). Humans, in contrast, ingest their folate vitamins and do not even have a DHPS enzyme. Unfortunately, the increased occurrence of *P. carinii* infection, corresponding to the AIDS epidemic, has revealed that these antibiotics frequently result in toxic and allergic side-effects in patients who are very ill (Edman *et al.*, 1989b). It has also been discovered that trimethoprim targets human DHFR more potently than that of *P. carinii* (Edman *et al.*, 1989a). There is also a distinct possibility that *P. carinii* may develop resistance to sulfonamides by overproducing p-aminobenzoate (PABA), the precursor to folate. This phenomenon has already been documented in *S. cerevisiae* (Edman *et al.*, 1993). Considering the above information, new antibiotics must be developed to combat *P. carinii*, and a likely target for such drugs would be the enzymes responsible for PABA biosynthesis.

Characterization of *P. carinii* biochemistry and enzymology has proven difficult because this microorganism can not yet be cultured outside of a host (Edman *et al.*, 1989b). It is therefore useful to study *Pneumocystis* by the investigation of analogous model systems. Genetic studies, based on DNA and RNA obtained from infected lung tissue, have proven useful in defining a suitable model organism.

Electrophoretic karyotyping has shown that *P. carinii* is likely haploid, like *Neurospora crassa* (Stringer, 1996; Cushion *et al.*, 1993). Phylogenetic analysis using 16-S-like rRNA has shown *P. carinii* most closely related to the ascomycetes *N. crassa* and *S. cerevisiae* (Edman *et al.*, 1988; Stringer *et al.*, 1989). Sequence comparisons and phylogenetic analysis of the actin I gene from *P. carinii* indicated the most similarity to fungal actins (Fletcher *et al.*, 1994). Close matches to actin from *Schizosaccharomyces pombe* (91% identity), *Aspergillus nidulans* (95% identity), and *S. cerevisiae* (94.7%) have been found. Christopher *et al.* (1995) isolated a second actin-related gene from *Pneumocystis*. The gene was homologous to the Arp1 class of actin-related proteins. The *P. carinii* Arp1 had 87.6% similarity with Arp1 of *Neurospora crassa*, and 71.2% similarity with Act5p of *Saccharomyces cerevisiae*. Characterization of the *pcg1* gene, which encodes the α -subunit of a G protein, showed it shared the most homology (52.4% identity) with *N. crassa*'s Gna-2 (Smulian *et al.*, 1996). Considering the above information, it is plausible to use *N. crassa* as a model for enzyme pathways, like PABA biosynthesis, in *P. carinii*.

Objectives and Hypotheses:

The objective of this research is to clone and characterize both the mutant and wild-type alleles of *pab-2* in *Neurospora crassa*, and thereby develop the potential of this locus for use as a selectable marker. It is my hypothesis that the biosynthesis of PABA in *Neurospora crassa* occurs via a short, linear pathway similar to that found in bacteria and that homologous enzymes are required. Furthermore, I believe that that the *pab-2* locus codes for a biosynthetic enzyme, and that this enzyme is constitutively expressed at low levels.

II. METHODS AND MATERIALS

1. Strains of Interest and Culture Conditions

The organism investigated in this research was *Neurospora crassa*. Two different *N. crassa* strains were studied in the current set of experiments. The wild-type strain used was 74-OR23-1VA (FGSC# 987; Oak Ridge background). The PABA auxotroph analyzed was designated *pab-2* (FSGC#: 4076; allele#:H193; SL9 background). These strains were cultured in VMM + 1.5% sucrose (Davis and deSerres, 1970); media for the mutant strain was supplemented with 200 µg/ml PABA.

The following *Escherichia coli* strains, were used for cloning and phage work.

DH5α: F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1*

One Shot™ TOP 10 F': F' {*lacI^f* Tn 10 (Tet^R)} *mcrA* Δ(*mrr-hsdRMS-mrcBC*)
Φ80*lacZ*ΔM15 Δ*lacX74recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*

XL1-Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^fZ*ΔM15 Tn10 (Tet^f)]^c

2. Preparation of Cosmid DNA

A sterile loop was used to transfer the stock bacteria from a microtitre plate to a LBA agar plate. After growth at 37°C O/N, an isolated colony was streaked onto the entire surface of four new plates. After another O/N growth at 37°C, each plate was flooded with 4 ml of LB containing 01. mg/ml ampicillin and 1 mg/ml chloramphenicol to amplify cosmid copy number. The plates were incubated again O/N at 37°C. To each plate was added 5 ml of STE (100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA). The cells were subsequently resuspended from the plate surface using a flamed spreader. The solution was transferred to a SS-34 PP centrifuge tube. A further 5 ml of STE was used as a wash and collected in the same tube. After spinning at 5 000 rpm for 10' in a Sorvall RC-5B centrifuge, the supernatant was decanted and discarded. The cells were then resuspended, in 8 ml of cold STE, by vortexing. The pellet from a further 10' centrifugation at 5 000 rpm was resuspended thoroughly in 5 ml of solution I (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA). The cell suspension was stored at -20°C until the next step.

The following DNA extraction procedure was followed for each 5 ml cell suspension. Cells were defrosted, resuspended by vortexing, and transferred to 50 ml polypropylene centrifuge tubes. To each tube was added 500 µl of freshly prepared lysozyme (10 mg/ml in solution I, pH 8.0), followed by 10 ml of freshly prepared solution II (0.2% NaOH, 1% SDS). The centrifuge bottle was capped, the contents mixed by gentle inversion several times, and left at RT for 5-10'. A 7.5 ml volume of ice-cold

solution III (3M potassium acetate, pH 4.8) was added, and the contents of the tube mixed by vigorous shaking. The bottle was then stored on ice for 10' (while a flocculent white precipitate formed). The bacterial lysate was centrifuged at 8 000 rpm in an SS-34 rotor for 15' at 4°C. The supernatant was filtered through one layer of miracloth into another 50 ml PA tube; to this was added 0.6 volumes of isopropanol (~14 ml), which was well mixed with the filtrate and stored at RT for 10'. The precipitating nucleic acids were collected by centrifugation at 10 000 rpm for 15' at 4°C. The supernatant was decanted carefully, and the tube inverted to allow the last drops of supernatant to drain away. The pellet and walls of the tube were rinsed with 70% ethanol at RT. This was followed by further centrifugation at 10 000 rpm for 10', attempting to reposition the pellet. The ethanol was drained for 5-10', after which the pellet was dried under vacuum. The cosmid pellet was then dissolved in 3 ml of TE (pH 8.0).

Precipitation with polyethylene glycol was necessary for purification of the cosmid. The first step was to add 3 ml of ice cold 5 M LiCl to the 3 ml of cosmid in TE. After thorough mixing, this was centrifuged at 10 000 rpm for 10' at 4°C. The supernatant was transferred to a 15 ml PP tube and an equal volume of isopropanol was added (~6 ml). This was mixed well and left for 30' at -20°C. The precipitating cosmid was recovered by centrifugation at 10 000 rpm for 10' using an SS-34 rotor. The tube was carefully drained of supernatant and inverted such that the last drops of supernatant were removed. The pellet and the tube walls were rinsed with ~10 ml of 70% ethanol at RT and centrifuged at 10 000 rpm for 10', repositioning the pellet. The ethanol was then drained and the tube inverted on a paper towel for 5'. The tubes were tapped to loosen

the pellet and which was then dried under vacuum. The pellet was resuspended in 500 μ l of TE (pH 8.0) and left O/N.

The solution was transferred to an 1.5 ml Eppendorf μ tube and mixed well with 500 μ l of 1.6 M NaCl containing 13% (w/v) polyethylene glycol (PEG 8000). The cosmid DNA was recovered by centrifugation, using an Eppendorph 5415 C microfuge, at 12 000 rpm for 5' at 4°C. The supernatant was removed by aspiration and the pellet dissolved O/N in 400 μ l of T₁₀E₁ (pH 8.0). RNase A (10 μ g/ml) treatment was at RT for 60'. The solution was then extracted once with 400 μ l phenol:chloroform:isoamyl alcohol and again with chloroform:isoamyl alcohol. Each extraction involved gentle shaking to mix and centrifugation at 12 000 rpm for 5'; after which the aqueous phase was added to a fresh microfuge tube. The solution was mixed well with 1/10 volume of 3 M Na-acetate (pH 5.2) and combined with 2 volumes of (~1 ml) of absolute ethanol. This was mixed and kept at -20°C for at least 20'. The precipitated DNA was obtained by centrifugation at 12 000 rpm for 10' in a microfuge. This was followed by a wash with ~1 ml of 70% ethanol for another 2'. The supernatant was discarded and the microtubes drained on paper towels for 5-10'. The last traces of ethanol were removed under vacuum. Finally, the cosmid pellet was resuspended in 250 μ l of T₁E_{0.1} (pH 8.0) and stored at -20°C. Concentration was determined using a 1/100 dilution at OD₂₆₀.

3. Restriction Digestions

Restriction digests were performed according to the specifications of the manufacturer. Restriction enzymes used were supplied by NEB, GIBCO BRL and Boeringer-Mannheim.

4. DNA Band Isolation from Agarose Gel: Gene Clean

The desired DNA band was isolated from an agarose gel using a razor, weighed, and put in a microfuge tube. After addition of 2.5-3 volumes of 6N NaI, the tubes were incubated at 50°C for at least 5' to dissolve the agarose. Glass Milk (5 µl) was then added and the tubes left on ice for 5'. Pellets were obtained by centrifugation for 6 sec (s) at 14 000 rpm; the supernatant was discarded. The pellet was washed with 400 µl of New Wash (buffered EtOH), centrifuging for 6 s at 14 000 rpm. This was repeated twice more for a total of three washes. The pellet was dried at 37°C then resuspended in 15 µl of sdH₂O. DNA was released by incubation at 50°C for 3-5'. The tubes were centrifuged for 1', after which the supernatant was transferred to a fresh microtube.

5. Phenol Extraction of DNA

Some occasions required the extraction of restriction digested cosmid for use in spheroplast transformations. Restriction digest mixture volume was increased to 100 µl

with $T_{10}E_1$ or sdH_2O and combined with 100 μl of phenol. This was mixed by vortex then centrifuged for 5' at 14 000 rpm. A 80 μl aliquot of the aqueous layer was transferred to a fresh microtube. Another 80 μl of $T_{10}E_1$ or sdH_2O was combined with the phenol mixture and the above repeated (yielding 160 μl of aqueous phase). To the aqueous extract was added 2.5 volumes (~ 450 μl) of absolute EtOH. Precipitation occurred O/N at $-20^\circ C$. The DNA was pelleted by centrifugation at 14 000 rpm for 10'. The pellet was dried and mixed with 5 μl of sdH_2O . This solution was used for spheroplast transformation.

6. DNA Ligations

Ligations were performed according to the protocol of the manufacturer (NEB). They were typically done in 20 μl volumes using trace amounts of T4 DNA ligase (0.2 μl for cohesive ends; 1 μl for blunt ends), 2 μl DNA ligase buffer (10x stock w/ 10 mM ATP), and varying amounts of vector and DNA. Total volume was made up with sdH_2O . Incubation was typically O/N at $14^\circ C$ or RT.

7. PCR Product Polish and Ligation

The following method is a one-step protocol for polishing and ligating a PCR product into a vector. Approximately 10 ng of glass milk purified PCR product was combined with 0.5-1 ng of uncut pBSKS+, 1 μl of 0.6 mM dNTP and 2 μl of 10x ligation buffer w/ 10 mM dATP (NEB). The following enzymes were also added: 2U *SmaI*

(Boehringer-Mannheim), 0.2 μ l T4 DNA ligase (NEB), and 1U of T4 DNA polymerase (NEB). Sterile water was used to bring the total volume to 20 μ l. The ligation solution was allowed to incubate O/N at RT.

8. TA Cloning of RACE PCR Products

RACE PCR products were cloned using the TA Cloning system by InvitrogenTM. The RACE PCR products (~0.5 Kb) were excised from an agarose gel and gene cleaned. Ligation was performed, as specified by the manufacturer, by combining the following: 2 μ l of pCR 2.1 vector (25 ng/ μ l), 1.5 μ l isolated RACE PCR product (~7 ng/ μ l), 1 μ l 10X NEB ligation buffer (w/ 10 mM ATP), 4.5 μ l sdH₂O, and 1 μ l T4 DNA ligase (NEB). The ligation solution was mixed, and incubated at 14 °C for 2 days.

The plasmid construct was recovered using Invitrogen's recommended transformation procedure. One ShotTM TOP 10 F' *E. coli* were thawed on ice, to which was added 2 μ l of 0.5 M β -mercaptoethanol. A 2 μ l aliquot of the ligation was also mixed in with the cells. Cells were subsequently incubated on ice for 30', heat shocked at 42°C and again iced for 2'. SOC media (250 μ l) was added to the cells, which were then incubated for 20' at 37°C w/o shaking, followed by agitation at 37°C for 40'. The *E. coli* were plated on LB + kanamycin (50 μ g/ml) with XGAL and IPTG for blue/white selection. After overnight growth at 37°C, colonies were used to inoculate standard LB + Amp (100 μ g/ml) minipreps and recombinant plasmids were recovered.

9. Preparation of Competent *E. coli* (CaCl₂ Method)

This method was based on that of Sambrook *et al.* (1989). A 1 ml aliquot of a fresh O/N culture of DH5 α *E. coli* was used to inoculate 100 ml of LB in a 250 ml flask. This was incubated in a shaker at 37°C until an OD₅₅₀ was reached. The culture was aseptically transferred to two sterile, ice cold, 50 ml PA centrifuge tubes and kept on ice for 10'. The cells were then spun at 4000 rpm in an SS-34 rotor for 10' at 4°C. The pellets were gently resuspended with 10 ml of ice cold, 0.1M CaCl₂, and stored on ice for 10'. The cells were spun again at 4000 rpm for 10' at 4°C; the tubes were inverted for 1' to drain the supernatant. The pellets were gently resuspended with 2 ml of ice cold, 0.1M CaCl₂ and 35 μ l of DMSO was added per 1 ml cells. Sterile 75% glycerol was added to the cells for a final concentration of 15%. The competent cells were then aliquoted into μ tubes and stored frozen at -70°C.

10. Transformation of *E. coli*

Competent *E. coli* were thawed on ice. DNA was added to cells (typically 5 μ l of ligation mixes). The cell suspension was mix by tapping/inversion and kept on ice for 30'. Cells were then heat shocked at 37-42°C for 90 s, then cold shocked on ice for 90 s. The mixture was then combined with 800 μ l of LB and allowed to recover for 45-60' at 37°C. The transformed cells were then plated on LB (100 μ g/ml Ampicillin was used for selection through out the project) 90 mm agar plates in 200 μ l aliquots; which were

spread with a glass rod. Blue/white selection was accomplished by the addition of 50 μ l XGAL (2% in DMF) and 10 μ l IPTG (100 mM) to the plates.

11. Rapid Plasmid Preps (Alkaline Lysis)

A single colony of *E. coli* was used to inoculate 1.5 ml of LB (+Amp 100 mg/ml) and cultured overnight with shaking at 37°C. The cultured cells were collected by centrifugation for 1' at 14 000 rpm. The supernatant was removed and the cells resuspended in 100 μ l of T₁₀E₁ buffer. The cells were lysed by the addition of 200 μ l of solution II (0.2N NaOH, 1% SDS) and mixing by tapping/inversion. The solution was then combined with 150 μ l of 3M K-Ac pH 4.8 (sol'n III), mixed briefly by inversion and brief, gentle vortexing, then left on ice for up to 5'. The precipitate was pelleted by centrifugation at 14 000 rpm for 4'. The supernatant was transferred to a fresh microtube and mixed with 800 μ l of absolute EtOH; the solution was then left at -20°C for at least 20' to allow precipitation of plasmid DNA. The DNA was pelleted by centrifugation at 14 000 rpm for 10' at RT. The pellet was washed with 500 μ l of 70% EtOH and then allowed to air dry. Pellets were typically resuspended in 50 μ l of sdH₂O + 0.1 mg/ml RNase A.

12. *Neurospora* Spheroplast Preparation

Spheroplast preparation was accomplished by essentially the same methods as those used by Vollmer and Yanofsky (1986). This method required the collection of 2×10^9 conidia (approx. 1 000 A_{260} units) using 100 ml sterile water from four, 10 day old, culture flasks containing VMM (and PABA for *pab-2* mutants). Conidia were concentrated by centrifugation (4000 rpm; 5', GSA rotor) and then resuspended in 150 ml of VMM (w/ 1.5% sucrose) in a 500 ml flask and incubated at 30°C with shaking at 120 rpm for 4 to 6 hours. The germinated conidia were collected by centrifugation (6,900 rpm; 5', GSA rotor), and washed once with an equal volume of sterile water, and once with 1/4 volume of 1M sorbitol. The conidia were resuspended in 10 ml of 1 M sorbitol and mixed with 4 ml of Novozyme™ (5 mg/ml in 1M sorbitol; filter sterilized). This mixture was incubated at 30°C with shaking (approx. 60 rpm) for about 1 hour.

The efficiency of spheroplast formation was estimated by examining a 10 μ l aliquot under a microscope (40x objective). Efficiency was calculated by adding 10 μ l of water to the edge of the coverslip and determining the percentage of spheroplasts that swelled and lysed in a given microscopic field.

The spheroplasts were collected by centrifugation at 500 x g (2 000 rpm in SS-34 rotor) for 5'. The spheroplasts were then washed twice with 1 M sorbitol (30 ml) and once with 30 ml of STC (1 M sorbitol/50 mM Tris/50 mM CaCl_2 (pH 8)). They were then resuspended to a final volume of 16 ml in STC; to this was added ~ 4 ml of 40%

PEG 4000/50 mM Tris (pH 8.0)/50 mM CaCl₂ and 200 µl of DMSO. Spheroplasts were stored in 1 ml aliquots at -70°C until use.

13. Transformation of *Neurospora* Spheroplasts

The transformation protocol used was based on that of Vollmer and Yanofsky (1986). A 5 µl solution of DNA (1-2 µg uncut plasmid, cosmid or linearized DNA) and sterile water was mixed with 2 µl of 50 mM spermidine/3HCl. Note that when functional *hph* was not present in the transforming DNA, ~0.2 µg of pCSN43 was added to confer Hg^r phenotype. The preceding solution was mixed with 5 µl of heparin (5 mg/ml in 1 M sorbitol/50 mM Tris/50 mM CaCl₂/pH 8) and left on ice. Spheroplasts were thawed on ice; a 100 µl aliquot (~ 1 x 10⁷ spheroplasts) was added to the DNA mixture and left on ice for 30'. This was subsequently combined with 1 ml of 40% PEG 4000/50 mM Tris (pH 8.0)/50 mM CaCl₂ and incubated at RT for 20'. A 600 µl aliquot of this mixture was added to 10 ml of regeneration agar containing VMM + FIGS (containing 0.1 g/l fructose, 0.05 g/l inositol, 0.1 g/l glucose and 5 g/l L-sorbose) + 1 M Sorbitol at 48-50°C and poured onto a bottom plate of VMM + FIGS (w/ 0.2 mg/ml Hygromycin; no sorbitol). Note that the FIGS was autoclaved separately as a 10x stock. The plates were incubated at RT for 4-7 days before being assessed for colony formation.

14. Non-Radioactive Oligolabelling (DIG system)

Probe construction was accomplished using the random hexanucleotide priming protocol from the DIG System User's Guide (1993). Linearized template DNA was isolated from an agarose gel using the Gene CleanTM protocol described previously. A priming mixture of: 2 μ l isolated template (ideally 0.2 μ g of DNA), 2 μ l of 40 ng/ μ l random hexanucleotide primer mix {dp(N)₆} and 11 μ l of sdH₂O was combined. This was boiled for 5-10' then flash cooled on ice/NaCl. To this mix was added: 2 μ l OLB (10x), 2 μ l DIG DNA mix and 1 μ l Klenow enzyme. The labelling reaction was allowed to proceed for 60' and 37°C. The solution was then combined with 1 μ l of 0.25 M EDTA, 3 μ l of tRNA (10 ng/ml) and 76 μ l STE. The probe was then cleaned using a Sephadex G-50 spun column (Sambrook *et al.*, 1989) and stored at 4°C.

15. Radiolabelling of DNA Probes

Radioactive probes were synthesized using Ready To GoTM labelling beads from Pharmacia Biotech. Template DNA was purified using the Gene CleanTM protocol prior to use. Immediately before use, the template was denatured by heating at 100°C for 3' and then chilled in an ice bath for 2'. Denatured DNA (25-50 ng) was then combined with sdH₂O to a final volume of 45 μ l and added to 5 μ l α -³²P. This solution was mixed with the labelling beads by pipetting and stirring. The labelling mixture was incubated at 37°C for 30', after which 1 μ l of 0.25 M EDTA, 3 μ l of 10 mg/mL tRNA and 46 μ l of STE were added. The probe was purified by passing it through a Sephadex G-50 Spun

Column (Sambrook *et al.*, 1989); centrifugation was 4' in duration at 2 500 rpm (3/4 speed) using an IEC HN-SII swinging bucket centrifuge.

16. Radiolabelling of RNA Probes

RNA probes were synthesized using the MAXIscript™ *in vitro* transcription kit from Ambion. The template used in these reactions was pPAB-cDNA (this vector has no polyA+ tail, so amplification of carboxy terminus is irrelevant in terms of probe specificity), linearized with HindIII (this prevented transcription of vector sequence). The template was gene clean purified after digestion, then dried down in a Savant SC100 Speed Vac (with Savant VP 100 vacuum pump). The following 20 µl reaction mix was prepared on ice: 8 µl DEPC sdH₂O, DNA template (1 µg dried down), 2 µl 10x Transcription Buffer, 1 µl 10 mM ATP, 1 µl 10 mM GTP, 1 µl 10 mM UTP, 1 µl 10 mM CTP, 5 µl α-³²P CTP (Amersham) and 2 µl T3 RNA polymerase. This solution was well mixed and incubated at 37°C for 1 hour, after which it was heated to 95°C for 2 min, then kept on ice. DNase I (1 µl) was added and the solution was heated at 37°C for 15'. The volume was increased by adding 80 µl of 0.1 M NaOAc (pH 5.2), 0.1% SDS (DEPC treated). The probe was purified by running it through a Sephadex G-50 spun column.

17. Southern Blot Agarose Gel Transfers

Southern blot transfers were performed as outlined by Sambrook *et al.* (1989). Hybond N membrane was used.

17.1 Preparation of the Southern Membrane for DIG DNA Probing

A 0.1% w/v solution of gelatin (1 ml/cm^2) was prepared. The solution was heated to ensure the solution of the gelatin, then cooled to RT. The membrane (when intended for DIG reactions) was dipped in solution for 5 s then allowed to air dry. This step was replaced with a 2 hr pre-hybridization for radioactive southern blots,

17.2 Probing of Southern Blots

The following protocol is based on that of the Boehringer Mannheim DIG System user guide. Hybridization buffer ($\sim 25 \text{ ml/100 cm}^2$) contained the following in dH_2O : 20 $\mu\text{g/ml}$ sssDNA (boiled to denature, then iced), 6x SSC, 5x Denhardt's, and 0.5% SDS. The hybridization buffer was added to a rotary oven tube containing the blot and incubated for 20' at 65°C in a rotary oven. The buffer, excepting $125 \mu\text{l/cm}^2$, was discarded (a buffer volume of 12.5 ml was used as a minimum). To the remaining buffer was added 15 μl of DIG DNA probe, or 100,000-500,000 cpm/ml (of hybridization buffer) for radiolabelled probes; probes were denatured by boiling, then ice quenched. Hybridization was carried out at 65°C O/N.

The blot was washed with 0.5 ml/cm^2 2x SSC for 15' at 65°C ; then with 0.5 ml/cm^2 2x SSC + 0.1% SDS for 30-60' at 65°C ; then finally with 0.5 ml/cm^2 0.1x SSC for 10' at 65°C . The blot was then air dried briefly, wrapped in plastic film and stored at

4°C. Radioactive blots were exposed to film (KODAK BioMax X-OMAT™ AR), for varying lengths of time (depending on signal strength), at this stage

17.3 Visualization of DIG probed southernns

The hybridized blot was washed with 1 ml/cm² of washing buffer for 5' at RT, then incubated with 1 ml/cm² buffer 2 for 30' at RT. A 0.2 ml/cm² solution of anti-DIG-AP, diluted 1:10 000 in buffer 2, was prepared and incubated with the blot for 30' at RT. The membrane was washed twice for 15' at RT with 100 ml of washing buffer, then equilibrated 2-5' in 0.2 ml/cm² buffer 3. Lumigen PPD™ was diluted 1:100 in buffer 3 for a total volume of 0.05 ml/cm². The blot was sealed with Saran Wrap. Before exposure, the blot was incubated for 5-15' at 37°C. Exposure to film (KODAK BioMax X-OMAT™ AR) at RT, varied depending on the strength of the probe signal.

17.4 Developing Film

Film was treated for up to 4' in KODAK GBX developer, washed for 1' in tap water, fixed for 2-4' with GBX fixer, then flushed with tap water for about 5'.

18. Colony Blots

Colony lifts were performed essentially as outlined by Sambrook *et al.* (1989). *E. coli* plates were cooled at 4°C for at least 30' prior to blotting. Pre-cut MagnaGraph nylon transfer membranes were overlayed on the agar surface for 10' to allow colony transfer (orientation was marked with a needle). Double layers of 3MM paper, lining three separate glass baking trays, were saturated with: denaturing sol'n (0.4 M NaOH, 1M NaCl), neutralizing sol'n (1M Tris-Cl, pH 7.5, 1.5 M NaCl) or wash sol'n (2x SSC). The membranes were placed, for 5' face up, in the denaturing tray, followed by the neutralizing tray, and finally the wash tray. When going between trays, the membranes were briefly blotted on dry 3MM paper. The transfer membranes were finally dried at RT for 30', then baked at 80°C for 2 hours. The colony lifts were probed as per standard southern blot protocol.

19. Preparation of Fungal Mycelia for RNA or DNA Extraction

N. crassa total RNA was extracted using a protocol similar to that of Reinert *et al.*, 1981). Conidia from 10+ day old slant cultures were collected in 5 ml of sdH₂O. The amount of conidia was then determined spectrophotometrically at A₂₆₀. Approximately 2×10^7 conidia were used to inoculate 50 ml of VMM broth (occasionally supplemented with either 20 or 200 µg/ml p-aminobenzoate) contained in 250 ml Erlenmeyer flasks; the flasks were shaken at 200 rpm at 21-23°C. After 25 hours, mycelia were collected using a Buchner funnel and Whatman filter discs (No. 1; 7.0 cm).

Samples were washed with ~100 ml dH₂O. Fungal filter cakes were blotted dry with Whatman 3MM filter paper, peeled off and transferred to 15 ml falcon tubes, then frozen at -70°C O/N. Samples were then lyophilized under vacuum for 5-6 hours immediately prior to processing.

20. Genomic DNA Extraction

A lyophilized mycelia sample (~0.25 g) was combined with 1 g of acid washed sea sand and pulverized in a mortar and pestle (heated to 80°C O/N then allowed to cool to RT). The powder was transferred to a 15 ml falcon tube and resuspended in 2.5 ml of Extraction Buffer (0.2 M Tris pH 8.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS). Pronase was added to a final concentration of 0.5 mg/ml; the sample was incubated at RT for 10'. The slurry was vortexed briefly with 825 µl phenol (Tris buffered pH 8.0) and 150 µl chloroform and left to sit for ~30'. The sample was spun at 10,000 rpm in a SS-34 rotor for 60' at RT. The aqueous phase was immediately transferred to a new 15 ml tube and 150 µl of RNaseA (10 mg/ml) was added; the sample was incubated 30' at 37°C. The solution was subsequently extracted with 1 vol. C:I (without vortexing), centrifuging at 10,000 rpm for 10'. The aqueous phase was transferred to a new tube, and the DNA precipitated with 0.54 vol. of isopropanol (tube was inverted to mix). Brief centrifugation (~5 s) pelleted the DNA; the supernatant was removed with a drawn out pasteur pipette. The pellet was rinsed with 3 ml of 70% EtOH, air dried, and resuspended in ~2 ml of T₁₀E₁. The sample was stored at 4°C.

21. Fungal RNA Extraction – pH 5.0

Lyophilized mycelia, from each sample culture, were pulverized using mortar and pestle (previously oven dried at 80°C for several days) and 1 g of acid washed sea sand. To the mycelial debris, a mixture of 10 ml Extraction Buffer (100 mM NaOAc, pH 5.0, 1 mM EDTA, 5% SDS) and 10 ml of unbuffered P:C:I. (acidic pH) was added and ground together thoroughly. The slurry was transferred to a labelled 50 ml PA tube and centrifuged in a SS-34 rotor (10 000 rpm, 10', 4°C). A bent Pasteur pipette was used to carefully transfer the aqueous phase to a new 50 ml PA tube containing 60 µl of pre-digested Pronase (20 mg/ml). This was left for 1 hour at RT. The aqueous phase was then extracted two more times with unbuffered P:C:I. Adding 2 volumes of absolute EtOH and leaving O/N at -20°C precipitated the RNA. RNA was pelleted by centrifugation in a SS-34 rotor (10 000 rpm, 4°C, 20'), resuspended in 1.8 ml of DEPC treated dH₂O at 4°C, and transferred to a 15 ml corex tube. The RNA was precipitated at second time by adding 3 volumes of 4 M NaOAc (pH 6.0), 10 mM EDTA and leaving at 4°C for 10'. Centrifugation (10 000 rpm, 20', 4°C, SS-34 rotor) pelleted the RNA, which was then rinsed with 1.8 ml of 70% EtOH. The pellet was air dried at RT then resuspended in 2 ml of DEPC dH₂O, 0.5% SDS. The concentration and purity of the RNA was checked by spectrophotometry (A_{260} and A_{280}). The RNA was precipitated a third time by adding 1/13 volume of 4M NaOAc (pH 6.0), 10 mM EDTA and 4 ml of absolute EtOH. Centrifugation (10 000 rpm, 20', 4°C, SS-34 rotor) pelleted the RNA. The pellet was resuspended in DEPC dH₂O to a final RNA concentration of 2 mg/ml.

22. Oligo (dT) Cellulose Column Purification of mRNA

Poly(A)⁺ RNA was isolated from total RNA preparations using affinity chromatography with oligo(dT)-cellulose columns. The method used was based on that in Sambrook *et al.* (1989). 1.0 g of oligo(dT) cellulose (from New England Biolabs) was suspended in 0.1 N NaOH + 10 mM EDTA and left for 1 hour at RT. The resulting slurry was added to 1.5 x 5 cm BIORAD Econo-Column Chromatography Columns (containing some 1x Column Loading Buffer) to obtain a packed column of ~ 1 cm height. Excess liquid was drained and the tubes filled with dH₂O + 0.1% DEPC (not autoclaved) for 30' at RT. The columns were drained again, then washed with three changes of DEPC (0.1%) treated sdH₂O. Columns were further washed with 1x Column loading buffer until effluent was less than pH 8.0.

The total RNA sample was heated to 65°C for 5' then cooled to RT using water; This was combined with an equal volume of 2x Column Loading Buffer. This solution was run through the column and the eluate was collected in a sterile test tube. This was followed by one column volume of 1x Column Loading Buffer, the eluate of which was collected in the same tube as the previous step. The combined eluates were heated to 65°C for 5', cooled to RT in water, and re-applied to the column (again collecting the eluate). The column was then washed with seven column volumes of 1x Column Loading Buffer.

The mRNA was eluted from the column with 2-3 column volumes of Elution Buffer. The eluates were collected in ½ column volume aliquots in sterile tubes. Two volumes of absolute EtOH were added to each tube, and samples were left to precipitate O/N at -20°C. The tubes were then centrifuged in an SM-24 rotor at 10,000 rpm for 20' at 4°C. Pellets were washed with 500 µl of 70% EtOH with a 10' spin at 10,000 rpm, then resuspended in 200 µl of DEPC sdH₂O. Samples were then evaporated with heat and vacuum and resuspended in 20 µl DEPC sdH₂O. Poly (A)⁺ RNA was precipitated a second time (to remove excess salt) with 1/10 volume 3 M NaOAc (pH 5.6), 2 volumes of absolute EtOH and 5 µg of yeast tRNA (as co-ppt agent) at -20°C O/N. The mRNA was spun down at 12,000 rpm for 20' at RT in a microfuge. The pellet was washed with 200 µl of 70% EtOH, and resuspended in 10 µl of DEPC sdH₂O.

23. Northern Blotting

23.1 Preparing, Loading and Running a Formaldehyde Denaturing Gel

The gel was prepared by melting the appropriate amount of agarose (1.2%) in dH₂O, cooling to 60°C, then adding formaldehyde (~12.3 M) and 5x formaldehyde gel-running buffer to give final concentrations of 2.2 M and 1x respectively. Once cast, the gel was allowed to set for at least 30'.

A given RNA sample was prepared in a 20.2 µl volume. The RNA (up to 30µg) was roto-evaporated and resuspended in 4.5 µl of sdH₂O. This was combined with 2.0

μl 5x formaldehyde gel-running buffer, 3.5 μl formaldehyde, 10.0 μl formamide, and 0.2 μl ethidium bromide (10 mg/ml). Samples were incubated for 15' at 65°C and chilled on ice. Before loading, 2 μl of formaldehyde gel-loading buffer was added to each sample.

The gel was pre-run for 5' at 5 V/cm immediately before sample loading. After loading, the gel was run submerged at 4 V/cm in 1x formaldehyde gel-running buffer. After 1-2 hours the buffer from the reservoirs was collected and mixed. The typical run time for a 30 ml mini-gel was 3 hours.

23.2 Transfer of Denatured RNA to Nylon Filters

In advance of transfer, denaturing RNA gels were soaked in 0.05 N NaOH for 20', rinsed in DEPC-sdH₂O, and soaked again in 20x SSPE for 45' (note that all solutions used for Northern blotting were DEPC treated). The gel was then set up on a solid support for capillary transfer in the same manner as a southern blot (Sambrook *et al.*, 1989); 20x SSPE was used as transfer buffer. Maximum Strength Nytran Plus (Schleicher & Schuell) nylon membranes were used to immobilize eluted RNA. Transfer was allowed to proceed for 16-24 hours after which time the lanes were marked and the membrane was soaked in 6x SSPE for 5' at RT. The membrane was then air dried for 30' at RT and baked for ~2 hours at 80°C to fix the RNA.

23.3 RNA Blot Pre-Hybridization and Hybridization

RNA membranes were pre-hybridized using 125 $\mu\text{l}/\text{cm}^2$ hybridization solution; a minimum volume limit of 12.5 ml was observed. The hybridization solution (for 12.5 ml) consisted of 3.125 ml 20x SSPE, 6.25 ml deionized formamide, 0.625 ml 100x Denhardt's, 2.5 ml 50% (w/v) dextran sulphate and 125 μl heat denatured sssDNA (2 mg/ml); in some cases SDS was included to a final concentration of 0.5%. Blots were pre-hybridized in a rotary oven at 45°C for 1-2 hours. The RNA probe (500,000 -1,000,000 cpm/ml hyb. sol'n) was added directly to the rotary tube; hybridization proceeded for at least 12 hours at 42-45°C.

23.4 Washing, Exposing and Stripping RNA Blots

Immediately after hybridization, wet RNA blots were washed twice with 50 ml of 5x SSPE at 45°C for 20'. Blots were then washed once with 50 ml of 0.1x SSPE, 0.5% SDS for at least 60' at 65°C, and washed again with 200 ml of 0.1x SSPE, 0.1% SDS at RT for 25'. If membrane was still very radioactive, it was repeatedly washed 0.1x SSPE, 0.5% SDS at 65°C for as long as 48 hours. Exposure of membranes to Kodak X-OMAT film took place at -20°C for 1-7 days.

Stripping of RNA blots was accomplished by immersing membranes in ~100 ml of boiling 0.1% SDS solution. After allowing the solution to cool to room temperature, stripped membranes were pre-hybridized and re-probed as normal.

24. Screening of λ Phage Library

24.1 Preparation and Plating of Phage

The following was the basic method used for all phage manipulations and is based on the method of Sambrook *et al.* (1989). The cDNA for *pab-2* was obtained using a nitrate induced, cDNA library in lambda ZAP version II+ obtained from the FGSC (Exley *et al.*, 1993). A colony of XL1-MRA *E. coli* from a fresh (day-old) LB plate was used to inoculate 5 ml of LB + 0.2% maltose + 10 mM MgSO₄; this was incubated at 37°C until an OD₆₀₀ of 1.0 was reached (~5 hours). The cells were transferred to a sterile 50 ml PA tube and spun down at 4000 rpm for 10' at 4°C in an SS-34 rotor. The pellet was resuspended by vortexing in 10 mM MgSO₄ to an OD₆₀₀ = 0.5. The suspension was divided into μ tubes; one 200 μ l aliquot for each phage sample to be tested. Each aliquot was inoculated with 1-10 μ l of phage (diluted to desired PFU in SM buffer) and incubated for 20' at 37°C. It was subsequently added to 50°C, 0.7% top agarose (4 ml or 9 ml for 90 mm or 150 mm plates respectively), briefly vortexed, then poured on to pre-warmed LB plates. The plates were grown O/N at 37°C until plaques reached ~ 2 mm in diameter.

24.2 Immobilization and Screening of Bacteriophage Plaques on Nylon Membranes

Plaque plates were chilled at 4°C for at least one hour to allow top agarose to harden. Circular HybondTM-N nylon membranes were then placed in contact with the

plate's surface for 2 min to adsorb phage (membrane orientation was marked with needle); the contact time for a second blotting was extended to 4 min. The membrane was peeled off and immersed, DNA side up, in denaturing solution (0.4 N NaOH, 1.0 M NaCl) for 5', transferred to neutralizing solution for 5', rinsed for 30 s in 2x SSC, then allowed to dry. The DNA was fixed by baking in an 80°C oven for 1-2 hrs. The membranes were examined using ³²P-labelled, probes constructed from wt *pab-2* template DNA. The protocol for southern blotting was followed for pre-hybridization, hybridization and washing steps.

24.3 Amplification of *pab-2* phage isolate

Plaques which hybridized to the probe, as indicated by exposure of membrane to X-ray film, were taken as plugs from the agar plate. SM buffer (750 µl) was added to elute the phage from the plug. This new phage stock was titred and used to generate successive, low density plaque plates (~150-300 CFU/plate). The plates were again screened as described above. This process continued until all plaques gave positive hybridization signals, indicating a pure phage stock.

25. Excision of Phagemids (pBSSK-)

The following method used for excision of the phagemid from the λ ZAP II+ phage was based on those found in Short *et al.* (1988) and Short and Sorge (1992). A fresh culture of XL1-Blue (RecA⁺ F') *E. coli* was grown in an O/N, 5 ml LB culture at

37°C. The cells were spun down for 10', 4000 rpm at 4°C, and resuspended to an $OD_{600} = 1.0$ in 10 mM $MgSO_4$. A 200 μ l aliquot of this cell suspension was combined with 200 μ l of phage isolate (containing 1×10^5 PFU in SM buffer), and 20 μ l R408 helper phage (1×10^9 PFU in SM buffer); this mixture was incubated at 37°C for 20' in a μ tube. The contents of the μ tube were then transferred to a 50 ml PA tube, containing 5 ml of LB, and gently shaken at 37°C for 3.5 hrs. A 70°C heat treatment for 20' was then used to kill the bacteria and parent phage. Centrifugation for 10' at 5000 rpm allowed removal of the pBluescript phagemid (packaged as filamentous phage particles) contained in the supernatant.

The phagemid was removed from the packaging phage by infecting 200 μ l of $OD_{600} = 1.0$ XL1-Blue (F') *E. coli* (suspended in 10 mM $MgSO_4$) with 200 μ l of the phagemid particle solution (10^0 and 10^{-2} dilutions in 10 mM $MgSO_4$) and incubating at 37°C for 15'. The infected bacteria were then plated on LBA media and incubated O/N at 37°C. Colonies formed contained the pBluescript SK- (pBSSK-) double stranded phagemid with the cloned DNA insert in flanking *EcoRI* sites. Plasmid preparations were performed using standard mini-prep protocols.

26. PCR Protocols

Standard PCR reactions were performed using a Perkin Elmer Cetus 480 DNA Thermal Cycler and GIBCO BRL reagents. Typical PCR reactions contained: 2.5 U of Taq DNA Polymerase, 5 μ l of 10x PCR buffer, 1.5 μ l MgCl_2 (50 mM), 1 μ l dNTP (10mM), primers (25 pmoles of each), template (varying concentrations; see below), and sdH_2O (up to 50 μ l). In some cases, 1 μ l of ^{35}S dATP (Amersham) was included. After thorough mixing by pipette, 50 μ l an overlay of sterile mineral oil was added.

The standard reaction conditions were: denaturation at 95 °C for 5', followed by 30 cycles of 1' denaturation at 95°C, 1' annealing at optimal temperature for primer (see below), 1' extension at 72 °C. A final 10' extension at 72 °C was usually included. Optimal primer annealing temperature was calculated by adding 4 °C for each G/C and 2°C for each A/T present in the primer; this value was then reduced by 0-4 °C depending on PCR results.

The amount of template used was highly variable. Amplifications from genomic DNA required approximately 25 ng of DNA; ~1 ng was needed for plasmid DNA reactions. Amplification directly from a λ phage library (Raju *et al.*, 1995) required approximately 5 μ l (5×10^7 PFU) of phage which was boiled for 5' before addition to the PCR mix.

27. Sequencing

Sequencing reactions were prepared using either the FIDELITY™ DNA sequencing system (Oncor) or the T7 Sequenase (v2.0) Quick-Denature plasmid sequencing kit (Amersham). The quick alkaline denaturation method was used for both kits. Typical sequencing reactions contained 0.5-1.0 µg of plasmid template, and 4-5 pmole of primer.

Sequencing gel runs of 2-5 hours were carried out on a Model S2 (BRL) sequencing apparatus using a BIORAD Model 3000 xi Computer Controlled Electrophoresis Power Supply set to maximums of: 1900 V, 45 mA, 60 W. Glycerol tolerant 6% polyacrylamide gels (as described in the FIDELITY kit) were used for the electrophoretic matrix.

28. Cycle Sequencing

The frameshift mutation in *pab-2* was verified using the CircumVent™ Thermal Cycle Dideoxy DNA Sequencing Kit with Vent_R (exo⁻) DNA polymerase (NEB). Typical reactions included ~70 ng of template (PCR product from genomic DNA), 1.2 pmole of primer, 2 µl ³⁵S dATP (Amersham), and 2 U of Vent_R Exo⁻. Cycle sequencing reactions were done for 20 cycles consisting of 95°C for 20 sec, 52°C for 20 sec (primer optimum), 72°C for 20 sec using a Perkin Elmer Cetus 480 Thermal Cycler. The results were electrophoretically resolved as with standard sequencing.

III. RESULTS

Isolation of *pab-2*⁺

Robb *et al.* (1995) isolated the *ro-4* gene from the Orbach/Sachs cosmid library of *Neurospora crassa* DNA (Fungal Genetics Stock Center) based on complementation experiments involving the closely linked *pab-2*⁺ gene. The DNA contained in this pMOcosX library was taken from *N. crassa* strain 74-OR23-1VA (FGSC# 987). The cosmid G9:10F, containing both these genes, was used as a starting point for the isolation of *pab-2*⁺. The cosmid was digested with several restriction enzymes, and the fragments tested for the ability to complement growth of auxotrophic *N. crassa pab-2* (FGSC# 4076) spheroplasts (Table 1). Results from these experiments were used 1) to determine which enzymes were most suitable for cloning an intact *pab-2*⁺ gene, and 2) to facilitate the subcloning process by determining the precise location of *pab-2*⁺ via functional disruption.

The restriction fragments from an *EcoRI/HindIII* double digestion of G9:10F were individually isolated from an agarose gel. The fragments were purified by gene cleaning and tested for their ability to transform *pab-2* spheroplasts. It was concluded from these experiments that the *pab-2*⁺ gene resided on the largest (6.8 Kb) *EcoRI/HindIII* fragment of G9:10F (Figure 3a). This portion of the cosmid was subsequently sub-cloned into the MCS of pBluescript II KS+ creating pPAB1-EH6.8.

Table 1 - Complementation of *pab-2* spheroplasts with restriction digests of G9:10F. Growth (+) indicates that *pab-2*⁺ remains intact. The data represent a summary of experiments involving growth on VMM + hygromycin (0.2 mg/ml) both with and without sorbose. The hygromycin phosphotransferase B gene carried on pCSN43 was co-transformed to provide Hg^r. Positive control was uncut G9:10F; negative control was pCSN43 only.

Restriction Enzyme	Growth of <i>pab-2</i> spheroplasts
<i>Bam</i> HI	+
<i>Eco</i> RI	+
<i>Eco</i> RV	+
<i>Hind</i> III	+
<i>Kpn</i> I	-
<i>Pst</i> I	+
<i>Sac</i> I	+
<i>Sal</i> I	-
<i>Sma</i> I	+
<i>Xba</i> I	+
<i>Xho</i> I	+

Figure 3 - Restriction digests of the G9:10F cosmid and southern indicating fragments harbouring *pab-2⁺*.

a: Restriction fragments of G9:10F resulting from individual and double digestion using *Not* I, *Xba*I, *Eco*RI and *Hind*III.

b: Southern blot showing the localization of *pab-2⁺* to the *Eco*RI/*Hind*III 6.8 Kb and the *Eco*RI 13.3 Kb restriction fragment; the DIG probe was constructed from the *Eco*RV insert of pPAB3-E1.6.

a

b

The pPAB1-EH6.8 plasmid was reduced in size by successive restriction enzyme shrinks (Figure 4). Constructs containing putative *pab-2*⁺ gene sequence were tested for their ability to transform *pab-2* spheroplasts (Table 2). Complementation tests were carried out on colony forming media to better characterize successful complementation of the *pab-2* mutation. Evidence of complementation was observed in the formation of ~25+ colonies after 4-7 days. A complete lack of growth, after 4-7 days, indicated a construct containing functionally inactive, or absent, *pab-2*⁺. The purpose of these experiments was to verify the earlier data (Table 1), and to ensure that the *pab-2*⁺ gene had indeed been isolated.

Among the many sub-clones tested, pPAB4-S1.3 contained the smallest fragment (1.3Kb) with a functional *pab-2*⁺ gene. Sub-clones with larger fragments containing this region intact showed similar ability to complement the mutation. When this region was interrupted using internal *KpnI* and *SaII* sites, the ability to complement was entirely lost. These results corroborated the conclusions drawn from earlier experiments (Table 1). A probe was constructed from the pPAB3-E1.6 insert and used to identify the fragment containing *pab-2*⁺ in an *EcoRI* digest of G9:10F. The uppermost, 13.3 Kb fragment hybridized (Figure 3b) and was sub-cloned, creating pPAB-1E. The aforementioned sub-clones were used, in conjunction with synthetic primers (Figure 5), to sequence *pab-2*⁺ (Figure 6).

Submission of *pab-2*⁺ sequences to the BLAST data base, produced no reliable matches on the basis of similarity. However, sequencing of pPAB-E1.6, with the reverse

Figure 4 - A restriction map of the genomic DNA insert for pPAB-1E; note that the region downstream of *pab-2*⁺ remains largely uncharacterized. The relative placement of several sub-cloned regions are also shown. The restriction enzymes used for sub-cloning, as well as the location of universal primers in the construct, have been added for clarity.

Table 2 - Transformation of *pab-2* spheroplasts with plasmids containing DNA belonging to, or neighbouring, the *pab-2* locus. Growth (+) indicates intact *pab-2*⁺; no growth (-) indicates disrupted or absence of *pab-2*⁺. Experiments were performed, in at least triplicate, on colony forming VMM + Hygromycin media. pCSN43 was co-transformed to confer Hg^r.

Plasmid	Growth of <i>pab-2</i> spheroplasts
pPAB1-EH6.8	+
pPAB2-K6	-
pPAB2-K0.8	-
pPAB2-B3.4	+
pPAB3-E1.6	+
pPAB3-P1.9	+
pPAB3-P1	-
pPAB4-SP1	-
pPAB4-SP0.8	-
pPAB4-S1.3 (R&L)	+

Figure 5 - The relative locations of primers used for sequencing and PCR involving *pab-2*⁺. The grey filled regions indicate *pab-2*⁺ exons; the single intron is shown in white. Note that PAB-16 was designed to amplify only a spliced form of *pab-2*⁺, and is therefore shown spanning the intron.

100 BP

Figure 6 - The DNA sequence of *pab-2⁺* from *N. crassa* (FGSC#987). The coding region of the gene is indicated in capitals; non-coding sequence, including the intron (as for pPAB-polyA6; the major transcript), is shown in lowercase letters. The 5' and 3' intron splice sites, in addition to the lariat sequence, are bolded (Bruchez *et al.*, 1993b, Mann *et al.*, 1989). An alternative splice site (as for transcript pPAB-cDNA; the minor transcript) is italicized and in bold. The CAAT box is underlined by dashes (Mann *et al.*, 1989); the TATA box is double underlined; a transcriptional start site is marked by wavy underline (Bruchez *et al.*, 1993b). A dotted underline marks the Kozak sequence (Bruchez *et al.*, 1993a). The stop codon for the minor transcript is single underlined.

-358 ctgcagtgatttcagttttatacatccgtctgtatctttttgtttacatgtttcattcctg
 -298 ccatcacccggcggtcttctttttaccaatcatcggtcatgtcggcgcggaaggattggtgatt
 -238 tatcggggcccatcaggtatgaggaaacctaaagctgatagattttatacattatattcccc
 -178 atgaaacgaccacagagagcaatgagtcagcaagctggattgcatgtcatataaaacaga
 -118 taatacattacggatatcgtatttcttcaccattcaccttcactcttcggagctctaatc
 -58 catcgagtctatgctgttcctccatctaggtaaccttcaaagacaggcacccctcacaATG
M

4 GCGACCACGACAGAAGCTCCCTCCTCCTCTTCACCACCCTGCGTTACGACCCTCTCCTG
 2 A T T T E A P L L L F T T L R Y D P L L

64 CTTCCCGGCTTCGTCTCTCCCACTGAATCCTCCCGTTCGCTCCATTGTACATGCTTCCT
 22 L P G F V S P T E S S P F A P L Y M L P

124 TACCATCGCGATCGCATCCTGCGCGCCGCACTCACTTCAAATGGCCCGCGTAGTCTCT
 42 Y H R D R I L R A A T H F K W P A V V S

184 CTCCTCTCCGGTGACAGCGGTCTCGAAACGCTCTCCTCCTTCATTTTCTCCTCCCTCTCC
 62 L L S G D S G L E T L S S F I F S S L S

244 GACGAGCAGCGATCTCACCCAAACCGCATCCGTGTAACCGTCTCTGAGAAGGGAGAGCTA
 82 D E Q R S H P N R I R V T V S E K G E L

304 GGCATCACCATCTCACCAGCACCACAAGTCCCTTCAACAGCCAACTTCTTCCCAAATTCC
 102 G I T I S P A P Q V P S T A N F F P N S

364 CTTCCCCAGCCGGGTACCAATATCCCTAGTCTATGCCTGATGGGAACCTCGACGCCGTTCC
 122 L P Q P G T N I P S L C L M G T S T P F

424 GAGGTCCTAGTCGACACCCAAAGGTCCAAAAGTCAGCATAACGCACTTTAAGACTACG
 142 E V L V D T Q R S K K S A Y T H F K T T

484 AAGCGCCAAATCTACGACGAAGCGAGAAAGAGGCACGGGATCGCGTTGACGGATCGGAAG
 162 K R Q I Y D E A R K R H G I A L T D R K

544 GAGGTGTTGATTGTTAGCGAGGATGGGGAGGTGATGGAAGGGAGTATTGCCACGCCGTAC
 182 E V L I V S E D G E V M E G S I A T P Y

604 TTTTGGAGAGACGGGAGATGGGTGACACCGCCGGTTGGGGAAGGTGAGGGAGAGACAGAA
 202 F W R D G R W V T P P V G E G E G E T E

664 GGGGATTGGGGAAGTGGTGGAAATGCGGGAACCTACGAGGAGATGGGCTTTGGAAAAGgtg
 222 G D W G S G G N A G T T R R W A L E R

724 gtttctcctgctttgagatgagatgattattattgggcagttctgctaacggcgcgctg

784 ggtagGGGAATTGCGGTTGAGCAGGTTGTACGGGCAAACCTCGCTGGTTGATGAAGAAGAG
 241 G I A V E Q V V R A N S L V D E E E

844 TGCTGGTTGAGTAATGGGGGCAGGGGATTCTACTTTGGAAAGGTCAAGTTGGGCTAGgtc
 259 C W L S N G G R G F Y F G K V K L G *

904 ctgagatggcaagcggatgagcgttaagggcatgattccgtgatgctttcattgogccag
 964 gataccgtttctgaaagtgcggttaccataagatatggcttgtaatcgctcggttcggtgc
 1024 taccgaggctgaacaggggcaatgtttatgtctcccggtcccgggcatcactagggcaac
 1084 acaccaggctgggctgtttcccatcgtgagacttcggggattttgcgaggcaatgtatca
 1144 cgggtgcgcaagcca

primer (precedes T3), identified the promoter region of *ro-4*. It was determined that the *EcoRV* restriction site present in this pPAB3-E1.6 sequence is the same as that found 724 bp upstream from the *ro-4* start codon.

Cloning of the Mutant *pab-2* Allele (pPAB-MUT)

The mutant allele *pab-2* was obtained by performing a PCR on genomic DNA prepared from *pab-2 N. crassa* cultures (FGSC# 4076). The PAB-7 and PAB-8 primers (Figure 5), which flank the wild-type gene, were used for the amplification process. The product, a single 1.1 Kb band (migrating the same as the positive control), was positively identified as *pab-2* via southern blot. The pBSKS+ vector was used to clone the PCR product using a simultaneous *SmaI* digestion, fill in and ligation reaction; polishing of the PCR product was necessary to eliminate any non-template, 3' dATP additions generated by Taq polymerase (Clark, 1988). The resulting construct, pPAB-MUT, was sequenced (Figure 7) using synthetic primers homologous to both the wild-type and mutant *pab-2*.

Comparison of mutant to wild-type sequence revealed a single base deletion (combined with a T to C transition) in the tail end of the first exon, a five base deletion in the intron, and several silent mutations. Cycle sequencing, using PAB-3 and PAB-5 primers, verified that the single base deletion/transition was not a PCR artifact (Figure 8). The single base deletion, which causes a frame shift, was found to scramble the amino acid sequence after codon 201 and to create a premature stop codon at 209.

Figure 7 - The DNA and amino acid sequence of the *N. crassa* mutant (FGSC# 4076) *pab-2* gene. Coding DNA regions are shown in uppercase, non-coding regions are shown in lowercase. Single bases that differ from wild-type (i.e. point mutations) are shaded, the corresponding wild-type base is shown above in lowercase italics. Possible intron splice sites and lariat sequence (as identified in *pab-2*⁺) are indicated in bold. The frameshift/transition of "TT" to "C" is marked by a double underline. Amino acids that differ from wild-type, due to this frameshift mutation, are shown in italics. Underlining shows the replacement of "ACCA" by six bases. A dotted underline delineates where 5 bases have been deleted from the wild-type sequence "GAGATGAT".

-35

catctaggtaaccttcaagacaggcaccctcaca

1 ATGGCGACCACGACAGAAGCTCCCTCCTCCTCCTCACCACCCTGCGTTACGACCCTCTC
1 M A T T T E A P L L L F T T L R Y D P L
61 CTGCTTCCCGGCTTCGTCTCTCCCACTGAATC^CTCCCCGTTTCGCTCCATTGTACATGCTT
21 L L P G F V S P T E S S P F A P L Y M L
121 CCTTACCATCGCGATCGCATCCTGCGCGCCGCCA^CTCACTTCAAATGGCCCGCCGTAGTC
41 P Y H R D R I L R A A T H F K W P A V V
181 TCTCTCCTCTCCGGTGACAGCGGTCTCGAAACGCTCTCCTCCTTCATTTTCTCCTCCCTC
61 S L L S G D S G L E T L S S F I F S S L
241 TCCGACGAGCAGCGATCTCACCCAAACCGCATCCGTGTAACCGTCTCTGAGAAGGGAGAG
81 S D E Q R S H P N R I R V T V S E K G E
301 CTAGGCATCACCATCTCACCAGCACCACAAGTCCCTTCAACAGCCAACTTCTTCCCAAAT
101 L G I T I S P A P Q V P S T A N F F P N
361 TCCCTTCCCCAGCCGGGTACCAATATCCCTAGTCTATGCCTGATGGGAACCTCGACGCCG
121 S L P Q P G T N I P S L C L M G T S T P
421 TTCGAGGTCTAGTCGACACCCAAAGGTCCAAAAA^gTCAGCATAACGCACTTTAAGACT
141 F E V L V D T Q R S K K S A Y T H F K T
481 ACGAAGCGCCAAATCTACGA^CGAAGCGAGAAAGAGGCACGGGATCGCGTTGACGGAT^CGG
161 T K R Q I Y D E A R K R H G I A L T D R
541 AAGGAGGTGTTGATTGTTAGCGAGGATGGGGAGGTGATGGAAGG^gAGTATTGCCACGCCG
181 K E V L I V S E D G E V M E G S I A T P
601 TACT^{tt}CTGGAG^aGACGGGAGATGGGTGAC^accgccggttggggaaggtgagggagagacag
201 Y S G G T G D G *
661 aaggggattggggaagtggtggaaatgcgggaactacgaggag^atgggctttgga^aaggt
721 gcg^ttttctcctgct^ttgaga^tgat^tattattgggc^{ag}tt^ctgctaacggcg^cgc^ctg^ggt
781 aggggaattgcggttgagcag^gttgtacgggcaaactcgctggttgatg^aagaagagtgc
841 tgggttgagtaatgggggcaggggattctactttggaaaggtcaagttgggctaggtcctg
901 agatggcaagcggatgagcgttaagggcatgattccgtgatgctttcattgcgccaggat
961 accg^gtttct^gaaagtcgc^ggt^ttg^tttaccctaagatatggcttg^taatcgctcgttcggtgc
1021 ta

Figure 8 – Autoradiographs of sequencing gels showing the frameshift/transition mutation that occurs in *pab-2* where two thymadines are converted to one cytosine.

a: Wild-type *N. crassa* sequence.

b: Sequence of *pab-2* mutant allele obtained from pPAB-MUT subclone.

c: PCR cycle sequencing of genomic DNA prepared from the *Neurospora pab-2* mutant.

a

b

c

Isolation of *pab-2*⁺ Transcripts from cDNA

A NO₃⁻ induced, cDNA phage library (from FGSC) was used to isolate the transcript sequence of *pab-2*⁺ (Exley *et al.*, 1993). The library was screened, in four successive rounds, using the *EcoRV* insert from pPAB4-S1.3L as a *pab-2*⁺ probe. Two clones were initially identified in the primary screening. Only one of these, however, was successfully amplified and isolated. The phagemid excised from this phage clone was named pPAB-cDNA. Sequencing of this clone revealed that the putative intron, identified by consensus splicing signals in the genomic sequence, had indeed been spliced out. It was also noted that pPAB-cDNA did not possess a 3' poly-A⁺ tail, and that there was no recognizable polyadenylation signal.

A cDNA pool, prepared from *ro-2B N. crassa*, was used as template for a PCR reaction in an attempt to isolate a *pab-2*⁺ cDNA with an intact poly-A⁺ tail. The PAB-6 and adapter primer (the complement of which is common to all cDNA in the pool) were used for this procedure (Figure 9a). Southern blotting, using the *EcoRV* fragment of pPAB-1.3L as a probe, was used to determine which of the multiple PCR products should be cloned (Figure 9b); two small bands (~0.5 Kb) hybridized. The PCR product mix was used as the template for a successive PCR reaction using PAB-11 and the adapter primer. The second, nested PCR reaction, yielded an amplification of two bands matching those identified in the earlier southern blot (Figure 9c). Fragments of the same, approximate size were isolated from an agarose gel and sub-cloned, via the TA-cloning system, yielding pPAB-polyA5 and pPAB-polyA6.

Figure 9 - PCR amplification of the 3' end of *pab-2*⁺, using a *ro-2B* cDNA pool, to obtain DNA sequence for analysis of intron splicing and polyadenylation.

a: PCR using the adapter primer and PAB-6 generates several bands. Lane 1: lambda *Hind*III standard; Lane 2: *Eco*RV fragment from pPAB3-E1.6; Lanes 3-5: PCR products using increasing amounts of template.

b: Southern blotting of the PCR products, using a *pab-2*⁺ probe, reveals two bands of approximately 0.5-0.6 Kb in size. Lane designations same as **a**.

c: A nested PCR using the adapter primer and PAB-11, and the PCR product from **a**, amplifies the two bands identified in **b**. Lane 1: lambda *Hind*III standard; Lane 2: nested PCR product. The two lower bands were excised for cloning.

a **b** **c**

The two PCR based constructs, after sequencing, were shown to have differential splicing of the intron and different polyadenylation sites. The pPAB-polyA5 construct was spliced just like pPAB-cDNA (i.e. using the consensus 3' splice junction). pPAB-polyA6, however, had the intron removed using a previously unidentified 3' junction. This 3' splice signal was unusual because it contained a guanine at the first position; this residue is normally conserved in its absence (Bruchez *et al.*, 1993b). The use of the non-consensus 3' splice site resulted in the removal of a smaller intron and the creation of a frame shift of the carboxy-terminal of the putative protein product (cf Figure 6 and Figure 10). Additionally, pPAB-polyA6 had an additional 47 bp of sequence before the start of its poly-A⁺ tail, when compared with pPAB-polyA5. Neither construct had any recognizable polyadenylation signal.

Which *pab-2*⁺ Transcript is Expressed?

Given the apparent presence of two types of *pab-2*⁺ mRNA transcripts, experiments were devised to determine the relative levels of each transcript and if those levels varied in *Neurospora*. PCR reactions were set up using the following *N. crassa* cDNA λ libraries (available from the FGSC) for templates: conidial, mycelial, perithecial, glutamine repressed, and nitrate induced (Nelson *et al.*, 1997a; Exley *et al.*, 1993). The primers (PAB-11, 15 and 16) were designed to amplify across the intronic region of *pab-2*⁺. PAB-11 was upstream of the intron, and was present in all reactions. The PAB-15 primer was downstream, but adjacent to the intron. PAB-16 was designed such that it actually spanned the intron if it was spliced out using the consensus 3' splice

Figure 10 - The amino acid sequence of the *pab-2*⁺ based on splicing of pPAB-cDNA (the minor transcript). Amino acids that differ from those coded by the major transcript are shown in bold.

1 MATTTEAPLL LFTTLRYDPL LLPGFVSPTE SSPFAPLYML PYHRDRILRA ATHEKWPAVV
61 SLLSGDSGLE TLSSFIFSSL SDEQRSHPNR IRVTVSEKGE LGITISPAPQ VPSTANFFPN
121 SLPQPGTNIP SLCLMGTSTP FEVLVDTQRS KKSAYTHEKT TKRQIYDEAR KRHGIALTDR
181 KEVLIVSEDG EVMEGSIATP YFWRDGRWVT PPVGEGET EGDWGSGGNA GTTRRWALER
241 LYGQTRWLMK KSAG*

site (i.e. as in pPAB-cDNA). The resulting PCR products were intended to vary visibly in size, depending on how the intron had spliced out. The PAB-11/PAB-15 primer combination should give either a 269 bp or 248 bp product for the "non-consensus" and "consensus" splicings respectively. The PAB-11/PAB-16 combination was designed to produce a 231 bp fragment. These fragment sizes were resolvable on 1.2-1.5% agarose MP gels.

PCR amplification of the perithecial, conidial and glutamine repressed libraries using PAB-11 and PAB-15 yielded fragments corresponding in size to the "non-consensus" spliced transcript only (Figure 11). This transcript will hereafter be referred to as the "major" transcript. The same fragment appeared for the nitrate induced template, but the band was very faint (data not shown). There were no products for the mycelial library. PCR using PAB-11 and PAB-16 produced only a faint band, of the size for the "consensus" splice product, for the conidial and nitrate induced libraries (Figure 11). This latter transcript will hereafter be referred to as the "minor" transcript. The ~300 bp band (Figure 11a) appearing in the +ve control and conidial lanes is most likely a PCR artifact due to non-specific primer hybridization.

Figure 11 - PCR amplification, using the primers PAB-11, -15, and -16, of several λ cDNA libraries to determine the dominant form of differentially spliced *pab-2⁺* mRNA.

a: An 1.5% agarose gel showing separated PCR products. Lane 1: pBR322 *Bst*NI standard; 2: -ve control; 3: +ve control (pPAB-polyA6 w/ PAB-11/15); 4: +ve control (pPAB2-cDNA w/ PAB-11/15); 5: +ve control (pPAB2-cDNA w/ PAB-11/-16); 6: NO₃⁻ induced (PAB-11/-16); 7: Mycelial (PAB-11/-15); 8: Mycelial (PAB-11/-16); 9: Perithecial (PAB-11/-15); 10: Perithecial (PAB-11/-16); 11: Glutamine repressed (PAB-11/-15); 12: Glutamine repressed (PAB-11/-16); 13: Conidial (PAB-11/-15); 14: Conidial (PAB-11/-16).

b: Autorad showing significant separation of PCR products on a 6% sequencing gel. Lane 1: +ve control - major transcript (pPAB-polyA6 w/ PAB-11/15); 2: +ve control - minor transcript (pPAB2-cDNA w/ PAB-11/15); 3: +ve control - minor transcript (pPAB2-cDNA w/ PAB-11/-16).

c: Graphic detailing the placement of primers PAB-11, -15 and -16 relative to the *pab-2⁺* intron, and how it relates to PCR product size. The filled region indicates coding sequence; the unfilled region represents the introns of the major and minor transcripts.

❶: 3' splice junction of the major transcript; ❷: 3' splice junction of the minor transcript.

Note how PAB-16 is effectively disrupted unless the template is spliced as the minor transcript.

a

b

c

Testing for the Effects of PABA Concentration on *pab-2*⁺ Expression

The possibility that *pab-2*⁺ transcript levels are influenced by the concentration of PABA in the media, was investigated by way of Northern blotting. Total RNA was isolated from wild-type *N. crassa* broth cultures, grown in 0, 20 and 200 µg/ml PABA, and *pab-2* *N. crassa*, grown in 200 µg/ml PABA. The mRNA portions of the preparations were enriched using Oligo-dT cellulose columns. A northern blot was prepared using these samples (Figure 12a); a RNA probe was prepared from the *pab-2*⁺ cDNA insert of the pPAB-cDNA construct. The probe identified a band that co-migrated with the 17S (1.7 Kb) ribosomal RNA fragment (Figure 12b). The intensity of the *pab-2*⁺ hybridization signal on the northern (at ~ 1100 bp) did not vary significantly among the samples. This indicated that the expression of *pab-2*⁺ did not correlate with the exogenous concentration of PABA.

As a control for constitutive expression, the membrane was stripped and re-hybridized with a DNA probe synthesized from the highly expressed ribosomal protein gene *crps7* (Vierula, 1997). Since *crps7* is known to be constitutively expressed, its hybridization signal should have been equivalent in all lanes. Since the intensity of the *crps7* hybridization signal (at ~ 900 bp) varied by a factor of 2 (Figure 12c), this suggests that the 1.5 fold variations in *pab-2* hybridization signal intensity (Figure 12b) were likely due to experimental error rather than transcriptional regulation. It should be noted, however, that the possibility of some limited modulation of transcription can not be

Figure 12 - The levels of *pab-2*⁺ transcript do not significantly vary in relation to PABA concentration in the growth media.

a: A denaturing formaldehyde gel showing mRNA (25 µg/lane) prepared from *N. crassa* (wild-type or *pab-2*) VMM cultures with differing concentrations of PABA. Lane 1: wt *N. crassa* w/ 0 µg/ml PABA; Lane2: wt *N. crassa* w/ 20 µg/ml PABA; Lane3: wt *N. crassa* w/ 200 µg/ml PABA; Lane 4: *pab-2 N. crassa* w/ 200 µg/ml PABA.

b: Northern blot, prepared from gel **a**, hybridized with RNA probed synthesized from pPAB-cDNA.

c: Northern blot, prepared from gel **a**, stripped and re-probed with *crps7* (~900 bp fragment).

d & e: Denaturing formaldehyde gel and corresponding Northern blot of *N. crassa* mRNA and total RNA. The mRNA was prepared from wild-type (FGSC#987) cultured w/ 200 µg/ml PABA. Total RNA was prepared from wild-type (FGSC#987) grown with and without 0.05 M thiamine supplementation. Lane 1: 25 µg mRNA; Lane 2: 5.8 µg total RNA; Lane 3: 25 µg total RNA; Lane 4: 5.2 µg total RNA (thiamine); Lane 5: 25 µg total RNA (thiamine).

excluded. Furthermore, translational or post-translation regulation of *pab-2* remains a possibility.

A second northern was prepared to verify that the *pab-2*⁺ probe had indeed hybridized specifically to *pab-2*⁺ transcripts and not the co-migrating 17S rRNA. As expected, the signal was much less intense in the total RNA lanes, compared to the mRNA lane, despite the fact that there was proportionately more rRNA present (Figure 12d & e). Had there been non-specific hybridization to rRNA, the signal would have been proportionate to the amount of rRNA present, i.e., less signal in the mRNA lane where rRNA had been selectively removed with oligo-dT purification.

PABA Toxicity Test

Zalkolar (1948) investigated the *sfo* mutant strain of *Neurospora* that required sulfanilamide for growth. The author hypothesized that the mutant was overproducing PABA, such that it accumulated to toxic levels. The sulfonamides were required by the mutant to inhibit PABA utilizing enzymes, and thus avoid toxic effects. Toxic effects from excess exogenous thiamine have also been observed in *N. crassa* (McColl, 1997). To test if large amounts of exogenous PABA could also be toxic, wild-type *Neurospora* silica stocks were used to inoculate VMM culture tubes containing 0, 20, 200, 500, 2000, and 5000 µg/ml PABA. At high PABA concentrations, in the first few days development (i.e. degree of conidiation) was slightly impaired. However, this trend was no longer evident after nine days (Table 3).

Table 3 - The effect of various concentrations of PABA and its effect on the vegetative development of wild-type *Neurospora crassa*. Development is measured by the relative extent of conidiation over time. The absence of conidiation is indicated by (-); degree of conidiation is indicated by (+).

[PABA] $\mu\text{g/ml}$	Extent of Conidiation		
	Day 3	Day 4	Day 9
0	+	+++	++++
20	+	+++	++++
200	+	+++	++++
500	-	++	++++
2000	-	++	++++
5000	-	+	++++

IV. DISCUSSION

The *pab-2*⁺ Gene

The *pab-2*⁺ gene was cloned by functional complementation of *N. crassa pab-2* spheroplasts; the smallest cloned fragment capable of complementing the mutation was 1.3 kb in length. Since transformation with the cloned gene eliminated PABA auxotrophy, *pab-2*⁺ must be required for biosynthesis of PABA. Analysis of the genomic sequence revealed the existence of a putative intron (88 bp). This falls within the 50-100 bp size range most typical of introns in *N. crassa* genes (Edelmann and Staben, 1994). Very good matches were found for the 5' and 3' splice site consensus sequences (Bruchez *et al.*, 1993a). A lariat sequence was found which was an excellent match with one of the two elements of the *pho-4* gene (Mann *et al.*, 1989).

The GC content of *pab-2*⁺ is 49%. This surprisingly low given that while non-coding regions of *Neurospora* are generally regarded as having 50% GC content. Protein coding sequences, in contrast, have an average GC content of 59% (Edelmann and Staben, 1994). Edelmann and Staben (1994) point out that the increase in GC content for sequence coding functional genes tends to be higher due to a 71.3% GC bias at the third codon position. Analysis of *pab-2*⁺ showed that only 63.4% of its codons had a G or C at the third position. *Neurospora* genes, on average, also show a preference (62.5%) for codons with at least two of the three positions filled by G or C; this preference is only 58.7% for the *pab-2*⁺ gene.

The putative gene also has several features which indicate it does indeed code for a product (Figure 7). A good match to the consensus Kozak sequence, typical of functional *Neurospora* genes, was found to encompass the ATG start codon (Bruchez et al, 1993a). A consensus transcriptional start site was also found just upstream at nucleotide -96. This sequence differed by only one nucleotide from that of *N. crassa*'s *pho-4* (Mann *et al.*, 1989). Potential TATA and CAAT boxes were also found starting at -129 and -176 respectively. TATA boxes and CAAT boxes are usually found at 30 bp and 75-88 bp, respectively, from the transcription start site in *N. crassa* (Bruchez *et al.*, 1993b). Although an in frame stop codon was found to terminate the gene, there was no evidence of a sequence coding for a polyA⁺ tail discovered in the genomic sequence.

The mRNA of *pab-2*⁺

Sequencing of the cDNA clone obtained from the NO₃ induced λ library (pPAB-cDNA), revealed that the intron, predicted from the genomic sequence, had indeed been spliced out. Although the clone obtained had an intact stop codon, it had neither a polyadenylation consensus sequence nor a polyA⁺ tail. Faced with the possibility that the clone could represent a transcript truncated at the 3' end, a second set of clones were obtained via PCR from a cDNA pool from the *N. crassa ro-2B* mutant (Figure 9). Analysis of these latter clones showed that the clone obtained from the NO₃ induced λ library had not been significantly truncated. The PCR clones both had polyA⁺ tails, but starting at different locations (+1109 and +1157). Neither of these clones possessed the

consensus polyA⁺ signal. The latter finding is not surprising since only a small percentage (~20%) of *Neurospora* genes appear to possess a match, within one nucleotide degeneracy, for the AATAAA polyadenylation signal sequence (Bruchez *et al.*, 1993b; Mann *et al.*, 1989).

More surprising than the lack of polyA⁺ signals, however, was the revelation that the intron first identified in the pPAB-cDNA clone, was differentially spliced. The alternatively spliced intron of the PCR clone pPAB-polyA6 was shorter (69 bp) than that of clones pPAB-cDNA or pPAB-polyA5 (88 bp) due to a different 3' splice site. According to Bruchez *et al.* (1993b), the newly identified 3' splice junction was an extremely rare form due to the presence of a guanine at the first position of the consensus sequence. The differential splicing also resulted in a frameshift, such that the downstream amino acid sequence of the two possible gene products was substantially different (cf Figure 7 and Figure 10). The two possible *pab-2*⁺ transcripts code for proteins 254 (minor product) or 276 (major product) amino acids in length. The predicted sizes of the two proteins are 28 143 Da and 30 430 Da, respectively. The size of *E. coli* PabC, by comparison, is 29 700 Da (Green *et al.*, 1992).

Examples of Differential Splicing in Fungi

Alternative splicing is a rare phenomenon in fungi, the mechanism and significance of which is not yet fully understood (Sachs, 1998). The hyphomycete *Tolyposcladium inflatum* differentially splices the RNA of its transposable element *restless*

using different 3' splice junctions (Kempken and Kuck, 1996). The splice products code for different proteins, but the functionality of these proteins remains to be determined. The *fox-2* gene of *N. crassa*, which encodes a multifunctional β -oxidation protein, is processed using multiple 3' splice sites (Fossa *et al.*, 1995). However, the physiological effects of this also remain unknown.

Differential splicing can also be accomplished by the selective removal of introns, rather than a change in splice junction within an intron. Birch *et al.* (1995) found that the *cbhI.1* and *cbhI.2* transcripts of *Phanerochaete chrysosporium* are spliced according to the type of cellulosic substrate present in the media; presumably this alters substrate specificity of the enzymes. This form of splicing also takes place in the *mt A-1* (mating type) and *asd-1* (ascus development) genes of *N. crassa* (Saupe *et al.*, 1996; Nelson *et al.*, 1997b). Although its significance is obscure, the splicing of *asd-1* appears to be efficient in vegetatively growing cells, but less efficient in perithecia.

The Preferred Transcript

Given that two possible mRNA species could result from transcription of *pab-2*⁺, experiments were conducted to determine if both products were, or were not, equally abundant. This determination was carried out using PCR with primers that amplified across the intronic regions. In case the form of the transcript was influenced by changing cellular conditions, cDNA stocks prepared from *N. crassa* grown in various conditions were used as templates. The results indicated that the most abundant transcript was the

rare 3' splice junction coding for the 30 430 Da protein. This was dubbed the "major" transcript, while the other species was denoted the "minor" transcript.

The absence of any PCR product in the *myc* cDNA library (Figure 11), likely represents a cloning bias during the packaging process, or a loss of the rare clones during amplification of the library, rather than a case of differential expression. This conclusion is based on the fact that the transcript was present in both the NO_3^- and Gln samples which are also prepared from mycelia. This supports the contention that *pab-2*⁺ must be expressed at low levels if its transcripts are poorly represented in cDNA libraries.

Sequence Comparison Between PAB-2 and Other Proteins: Assigning Function

Although Blast searches turned up no good matches ($\max P(N)=2.8 \times 10^{-4}$), a manual comparison of the major transcript gene product to *E. coli* PabC and *B. subtilis* PabC revealed 16% identity (79% similarity) and 21% identity (71% similarity) respectively (Figure 13). By way of comparison, the PabC enzymes of *E. coli* and *B. subtilis* share 27% sequence identity (71% similarity). The search also yielded a match to a hypothetical protein from *Schizosaccharomyces pombe* (e1251110). Alignment between this protein and PAB-2 showed that a region of high homology was indeed shared (Figure 13); the sequences were 26% identical (68% similar). It is possible, therefore, that these proteins share a common function. PAB-2 did not match any of the consensus sequences for PabA or PabB from other microorganisms (Brown *et al.*, 1996). The lack of strong identity between the fungal and bacterial genes is not altogether

Figure 13 - Amino acid alignment amongst *N. crassa pab-2*⁺ major gene product, *E. coli pabC*, the putative *Bacillus subtilis pabC* and a hypothetical protein (e1251110) from *Schizosaccharomyces pombe*. The alignment was performed using DNAMAN v2.71 optimal alignment algorithms based on Feng and Doolittle (1987) and Thompson *et al.* (1994). PAB-2 has 16% and 21% identity with PabC of *E. coli* and *B. subtilis* respectively. The identity between PAB-2 and the *S. pombe* protein is 26%. UbiC (chorismate synthase) can complement mutations in *pabC* in *E. coli*. This implies that the two enzymes should be similar; no homology, however, was found (Nichols and Green 1992). It was verified that PAB-2 was also not homologous to *E. coli* UbiC.

B_S_PROT	MIYVNGRYMEIEKDAVLSPEHIGCHILYIGIGVFETIER.LYIEGCEPELIDWHIERIERAIEIKDIQIT	59
E_C_PROT	MFLINCHKQES...LAVSDRATQDGGQFIITAR.VIDCKVSILISAHIORIODACQIRIMI	55
S_P_PROT	MEE.....S.....NIFETI.LYDGHFLFLFSHIQRMKASANSIGY	35
N_C_PROT	MAITTEAPILL...FTTILRYDIHJLLEQFVSIFIESSPFAIHYMLHYHQRQIIRKATTHPKW	56
B_S_PROT	EYTVSKHEILEMIDKI.....IKLNDIKDCNARVKLNISAGISCKGEVAQTYDKETIVLCFV	115
E_C_PROT	S.....CDEFWQLEQE.....MKUJIANEQNGVLKVVLSRCSGGRGYSTLNSGPVIRIISV	106
S_P_PROT	S.....WPGEQ.....YIENKUREAVQDTSMAK...VKWEISKAGDVTVOIIVPI	76
N_C_PROT	PAVSLLSGDSGLETHSSFISSISDEQRSHPNRIRVTVS..EKGEIGITISPARQVHSY	114
B_S_PROT	NQIKPESLA.....LQKEGKVLISIR..RNTPEGSFRLKSIH.....YLNMYAKRE	159
E_C_PROT	TAYPAHYDR.....URNEGITIALSPVKLGRNPHILAGIKILN.....RLEQVLIIRSH	153
S_P_PROT	QTIJEKAPYT.....LTIIDKQPSSTIE..KNPSCINKMTNRAI.....YIEAMNRND	119
N_C_PROT	ANFEPNSLFPQPGTNIPSLQIMGTSTPEHVLVDIORSKKSAYTHFKYTKRQIYDEARKRUG	174
B_S_PROT	IIGNDPRVIEGIFLIEDGAVAEGITISNV..FWRKGRQITYTPSLDTGIIIDGVJIRKEIENAKDI	218
E_C_PROT	LEQTNADENIMLDSEGWVTECCANL..EWRKGNVYVTHRLDQAGVNGIMRQFCIRLLAQS	212
S_P_PROT	AQYSKQDDVLLYNHQGFVTEATFENVAHFNQGWIT..TPSIKHQIISCTMRKNLIENG..S	176
N_C_PROT	IJAUTDRKNEVIIVSEDGEVMEGSIATPFWRDRGWV..THPVGEDE..GETEGDWGSQQ...	228
B_S_PROT	GIJELKTGRYELEALITVADEAWMTNSVLEIIEHTKIEHVNYSQSSEATSAIQILYKKEIK	278
E_C_PROT	SYQLIVEVQASLEESIQADEMVICNATIMPMVMVCACGHSFSSAT.....LYEYLAP	263
S_P_PROT	IHEDDKGLLQKDNINKGEQVLLFNSFRKVCCKGLTIQPEKACEL.....IKKKDSS	227
N_C_PROT	.NAGTTIRWALERGIAVEQVVRANSIIVDEEE...CWLSN..GGRG.....FYFGKVK	274
B_S_PROT	NMIHEKGGRAWRSTQ	293
E_C_PROT	..LQE.....RPN	269
S_P_PROT	...E.....KLS	231
N_C_PROT	LG	276

surprising given the phylogenetic separation of these organisms. An example of this is the relatively low overall identity of the bi-functional PabAB enzyme from *S. cerevisiae* to the bacterial PabA and PabB enzymes (Edman *et al.*, 1993).

It was interesting to discover that although the *pab-2⁺* product was similar to sequences in *S. pombe*, there was no obvious equivalent in *Saccharomyces cerevisiae*. A search of the SGD database for matches to PAB-2 returned a putative protein from *S. cerevisiae* (GenPept# 530346), however, alignment analysis (Appendix V) showed that the regions of homology shared between *S. cerevisiae* and *N. crassa* were not the same as those shared between the bacteria and *N. crassa*. This is surprising given that yeast PabAB contained the same key conserved sequences when compared to the corresponding bacterial genes (Edman *et al.*, 1993). Given this ambiguity, it is not possible to clearly state whether GenPept# 530346 is the equivalent of PAB-2.

As discussed in the introduction, anthranilate and its intermediates are very similar to the compounds of PABA biosynthesis. It seemed likely that PAB-2 (PabC) might share some homology with TrpE since these lyase enzymes share the common functions of aromatization via pyruvate elimination (Ye *et al.*, 1990). Unfortunately, *trpE* has not yet been sequenced in *Neurospora* so the corresponding enzymes from other organisms were compared *in lieu*. No significant homology was found by comparing sequences of PAB-2 with TrpE of *S. cerevisiae* or *E. coli*. It has been concluded, therefore, that the trend towards homology established among PabA and Trp(G)D and PabB and TrpE, does not extend to PAB-2 (the PabC equivalent) and TrpE. This lack of

lyase homology was also evident upon comparison of bacterial lyases (Rayl *et al.*, 1996; Nichols *et al.*, 1989).

In the absence of data relating structure to function in the PabC proteins, a secondary structure prediction was undertaken to determine if the the PabC and PAB-2 proteins might share similarities at the secondary structure level (Figure 14). Comparison of PabC from *B. subtilis* and *E. coli* shows that both these enzymes, starting at about amino acid 25, possess some β -sheet followed by two stretches of α -helix (each separated by more β -sheet). This same basic pattern can be seen in PAB-2, although the helical regions are quite sparse. All three enzymes share a block of helix near amino acid 150 and near the carboxy terminus. However, the PabC enzymes have three regions of helix, starting at, or just preceding amino acid 200, that PAB-2 does not have. The *S. pombe* hypothetical protein e1251110 also shares a similar structural pattern to the PabC enzymes.

The preceding secondary structure analysis strongly supports the relationship between the PabC of *E. coli* and *B. subtilis*. It offers only weak support, however, for the similarity between these enzymes and PAB-2. This could be explained by the fact that bacterial enzymes are more likely to resemble each other than those from a eukaryote. The similarity of the *S. pombe* protein to proteins of *N. crassa* and the bacteria implies these proteins are likely related.

Figure 14 - Secondary structure prediction and comparison amongst *N. crassa pab-2*⁺ (major gene product), *E. coli pabC*, the putative *Bacillus subtilis pabC*, and a hypothetical protein sequence (e1251110) from *Schizosaccharomyces pombe*. The height of lines along the Y-axis (indicated on the *N.crassa pab-2* figure) represent a prediction of Helix, Sheet, Turn or Coil; the relative placement of amino acids is indicated along the X-axis of each diagram (amino terminus is at far left side). Structure predictions were performed using SOPMA (Geourjon and Deléage, 1995).

Protein Motif Analysis

The PAB-2 sequence was analyzed using the MOTIF prosite database in an attempt to ascertain some evidence of a specific function. Four possible myristolation sites were identified at amino acids: 174..179, 225..230, 227..232, and 228..233. Myristolation is the addition of myristic acid (a rare fourteen carbon fatty acid) to an N-terminal glycine, which allows the modified protein to anchor to the cytosolic face of membranes (Alberts *et al.*, 1994). It is unlikely there are true myristolation sites in PAB-2 since the glycine residues are towards the carboxy terminus, meaning that proteolytic cleavage to expose them would likely destroy the enzyme. PAB-2 also contains nine potential protein kinase C, and eight casein kinase II phosphorylation sites. These potential protein modification sites are not necessarily significant since these short, flexible consensus sequences could easily turn up by chance. Furthermore, phosphorylation is a general control mechanism of cells, thus having a potentially phosphorylated protein gives no real clues as to enzyme function. Searches of the protein databases did not identify additional motifs or domains.

Green *et al.* (1992) found that PabC in *E. coli* bore limited similarity to transaminases. The authors concluded that this similarity was not related directly to function, but rather because all these proteins were capable of binding pyridoxal phosphate (Pxy) as a co-factor. Green *et al.* (1992) went on to propose a mechanism for PABA synthesis involving this co-factor. There is no strong consensus sequence that determines a pyridoxal binding domain; only a slight preference for a -Ser-X-X-Lys(Pxy)-

motif (Tanizawa, *et al.*, 1989). Green *et al.* (1992) suggested that the *E. coli* lysine 140 may be responsible for binding pyridoxal phosphate *in vivo*, even though the neighbouring serine is absent, because of shared identity with the Pxy binding region in *B. sphaericus* YM-1 D-alanine aminotransferase. *Neurospora* PAB-2, however, has no common identity with PabC in this region. Given that there were no BLAST matches for PAB-2 with transaminases, it would be necessary to assay for pyridoxal phosphate binding before claiming its use as a cofactor in *Neurospora* PABA biosynthesis.

It has not been possible to determine the active site of PAB-2 by identification of particular motifs or domains. Furthermore, there have been no studies of bacterial mutant PabC genes, or attempts to localize functions to specific protein regions. Given the limited data, it is only possible to speculate that the functional domains of these proteins are limited to regions of high homology. One such region is that spanned by amino acids 108 to 212 (Figure 13).

Analysis of the *pab-2* Mutant Allele

The *N. crassa* mutant *pab-2* was created through the use of X-rays (Drake, 1956). X-rays are a form of high energy, ionizing radiation that can damage DNA and thereby induce mutations during the repair process. Ionizing radiation creates highly reactive free-radicals like (OH•), and can cause breakage of hydrogen bonds, oxidization of double bonds and destruction of ring structures (Prescott *et al.*, 1990). DNA damage may take the form of either single strand or double strand breaks, or nucleotide base

modification (Weaver and Hedrick, 1992). This is in contrast to UV radiation which causes mutation by forming covalently linked thymine dimers (Prescott *et al.*, 1990). It is therefore possible to get chromosome breakage, rearrangement and point mutations from X-rays; however, exposures to low levels of X-rays most often result in point mutations (Russel, 1996). These types of mutations are evident upon sequencing of the *pab-2* mutant allele.

Compared to the wild-type, the mutant *N. crassa pab-2* allele (Figure 7) had several single base mutations that still coded for the same amino acids (i.e. silent mutations). Although these could be point mutations caused by X-rays, they are more likely due to strain differences (Oakridge vs. SL9). The more severe replacement and deletion mutations likely represent examples of strand breakage or severe base modifications requiring radical repair. The frameshift/transition at nucleotide 605 (Figure 7) likely resulted from this type of damage. This mutation not only alters the amino acid code downstream, but also eliminates a large portion of the carboxy terminus by the introduction of a premature stop codon. It is interesting to note that this mutation disrupts the region of highest homology between PAB-2 and PabC identified earlier. This supports the assertion that the active site is located near this area. Alternatively the active site lie further toward the carboxy terminus. It is also possible that the severely truncated PAB-2 does not maintain any semblance of the wild-type tertiary structure. Therefore any active site, even one upstream of the mutation, is rendered non-functional.

Gene Regulation

The fact that some of the *trp* enzymes are regulated by feedback inhibition by tryptophan in *E. coli* (Ito and Yanofsky, 1969) suggested the need for tests to determine if there was feedback inhibition by PABA on the *pab-2⁺* gene. The Northern hybridizations of *Neurospora* mRNA showed that the levels of *pab-2⁺* transcript did not vary with PABA content of the growth media (Figure 12). The lack of regulation of *pab-2⁺* is not altogether unexpected. The *thi-4* gene, responsible in part for the synthesis of the vitamin thiamine, is also unregulated in *Neurospora crassa* (Akiyama and Nakashima, 1996). This is in contrast to the feedback repression exhibited by the thiamine biosynthetic genes in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Maundrell, 1990; Nishimura *et al.*, 1992; Zurlinden and Schweingruber, 1992; Fankhauser and Schweingruber, 1994; Praekelt *et al.*, 1994).

The level of expression of *pab-2⁺* was noted to be quite low, requiring mRNA isolation to be reliably detected. Furthermore, representation of this transcript in the NO₃ induced phage library was minimal (1 clone/~250,000). Considering the available data, i.e. low representation in phage libraries and the strength of northern hybridization signals, it appears that *pab-2⁺* is a constitutively expressed, low abundance transcript. These results agree with results in *E. coli*, where the enzymes involved in PABA biosynthesis exist at extremely low levels in wild-type cells (Ye *et al.*, 1989).

Interpreting PABA Toxicity

High levels of PABA (37.5 mM - 25x that needed to support the auxotroph) appeared to be somewhat toxic to *Neurospora*. This agrees with the earlier results of Emerson and Cushing (1946) who found that 10-100 mM PABA significantly retarded hyphae growth in wild-type *N. crassa*; it is at these levels that sulfonamides also become effective antibiotics. Extremely high concentrations (500 mM) of the thiamine vitamin are also toxic to *Neurospora* (McColl, 1997). It should be noted that this amount of thiamine is approximately 1000x that needed to support a thiamine mutant.

PABA toxicity was first investigated in connection to sulfonamide requiring (*sfo*) *Neurospora* mutants generated by growth on selective media (Emerson and Cushing, 1946). The *sfo* mutants required sulfonamides for growth at 35°C, but not at 30°C. Interestingly, low concentrations (1×10^{-3} mM) of p-aminobenzoate are toxic to the *sfo* strain at 35°C. At lower temperatures, however, the mutant tolerates much higher levels (~1mM) of PABA in the growth media (Emerson, 1947). This phenomenon leads to the hypothesis that the *sfo* mutant overproduces PABA and thus requires sulfonamides to avoid the toxic effects by preventing its uptake by downstream (i.e. folate) pathways (Emerson, 1947, Zalkalar, 1948).

“Future Considerations”

The next step in the characterization of *pab-2*⁺ will be to verify its catalytic function using enzyme assays. It will also be necessary to determine which gene product, that derived from the major or minor transcript, represents the functional enzyme. Such assays will invariably require purified PAB-2. If, as in *E. coli*, *pab-2*⁺ is expressed at very low levels, it may prove necessary to amplify the levels of transcription through the use of a more powerful promoter. This would require a replacement of the existing *pab-2*⁺ with a modified construct. It would also be interesting to determine if over expression of *pab-2* can confer sulfonamide resistance in *N. crassa*. In addition, it would be useful to have a knock out mutant of *Neurospora* available, to facilitate incorporation of the new construct. A scheme for the construction of a knock-out vector to aid in the creation of such a mutant is presented in Appendix I.

It has been established that complementation of *pab-2* spheroplasts allowed for stringent selection of transformants (J. Vierula, personal communication). This finding suggests that the use of hygromycin selection, as done in the current study, is not strictly necessary. Avoiding the use of hygromycin is advantageous since this antibiotic is both expensive and relatively toxic. Therefore, the *pab-2*⁺ gene holds promise for use as a selectable marker. This increases the need for a knock-out strain if *pab-2*⁺ is to be a useful marker for experiments in which the “RIP” phenomenon is a consideration (Fincham, 1989).

V. SUMMARY

1. A functional *pab-2*⁺ gene was successfully isolated from a cosmid containing *N. crassa* genomic DNA. This gene is 900 bp from start to stop codon (including the intron). It is unusual in that it has a surprisingly low %GC content compared to the average *Neurospora* gene.
2. Subcloning of *pab-2*⁺ via RACE PCR from a cDNA pool revealed that this gene has one intron that is differentially spliced using alternative 3' splice junctions. The two possible transcripts code for proteins either 254 or 276 amino acids in length; these proteins have different carboxy termini.
3. PCR of several λ cDNA libraries showed that the larger transcript encoding the 276 aa protein was more prevalent. This was dubbed the "major" transcript, while the other species was denoted the "minor" transcript.
4. PAB-2 was putatively identified as a 4-amino-4-deoxychorismate lyase based on primary and secondary structural similarities with the PabC enzymes of *E. coli* and *B. subtilis*. This suggests that *pab-2*⁺ codes for a biosynthetic enzyme and that PABA biosynthesis occurs via a pathway similar to that in bacteria.

5. The *pab-2*⁺ gene is not subject to feedback inhibition by p-aminobenzoate (PABA).
The level of gene expression was found to be quite low (based on low representation in cDNA libraries).
6. The *pab-2* mutation was found to be caused by a frameshift/transition at nucleotide 605. This mutation eliminates an area of significant PabC homology and introduces a premature stop codon which severely truncates the carboxy terminus of the gene product.

Appendix II

Appendix III

Type of Sequence	Consensus Sequence
Transcription Start Sequence	TCATCANC
Kozak Sequence	C ₅₇ NNNC ₇₇ A ₈₁ (A ₄₄ /C ₄₃)T ₃ A ₉₉ T ₁₀₀ G ₉₉ G ₅₁ C ₅₃
5' Intron Splice Site	G ₅₁ ⊗ G ₉₉ T ₉₉ (A ₇₇ /G ₁₇)(A ₅₀ /C ₂₃)G ₉₄ (T ₇₆ /C ₁₅)
Lariat Sequence	(G ₄₅ /A ₃₇)C ₉₄ T ₉₄ (A ₄₈ /G ₄₀)A ₉₃ C ₈₂
3' Splice Site	G ₄ (A ₅₆ /T ₂₀)(T ₆₂ /C ₃₃)A ₁₀₀ G ₁₀₀ ⊗ G ₄₀
PolyA Signal	AATAA
Protein Kinase C phosphorylation site	[ST]-x-[RK]
Casein Kinase II phosphorylation site	[ST]-x-x-[DE]
Myristolation Site	G-[EDRKHPFYW]-x-x-[STAGCN]-P

The nucleotide consensus sequences were taken from Bruchez *et al.* (1993a & b). Nucleotides shown with strike-through indicate a conserved absence of that nucleotide. Where possible, percentage conservation values are shown. ~~⊗~~ indicates exactly where intron splicing occurs within a sequence.

Consensus sequences of proteins with $\square$ indicate any single amino acid of the type shown in brackets; -x- indicates any amino acid.

Appendix IV

Primer	Sequence (5' to 3')
PAB-1	GGG CAG TTC TGC TAA CG
PAB-2	AGT ACG GCG TGG CAA TAC
PAB-3	TTC GAG ACC GCT GTC ACC
PAB-5	TTA CGA CCC TCT CCT GCT T
PAB-6	GGA TCG CGT TGA CGG AT
PAB-7	GGA GTC TCT AAT CCA TCG AG
PAB-8	TAG CAC CGA ACG ACG AT
PAB-9	CTC ATC TCA AAG CAG GAG
PAB-10	TGA GCG TTA AGG GCA TG
PAB-11	GGA AGG AGG TGT TGA TTG
PAB-12	AGC GAT CTC ACC CAA ACC
PAB-13	CCC TCA CAA TGG CGA CCA
PAB-14	AAG GGA CTT GTG GTG CTG
PAB-15	GCG AGT TTG CCC GTA CAA
PAB-16	CCC GTA CAA CCT TTC CAA

Appendix V

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N. crassa      MATTTEAPLLLETTTLRYDPLLLPGFVSPTESSPEFAPLYMLPYHRDRILRAATHFKWPAVV
S. cerevisiae -----

N. crassa      SLLSGDSGLETLSSFISSLSDEQRSHPNRIRVTVSEKGELGITISPAQVVPSTANFFPN
S. cerevisiae -----KMINERQ--CYKMRVLVSKTGDIRIEAIPMPMEPIKLTTDY
                        .  *..      ..** **  *..  *  *  *  *

N. crassa      SLPQPGTNIPSLC--LMGTSTPFEVLVDTORSKKSAYTHFKTTKRQIYDEARKRHGIALT
S. cerevisiae DSVSTYFIKTMLNGFLIDSTINWDVVVSSEPLNASAFTSEKTTSRDHYARARVRMQTAIN
                        *  *.. ..  .* * ..  **.* ***** *  *  *  *  *..

N. crassa      DRK-----EVLIVSEEDGEVMEGSIATPYFWRDGRWVTPPVGEGEGETEGDWGSG
S. cerevisiae NLRGSEPTSSVSQCEILFSNKSGLMEGSITNVAVIQKD-----PNGS-KKYVTPRLATG
                        .  *..  *  *.....  .  *  *  *  *

N. crassa      GNAGTTRRWALERGIAVEQVVRANSLVDEEECWLSNGGRGFYFGKVKLG-
S. cerevisiae CLCGTMRHYLLRLGLIEGDIDIGSLTVGNEVLLFNGVMGCIKGTVKTKY
                        .** * .  *  * .  *..  *  *  *  *  *  *

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CLUSTAL multiple sequence alignment between *N. crassa pab-2* and a hypothetical protein (530 346) from *Saccharomyces cerevisiae* obtained from the yeast genome database.

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