Regulation of Paxilline Biosynthesis in Penicillium paxilli

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For Ally & Ian

My best friends and my inspiration.

Abstract

Production of the indole-diterpenoid paxilline was examined in the filamentous ascomycete *Penicillium paxilli.* Paxilline is a secondary metabolite, that is synthesised via a specific secondary metabolite biosynthetic pathway. The primary precursors of paxilline biosynthesis, mevalonate and isopentenyl pyrophosphate, are synthesised via the isoprenoid pathway and the paxilline biosynthetic pathway branches from isoprenoid biosynthesis after the synthesis of farnesyl pyrophosphate. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co) reductase is the rate limiting step of the isoprenoid biosynthesis. Genes for (*hmg*) and β-tubulin (*tub-2*), were isolated from a genomic DNA libray and characterised by DNA sequencing and RT-PCR. The steady state mRNA levels of *hmg* and *tub-2* were compared with genes isolated from the paxilline biosynthetic gene cluster, using a semi-quantitative RT-PCR gene expression assay. A distinct pattern of expression was identified for genes involved in the biosynthesis of paxilline. Increased expression of these genes occurs 36 h prior to the detection of paxilline in liquid culture.

P. paxilli physiology and paxilline production was analysed in liquid culture after the development of reproducible growth conditions that results in the formation of homogeneous loose hyphal fragments and detectable paxilline after 72 h. The morphology of *P. paxilli* in paxilline-inducing media was examined microscopically and key physiological markers, culture pH and biomass accumulation, were also analysed. Paxilline levels in both mycelia and culture supernatant were analysed with HPLC and TLC. This confirmed that paxilline is not released into the media until 144 h when large scale autolysis is observed. Initial experiments to examine paxilline production in cultures supplemented with a biological buffer suggest that phase switching between primary growth and secondary growth may be triggered by changes in ambient pH. The presence of alternative carbon sources also affected the rate of paxilline production and preliminary results indicate that biosynthesis of paxilline may be under carbon catabolite repression by glucose.

Levels of HMG CoA reductase are known to be regulated at many levels, including mRNA transcription, protein inactivation and protein degradation, in response to excess sterols. A number of putative sterol response elements (SRE), which control transcription of *hmg* in higher eukaryotes, where identified in the 5' UTR of *hmg* from *P. paxilli*. In higher eukaryotes, the extremely complex 5' UTR of *hmg* has been proposed as the site of regulation for biosynthesis of non-sterol end-products. This complexity appears to be conserved in the 5' UTR of *hmg* from *P. paxilli* and another filamentous fungus *Neotyphodium lolii* Lp19. Intronic sequences are spliced from the 5' UTR of both genes and there are additional intronic sequences present that could produce alternative transcripts. At least two different *hmg* transcripts were identified from *P. paxilli* with 5' RACE. The mechanism by which these alternative transcripts arise is unclear at present, but could involve alternative splicing of the 5' UTR intron or initiation of transcription from alternative start sites.

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1.1 Secondary metabolism

The phenomenon of secondary metabolite formation is of great importance to many fields of human endeavour. Secondary metabolites, also referred to as natural products or products arising from dispensable pathways, are products non-essential for the primary metabolism, survival and reproduction of the producing organism. The organism is able to exist and reproduce in the complete absence of secondary metabolic functions. Examples of secondary metabolites include antibiotics, antifungal, antitumor and anti-AIDS agents, plant growth factors, insecticides and herbicides. Many secondary metabolites are beneficial, but many toxic molecules can also be produced, including compounds that contaminate important human and agricultural stock food resources and products that act as virulence factors during pathogenesis of many plants. The effects on other species, caused by secondary metabolites are obvious, however, the benefits to the producing organism are not always apparent.

There can be a selective advantage from synthesising some secondary metabolites. Antibiotics and antifungal agents help the producing organism compete for resources, whilst toxins such as the trichothecene 4,15-diacetoxyscirpenol (DAS) from *Gibberella pulicaris* (Desjardins *et al.*, 1992) and dothistromin from *Dothistroma pini* (Shain & Franich, 1981) may be essential during fungal invasion of some host species. *Aspergillus* species, that produce the carcinogenic aflatoxins, appear to have no biological advantage over their atoxigenic counterparts. Evidence has shown that atoxigenic strains of *A. flavus* can out compete toxigenic strains in field trials as a biocontrol agent in both maize and cotton (Brown *et al.*, 1991; Cotty, 1994). However, almost all strains of *A. parasiticus* isolated to date, produce aflatoxins, and it appears that *A. parasiticus* may be able to out-compete *A. flavus* in peanut crops in Australia (Carter *et al.*, 1997). *Penicillium paxilli* strains, that lack the genes for paxilline biosynthesis, have identical growth rates and morphology to wild-type paxilline producing strains (Young *et al.*, 1998). Reasons for the prevalence of dispensable pathways amongst fungi is still unknown.

1.2 Biosynthetic pathways

Many of the natural products produced, and the biosynthetic pathways that have evolved to produce them, are highly complex. It has been estimated that on average, approximately ten to thirty different structural genes, representing enzymes that perform successive transformations to a specific substrate, can be required for the production of a single secondary metabolic product. The structure and function of secondary metabolites can be as complex as the pathways that produce them and many organisms synthesise more than one product. The streptomycetes alone produce approximately half the known microbial antibiotics (Bibb, 1996). For example, *Streptomyces coelicolor* A3(2) produces a number of antibiotics via linked pathways (Hopwood, 1988). The sophisticated pathways required for secondary metabolite production, can be branched, causing the production of a range of closely related products. Alternatively, a primary biosynthetic pathway can branch into a secondary pathway at a point where an intermediate of the primary biosynthetic pathway becomes the precursor of a secondary biosynthetic pathway (Drew & Demain, 1977).

1.3 Gene clusters

Genes for secondary metabolite biosynthesis are frequently found in a single cluster. For a time, the D.A. Hopwood quote "there are currently no proven exceptions to the generalisation that genes controlling successive steps in antibiotic biosynthesis are clustered" could be assumed for other secondary metabolites as well, although a number of exceptions do appear in the paper by Martin & Liras (1989). The prevalence of fungal metabolic gene clusters was reviewed by Keller & Hohn (1997). Genes required for both primary and secondary metabolic functions, are frequently grouped together. A cluster of three genes involved in nitrate utilisation was located on a single genomic fragment of *Aspergillus fumigatus* (Amaar & Moore, 1998). In some bacterial species, genes are found clustered on a plasmid and can even form an operon, such as the eight genes for lantibiotic lactocin S from *Lactobacillus sake* (Skaugen *et al.*, 1997). Plasmids are mobile genetic elements that can be transferred horizontally between species, therefore resistance and regulatory genes for the specific antibiotic are

frequently located adjacent to the biosynthetic gene cluster. If the transfer of structural genes were to occur without the concurrent transfer of a resistance mechanism, the recipient organism commits "suicide" as soon as it starts producing the antibiotic. The same argument can be applied to vertical transmission of secondary metabolite pathways. If a resistance mechanism is separated from the structural genes during sexual recombination and is not inherited, the effect will be the same. Therefore, strong natural selective forces exist to keep not only the genes for the resistance mechanism linked, but the structural and regulatory genes as well (Martin & Liras, 1989). The synthesis of some products could prove deleterious if the timing of biosynthesis is not regulated. Examples of resistance mechanisms include the gene for β-lactamase, which gives protection against the β-lactam antibiotics, and transporter pumps, which export toxins. A gene recently isolated from the *Fusarium sporotrichioides* trichothecene gene cluster, *tri12,* has homology to toxin efflux pumps. Tri12 may be involved in protecting the producing organism from trichothecenes (Alexander *et al.*, 1999). In the rice blast fungus *Magnaporthe grisea*, an ABC transporter was isolated and shown to give protection against plant defence compounds, but did not play a role in transporting fungal compounds (Urban *et al.*, 1999). In some instances, the resistance mechanism alone can be transmissible, by way of a mobile gene cassette called an integron. Recombination takes place at a 59 bp DNA element associated with the integron (Stokes *et al.*, 1997).

Clustering of these secondary metabolite biosynthetic genes is extremely useful during the isolation these pathway (Kleinkauf & von Dohren, 1990). Once one gene has been identified, the other genes can usually been found on the same section of DNA (Gaisser *et al.*, 1997; Hohn *et al.*, 1993a; Tudzynski *et al.*, 1999). In fungi, gene clusters can be located on dispensable chromosomes, such as the cluster of *Nectaria haematococca* genes involved in pathogenicity to peas. The presence of transposable elements adjacent to the cluster has lead to the hypothesis that horizontal transfer of these 'pathogenicity islands' has occurred (Kistler *et al.*, 1999). In other cases, gene clusters have been located on non-dispensable chromosomes, which appear to contain certain regions that are dispensable. In *P. paxilli,* strains that contain large deletions of chromosome Va, encompassing the paxilline biosynthetic cluster, have normal growth and morphology (Young *et al.*, 1998). Conserved hexanucleotide sequences in *P. chrysogenum* mutants, frequently flank deletions in the penicillin biosynthetic gene

cluster (Fierro *et al.*, 1996). Conserved repeat sequences can increase the likelihood of a recombination event and could be a mechanism for the transfer of genetic material.

1.4 Evolution

If the products of secondary metabolism are potentially auto-toxic, there is a selective advantage for grouping resistance and regulatory mechanism with the biosynthetic genes. However, the clustering of biosynthetic genes that synthesise products of unknown function, is less well understood. Many hypotheses have been proposed to explain the clustering of secondary metabolite genes. The first theory, 'that genetic transfer promotes clustering', refers to the relatedness of fungal and prokaryote Isopenicillin N synthetase genes, cited in a number of examples by Keller & Hohn (1997). There is also similarity between genes involved in phenazine and polyketide biosynthetic pathways, which suggests that gene transfer between secondary metabolite producing species, has influenced the evolution of these pathways as well (Vining, 1992). The hypothesis, that horizontal transfer of pathway genes has occurred (and presumably still occurs) and confers a selective advantage, results in the selection of pathways that become clustered in a single region of a genome. Another theory predicts that the regulation of many biosynthetic pathways requires the structural genes to be clustered, so that a precise cascade of expression can be achieved and that chromatin structures may be involved (Cavalli & Thoma, 1993). This becomes increasingly important when a resistance mechanism is required prior to the production of potentially auto-toxic antibiotics.

Maplestone *et al.* (1992) and Stone & Williams (1992) presented an alternative hypothesis on the evolution of secondary metabolites. Clustering of biosynthetic genes and the products of biosynthesis must have a selective advantage. They proposed a mechanism for the evolution of secondary metabolite biosynthetic pathways. A gene for a primary metabolic enzyme duplicates, mutates, and becomes capable of catalysing the conversion of an intermediate product into a novel product. If this new product is beneficial to the producing organism, the mutation will be selected for. Subsequent duplication and further random mutation in the initially mutated gene may result in an

enzyme capable of converting the first novel product into a second product of increased benefit to the producing enzyme. Repeated gene duplications may be of no additional benefit or of adverse effect and that lineage would not be selected for, but some could be beneficial and the process could continue to give rise to the gene clusters present today. This would explain the sequentially ordered clusters we see in some secondary metabolic pathways (Martin & Liras, 1989). In the paxilline biosynthetic cluster from *P. paxilli*, there are two sets of genes with similar functions located adjacent to each other. *PaxP1* and *paxP2,* which have homology to P450 monooxygenases, and *paxR1* and *paxR2,* which have regions of homology to $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motifs (Young *et al.*, 2000). Stone & Williams (1992) also review the work of Marsh *et al.* (1989) that examines homology between two gene products of the propionic acid fermentation pathway in *Propionibacterium shermanii,* a methyl-malonyl-CoA mutase, and a transcarboxylase. A method for testing this theory is also discussed and examines the ability of sequential intermediates to bind to the specific receptor in the target organism. According to the "function-evolution" hypothesis, researchers would see increasing binding affinity with the subsequent pathway intermediates. Further evidence would be seen, as a high degree of sequence homology between genes of a single biosynthetic cluster.

A final theory on the evolution of secondary metabolites, specifically antibiotics, proposes that antibiotics were prebiotic effector molecules. That is, they were able to catalyse the synthesis of, and stabilise the structures of, such macromolecules as DNA and proteins. This would explain why modern antibiotics are able to bind to these structures and cause inhibition (Davies, 1990). However, once ribosomal and protein based synthesis of macromolecules had evolved, these prebiotic effector molecules would become redundant and evolve new functions to justify the evolution of their modern biosynthetic pathways. This, Stone & Williams (1992) argue, provides further support for their function-evolution hypothesis. Interesting speculations could be made with respect to the peptide toxins discussed by Kleinkauf & von Dohren (1993) that are synthesised via an RNA-independent, non-ribosomal process. Although the protein synthetases that catalyse the formation of the peptide toxins are themselves the product of standard ribosomal function.

1.5 Regulation

Regulation of gene expression can occur at many levels. Active repression or promotion of transcriptional machinery by regulatory proteins can affect the level of mRNA transcription. Post-transcriptional regulation can affect mRNA stability and splicing. Once a protein has been translated, it can be transported to different organelles, stored in an inactive form, phosphorylated or rapidly degraded (Day & Tuite, 1998). However, gene regulation in filamentous fungal is usually at the level of transcription (Gurr *et al.*, 1987). Biosynthesis of secondary metabolites is frequently seen after the cessation of primary biomass accumulation. Environmental cues often trigger the switch from primary to secondary metabolism, through global regulators. With respect to secondary metabolite gene cluster, there are frequently two levels of regulation, the first by environmental global regulators and the second by pathway-specific regulators (Keller & Hohn, 1997).

1.5.1 Nitrogen

Availability of a preferred nitrogen source regulates many different pathways in filamentous fungi, including genes for the assimilation of alternative nitrogen sources. The best examples of nitrogen global regulators are AREA from *A. nidulans* and NIT2 from *Neurospora crassa.* These positive-acting transcription factors have a Cys_2/Cys_2 zinc finger motif and are members of the GATA family of transcription factors (Marzluf, 1993). These proteins bind to GATA motifs in the promoters of nitrogen regulated genes with a DNA binding domain located next to the zinc finger (Wilson & Arst Jr., 1998). Binding sites for the homologous *A. fumigatus* nitrogen regulator AREA, are found in the promoters of the nitrate assimilation gene cluster (Amaar & Moore, 1998). High levels of nitrate are correlated with decreased aflatoxin production in *A. parasiticus* (Liu & Chu, 1998). AREA was shown to bind to the intergenic region of *aflR* and *aflJ* in *A. parasiticus,* suggesting that suppression of aflatoxin biosynthesis by nitrate may be abrogated by binding of AREA (Chang *et al.*, 2000). In *Penicillium chrysogenum*, the homologue of AREA and NIT2, NRE, regulates both nitrate assimilation and penicillin biosynthesis (Haas & Marzluf, 1995b) although there is no

evidence for similar regulation of penicillin biosynthesis in *A. nidulans* (Brakhage, 1998). A second global regulator protein, NMR, isolated from *N. crassa,* is also required for nitrogen metabolite repression. Analysis of protein-protein interactions showed that while NIT2 binds the promoter of nitrogen regulated genes, NMR binds to NIT2 (Pan *et al.*, 1997). A homologue of the *nmr-1* gene was recently isolated from *A. nidulans* (Andrianopoulos *et al.*, 1998), indicating that a two-component system for global nitrogen regulation may exist in other fungi as well.

1.5.2 Carbon

Carbon catabolite repression (CCR) also regulates many genes via global regulatory proteins. CREA, from *A. nidulans* is a negative-acting regulator that represses gene expression in the presence of a preferred carbon source. CREA binds to the sequence motif 5'-SYGGRG-3' in the promoters of repressed genes (Panozzo *et al.*, 1998). In *Saccharomyces cerevisiae*, the equivalent protein Mig1, binds the consensus sequence 5'-SYGGGG-3' with a Cys_2/His_2 zinc finger (Gancedo, 1998). In *N. crassa*, the CREA homologue is called Cre-1 (de la Serna *et al.*, 1999). Positive-acting carbon source regulatory proteins in yeast include the Hap2/3/4/5 complex, which binds to 5'-CCAAT-3' promoter motifs, Gal4 and Mal63. Gal4 and Mal63 activate genes involved in the catabolism of galactose and maltose and bind pairs of CG triplets separated by 11 and 9 nucleotides respectively (Gancedo, 1998). The presence of glucose often imposes CCR on secondary metabolite gene clusters (Drew & Demain, 1977), including the penicillin biosynthetic gene cluster from *P. chrysogenum* (Gutierrez *et al.*, 1999). Expression of penicillin biosynthetic genes is also under CCR control, as is the level of isopenicillin N synthetase transcript present under repressing carbon sources (Espeso & Peñalva, 1992). However, this repression is independent of the global carbon source regulator CREA (Peñalva *et al.*, 1998). Glucose is not always the preferred carbon source, even though filamentous fungi are only able to take up monosaccharides (Nielsen, 1992). If disaccharides are present, the enzyme invertase is released. Breakdown of invertase can lead to a temporary increase in nitrogen once disaccharides have been converted to monosaccharides. Sugar alcohols like mannitol, which functions as carbon storage molecules in the phloem of plants, can be the preferred carbon source of saprophytic or endophytic fungi. For example, substitution

of mannitol for glycerol resulted in 4-fold increase of the antibiotic pneumocandins A_0 by the fungus *Zalerion arboricola* in liquid culture (Tkacz *et al.*, 1993).

1.5.3 pH

Ambient pH is another environmental trigger that can affect gene expression via a global regulator protein. PacC, found in *A. nidulans*, *A. niger* and *P. chrysogenum* has three zinc finger motifs of the Cys_2/His_2 type and is known to increase transcription of genes under alkaline conditions (Espeso & Peñalva, 1996; Then Bergh & Brakhage, 1998). Penicillin biosynthetic genes in both *A. nidulans* and *P. chrysogenum*, have binding sites for PacC (Espeso & Peñalva, 1996; Suarez & Peñalva, 1996). The signalling cascade that induces *pacC* transcription, involves the *pal* genes in *A. nidulans* (Negrete-Urtasun *et al.*, 1999). Homologous genes have also been identified in *S. cerevisiae* (Denison *et al.*, 1998).

1.5.4 Pathway-specific regulators

Secondary metabolite pathways can also be regulated in a pathway-specific manner, usually by a product encoded within the cluster itself (Keller & Hohn, 1997). A good example of this is the regulation of aflatoxin and sterigmatocystin biosynthesis by *aflR* (previously *apa2* from *A. parasiticus* and *afl2* from *A. flavus*) which belongs to the $Zn(II)_2Cys_6$ binuclear cluster Gal4 family of transcription factors (Woloshuk *et al.*, 1994). Induced expression of *A. flavus aflR*, has been shown to initiate transcription of sterigmatocystin biosynthetic genes in *A. nidulans* grown under conditions that normally suppress toxin biosynthesis (Yu *et al.*, 1996). ALCR, the pathway specific regulator of the ethanol utilisation pathway in *A. nidulans,* is also a $Zn(II)_2Cys_6$ binuclear cluster transcription factor. Transcription of *alcR*, is repressed by CREA in the presence of a preferred carbon source (Panozzo *et al.*, 1998). The putative pathway regulators (*paxR1* and *paxR2*) of the paxilline biosynthetic pathway in *P. paxilli* also have homology to the $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motifs (Young *et al.*, 2000). In *Streptomyces griseus*, expression of streptomycin biosynthetic genes is controlled by *strR*, which is in turn controlled by the global regulator A-factor (Ohnishi

et al., 1999). A regulatory gene has also been isolated from the trichothecene biosynthetic pathway in *F. sporotrichioides*. *Tri6* has been shown to be temporally expressed in coincidence with trichothecene structural biosynthetic genes, and Tri6$^-$ mutants are unable to produce trichothecene, even when supplied with six different trichothecene intermediates. The transcription level of structural genes *tri4* and *tri5* was greatly reduced in the Tri6$^-$ mutant. The 217 residue protein specified by *tri6* was found to have regions of homology with the Cys_2/His_2 group of zinc finger proteins (Procter *et al.*, 1995). In filamentous fungi, the $Zn(II)_2Cys_6$ binuclear cluster group of transcription factors are extremely common, but not exclusively of this type (Schjerling & Holmberg, 1996). For example, PacC contains three Cys_2/His_2 type zinc finger domains (Tilburn *et al.*, 1995) and the nitrogen responsive regulatory proteins NIT2 from *N. crassa* and AREA from *Aspergillus* possess zinc finger motifs of the Cys_2/Cys_2 type (Marzluf, 1993).

1.5.5 Post transcriptional regulation of gene expression

Gene expression in filamentous fungi is generally regulated at the level of transcription, however, heterogeneity in the 5' UTR makes many post-transcriptional and post-translational regulatory mechanisms possible. The inclusion of certain sequences in the 5' UTR could alter mRNA stability. Translation initiation from alternate ATG codons could result in premature termination of translation, or the inclusion of specific sequences for the targeting of proteins to different cellular organelles. Multiple transcripts are produced from the arginase gene in *N. crassa*. One appears to be regulated by a basal promoter and a second transcript is produced in an arginine-dependent manner (Marathe *et al.*, 1998). Translation of the two transcripts is initiated from different ATG codons in each case. The genes for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), found in other species also produce multiple transcripts. Sixteen different forms of *hmg* mRNA were detected in the UT-1 cell line from Chinese hamsters. The multiple messages, which differ in their 5' UTR, are transcribed from a single gene at 8 different transcription initiation sites. Nine ATG codons are present in the genomic copy of this gene (Reynolds *et al.*, 1985). Primer extension also revealed heterogeneity in the 5' UTR of the *A. nidulans* isopenicillin N

synthase gene (Perez-Esteban *et al.*, 1993). Introns in the 5' UTR of transcribed genes are rare, but when present, they appear to play a role in the regulation of gene expression. Multiple introns and alternative splicing can determine the choice of ATG codon for translation initiation as is the case in the *hmg* gene of Chinese hamster (Reynolds *et al.*, 1985). In the human tenascin-C gene, distinct regions of an untranslated exon were shown to affect transcription (Gherzi *et al.*, 1995). Alternatively spliced messages from the rat estrogen receptor gene are found in different tissues (Hirata *et al.*, 1996). Sometimes, the alternative products are found in different ratios as is the case for the human α2 (VI) collagen gene (Saitta *et al.*, 1992), or in equal amounts in all tissues like the HMG CoA synthase gene from both humans and Chinese hamster (Gil *et al.*, 1987). The presence of alternative splicing mechanisms in the 5' UTR of genes is often highly conserved between homologous genes in different species, indicating an important role in regulation. A 5' UTR exon from the rat *Fgf-1* gene was used to isolate the homologous gene in mouse (Hackshaw *et al.*, 1996).

1.6 Growth of filamentous fungi in liquid culture

Reproducible physiology of filamentous fungi in liquid culture is notoriously hard to achieve. However, as a huge number of commercially important products can be isolated from fungi, a great deal of research has been directed at solving this problem. Some products, such as antibiotics are only produced under certain physiological or developmental conditions. Biosynthesis of primary products, such as citric acid has been easier to optimise. As discussed above, carbon, nitrogen and pH can affect the type and rate at which secondary metabolites are produced. However, another factor that relates directly to filamentous fungi in liquid cultures, is the availability of oxygen. Two factors that affect the availability of oxygen are culture aeration and culture morphology. Essentially there are two extreme morphologies that can appear in liquid cultures, discrete circular pellets, which are hollow (Clark, 1962) and homogeneous loose mycelia. Studies of culture kinetics show that as the diameter of mycelial pellets decreases, culture physiology resembles that seen in loose hyphal fragments and vice versa. Mycelial pellets produce a mass transfer gradient across the radius, of nutrients and oxygen into the pellet and of waste products and secreted metabolites out of the

pellet (Prosser & Tough, 1991). Using a miniature oxygen electrode, the concentration of dissolved oxygen was shown to exist in a gradient across the radius of mycelial pellets (Huang & Bungay, 1973). Prosser & Tough (1991) list a number of factors that can affect pellet formation including agitation and shear, growth medium composition, culture viscosity, inoculum concentration and type, specific growth rate, aeration, surfactants, pH and suspended solids. At higher shake speeds, mycelia are sheared from the outer surface of the pellet, reducing the maximum diameter of each individual pellet (Cui *et al.*, 1998). Larger culture volumes also decrease the pellet diameter presumably by increasing pellet interaction and shear effect. Mycelia on the outer surface of the pellet have greater access to nutrients and oxygen, while mycelia closer to the centre face a build up of waste products. Therefore, individual hyphae in pelleted cultures can have different physiological environments. Potentially, mycelia toward the centre of the pellets would cease primary growth much faster. With no access to nutrients and oxygen, autolysis occurs in the centre and the pellets become hollow (Cui *et al.*, 1998). However, in large-scale continuous cultures, pellets are frequently preferred, as loose mycelia can clog outlet lines and stirrers (Moreira *et al.*, 1996).

Loose filamentous hyphae produce very viscous cultures that need increased aeration to provide adequate oxygen, although all hyphae are in a homogeneous environment. The oxygen concentration in cultures of *Streptomyces parvulus*, was shown to affect the amount and type of secondary metabolites formed (Kaiser *et al.*, 1994). Pellet morphology, and thus oxygenation, has also been shown to affect penicillin biosynthesis in cultures of *A. nidulans*, with pelleted cultures producing no penicillin (Moore & Bushell, 1997). Under normal growth conditions, many natural products are only produced when the secondary growth phase or idiophase has commenced. However, the induction of secondary metabolism in liquid culture can be manipulated in numerous species with media supplements. Studies have revealed that different carbon sources can alter the growth kinetics of a culture. By growing cultures in optimal conditions, Liao *et al.* (1995) showed that a sufficient biomass is produced during trophophase to support secondary metabolite production after the switch to idiophase. Growth in sub-optimal conditions resulted in overlapping biomass accumulation and secondary metabolism, with lower levels of natural product biosynthesis.

1.7 Secondary metabolite structures

1.7.1 Peptides

Fungi are known to produce a range of peptidyl secondary metabolites that are toxic to plants, insects and nematodes. The interesting aspect of these products is the way in which they are produced. Biosynthesis is via a nonribosomal peptide system that completely bypasses the need for mRNA and tRNA and is catalysed by a single multi-functional synthetase enzyme, often encoded by large intronless genes (Kleinkauf & von Dohren, 1993). For example the proteins Hts-1 and Hts-2, involved in the synthesis of HC-toxin in *Cochliobolus carbonum,* were found to be part of a single protein. This single gene locus, designated *hts* contained no introns (Walton *et al.*, 1993). Sometimes further modifications, such as epimerisation or N-methylations, are required to produce the mature product. The genes encoding proteins that perform these modifications are often clustered with the peptide synthetase gene.

1.7.2 β-lactam antibiotics

Of the more common peptidyl secondary metabolites, the β-lactam antibiotics are the most widely recognised. Much work has gone into the elucidation and optimisation of the biosynthetic pathways that produce penicillins and cephalosporins (Peñalva *et al.*, 1998). The mode of action of the β-lactam antibiotics involves the inhibition of cell wall formation in Gram positive bacteria (Chopra *et al.*, 1998). Resistance to β-lactam antibiotics can be gained by expression of β-lactamase, which breaks the central lactam ring. This type of resistance can be thought of as aggressive, as opposed to passive resistance mechanisms that bypass the site of action of the attacking antibiotic. One of the initial steps in the biosynthesis of penicillin, is the conversion of the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), into isopenicillin N (IPN), by the enzyme isopenicillin N synthetase (IPNS). Espeso & Peñalva (1992) showed that in the presence of repressing sugars, that is sugars that induce the carbon catabolite repressed state, the amount of IPNS gene transcript was noticeably decreased. Further work showed that the *ipnS* promoter was predominantly regulated by three *cis*-acting negative

elements, one of which has a role in sucrose repression, that effect the levels of basal transcription (Perez-Esteban *et al.*, 1993). In *A. nidulans,* the gene for isopenicillin N synthase is called *ipnA*, the promoter of which possesses three binding sites for the PacC zinc-finger, transcription factor. Interestingly, one of the controlling factors that regulate the activity of *ipnA* in *A. nidulans,* is the pH of the environment. Thus, *ipnA* is an alkaline-expressed gene (Espeso & Peñalva, 1996).

1.7.3 Polyketides

Another class of secondary metabolites is the polyketides. These include the mycotoxins sterigmatocystin and aflatoxins B1 and B2 of *Aspergillus* species, a number of antibiotics, including actinorhodin, granaticin, oxytetracyclin, frenolicin, and tetracenomycin, immunosuppressants, and spore pigments, including a grey pigment from *Streptomyces coelicolor* A3(2) that is under the control of the *whiE* gene cluster (Yu & Hopwood, 1995). Polyketide biosynthesis is very similar to fatty acid synthesis, such that many structural similarities exist between the polyketide synthase (PKS) and fatty acid synthase (FAS) complexes. There are two classes of PKS and FAS complexes. Type I synthases (bacterial and fungal PKS and vertebrate FAS) are single large proteins with multiple catalytic domains and are divided into two subtypes. One type is transcribed from a single gene and the single product performs all the rounds of polyketide elongation. A second type, known as a modular PKS, is translated from several transcripts, resulting in a set of synthase units. A single synthase unit is proposed to catalyse a single round of polyketide chain elongation. (Yang *et al.*, 1996). For example the biosynthesis of the polyketide erythromycin is controlled by genes arranged as a set of six units, each unit responsible for a single round of polyketide elongation (Donadio *et al.*, 1991). Type II synthases (plant and bacterial FAS and PKS) are complexes of several proteins, with one or two functions per protein.

There appears to be a high degree of sequence homology between genes of the polyketide biosynthetic pathways, especially genes coding for polyketide synthases. In fact, probes designed on the basis of previously sequenced polyketide synthase genes are frequently used to isolate genes in the uncharacterised polyketide biosynthetic pathways of different species. The *actI* gene locus of the modular polyketide synthase

cluster, and the *actIII* polyketide reductase gene, both from the actinorhodin biosynthetic pathway in *Streptomyces coelicolor* A3(2), have been used singularly and in tandem to isolate a number of polyketide synthases from a range of species. Using an *actI* probe, the griseusin polyketide synthase gene cluster was isolated from *S. griseus* (Yu *et al.*, 1994) and polyketide synthase genes from the glycopeptide antibiotic ardacin biosynthetic gene cluster of *Kibdelosporangium aridum* (Piecq *et al.*, 1994). In combination, *actI* and *actIII* probes have been used to isolate polyketide synthase genes from the daunomycin-producing *Streptomyces sp.* strain C5 (Ye *et al.*, 1994) and the monensin producer *S. cinnamonensis* (Arrowsmith *et al.*, 1992).

1.7.4 Aflatoxin

The polyketides sterigmatocystin and aflatoxins B1 and B2 are three of the most potent carcinogenic toxins produced by fungi. Sterigmatocystin, the precursor of aflatoxins B1 and B2 is converted into aflatoxin in *A. flavus, A. nomius* and *A. parasiticus* via the pathways: sterigmatocystin (ST) → → O-methyl-sterigmatocystin (OMST) → → Aflatoxin B1 (AFB1); and dihydrosterigmatocystin (DHST) → → dihydro-O-methylsterigmatocystin (DHOMST) → → Aflatoxin B2 (AFB2) (Bhatnagar *et al.*, 1991). The biosynthetic pathway stops at ST in *A. nidulans* and many other *Aspergillus* species (Trail *et al.*, 1995). In *A. nidulans*, a FAS gene homologue was found linked to the ST biosynthetic cluster. Mutational analysis showed that this gene, in addition to a PKS, is required for ST biosynthesis. The FAS gene is specific for secondary metabolism and primary requirements are met by a second unlinked FAS gene (Brown *et al.*, 1996b). In *P. paxilli*, another example of gene duplication is seen. A copy of the gene for geranylgeranyl pyrophosphate (GGPP) synthase (*paxG*) was found within the paxilline biosynthetic gene cluster. A second, unlinked copy (*ggs-1*) fulfils the requirements of primary metabolism and is unable to complement *paxG* deletion mutants (Young *et al.*, 2000). The biosynthetic genes for AF and ST biosynthesis are clustered and the order of genes within the cluster often corresponds to the order of enzymatic steps. However, the sequential ordering of genes is more conserved in the AF pathway than the ST pathway (Brown *et al.*, 1996a; Woloshuk & Prieto, 1998). Gene expression is regulated by a pathway-specific, $Zn(II)_2Cys_6$ binuclear cluster, transcription factor designated AFLR (Woloshuk *et al.*, 1994)

1.7.5 Terpenes

Terpenes or terpenoids are another large group of secondary metabolites synthesised by a variety of organisms, particularly plants. The range of structures includes, monoterpenes, diterpenes and triterpenes, cyclic and noncyclic forms. Specific structural moieties can be included, such as indole-diterpenoids lolitrem B and paxilline, produced by the endophytic filamentous fungi *Neotyphodium lolii* (previously *Acremonium lolii* and *A. loliae* (Glenn *et al.*, 1996)). The functions of this class of metabolite are wide-ranging, and their synthesis involves both primary and secondary metabolic pathways. A number of important primary metabolites are synthesised via the isoprene pathway, including the steroid and retinoid hormones, insect juvenile hormones, and two important plant hormones, gibberellic acid and abscissic acid (Moore, 1990). The filamentous fungus *Gibberella fujikuroi* also produces the plant hormone gibberellic acid (GA) during secondary metabolism. The gibberellins are tetracyclic diterpenes that are synthesised via the isoprenoid biosynthetic pathway. The first committed step specific for GA biosynthesis is the cyclisation of GGPP by the enzyme copalyl diphosphate synthase. The gene, *cps* was recently cloned, and Northern analysis confirmed that a dramatic increase in expression of *cps* is associated with the onset of GA production (Tudzynski *et al.*, 1998). The primary isoprenoid biosynthetic genes for HMGR (Woitek *et al.*, 1997), GGPP synthase (Homann *et al.*, 1996) and farnesylpyrophosphate (FPP) synthase (Mende *et al.*, 1997) were also cloned from *G. fujikuroi.* Expression levels of the genes for HMGR and GGPPS were not affected by glucose or ammonia, while expression of the gene for FPPS was unaffected by light regimes.

Many of the terpene structures are toxic. An inhibitor of acyl CoA cholesterol acyltransferase was isolated from the liver and identified as a novel pentacyclic triterpene ester. It is hypothesised that the source of this inhibitor was plant triterpenes in the diet (Tabas *et al.*, 1990). Another terpene, the monoterpene d-limonene, inhibits small G proteins, thus affecting cell proliferation (Crowell *et al.*, 1995). Toxic terpenes are presumed to effect the cell membranes of microorganisms. Along with a vast range of other cyclic hydrocarbons, the lipophilic terpenes are capable of penetrating the lipid bilayer of biological membranes. Studies investigating this phenomenon reported that the membrane was seen to swell and have increased fluidity, which allowed for

uncontrolled flux of primarily protons across the membrane (Sikkema *et al.*, 1994). Cyclic monoterpenes can also cause damage to skin, as proliferation and survival of epidermal keratinocytes is greatly reduced by the addition of monocyclic terpenes, in a dose-dependent manner (Kitahara *et al.*, 1993).

The sesquiterpenoid alcohols are another group of toxic secondary metabolites. These toxins are produced in several genera of fungi, as well as by the higher plant species *Baccharis*. A group of these toxins, the trichothecenes, are synthesised via the cyclisation of the isoprenoid pathway intermediate FPP, into trichodiene, by the enzyme trichodiene synthase. Mutants of the fungal species *F. sporotrichioides,* that are deficient in the production of the trichothecene T-2 toxin, have been analysed by complementation studies with cosmids isolated from a *F. sporotrichioides* genomic DNA library. These studies show that a number of genes involved in the biosynthesis of T-2 toxin, are clustered in a single 30kb stretch of DNA upstream of a previously identified gene *tox5* (Hohn *et al.*, 1993a). The gene *tox5* encodes a trichodiene synthase and is now called *tri5* (Hohn & Desjardins, 1992). To further characterise the regulation of *tox5,* the promoter regions of *tox5* from high and low trichothecene-producing strains were compared. Promoters from the high-producing strains were found to contain a 42 nt tandem repeat. Low-producing strains only carried a single copy of this repeat in their promoters. However, once transformed into reporter constructs, expression of *tox5* was found to be independent of the 42 nt repeat (Hohn *et al.*, 1993b). Another gene from the trichothecene biosynthetic pathway, *tri4*, has recently been isolated from *F. sporotrichioides*. The predicted amino acid sequence of *tri4* shows significant homology to cytochrome P450 monooxygenases. Because Tri4$^-$ mutants only accumulate the unoxygenated intermediate trichodiene, *tri4* has been placed in a new cytochrome P450 subfamily, CYP58. Tri4$^-$ mutants were however, able to produce trichothecenes when supplied with oxygenated pathway intermediates, meaning it is unlikely to be involved in regulation of the pathway (Hohn *et al.*, 1995). The catalytic activities of cytochrome P450s are utilised in a vast range of metabolic capacities, and are thought to play an important role in the synthesis of many secondary metabolites, including isoflavonoids, terpenoids and alkaloids (Schuler, 1996). Similarities are also seen between the cytochrome P450s of different fungal species. For example, the eburicol 14-α-demethylase ($P450_{14DM}$) was isolated from *Penicillium italicum* by

heterologous hybridisation with the corresponding gene from *Candida tropicalis*. The $P450_{14DM}$ proteins from yeast and fungi are the targets of antimycotics and fungicides (van Nistelrooy *et al.*, 1996).

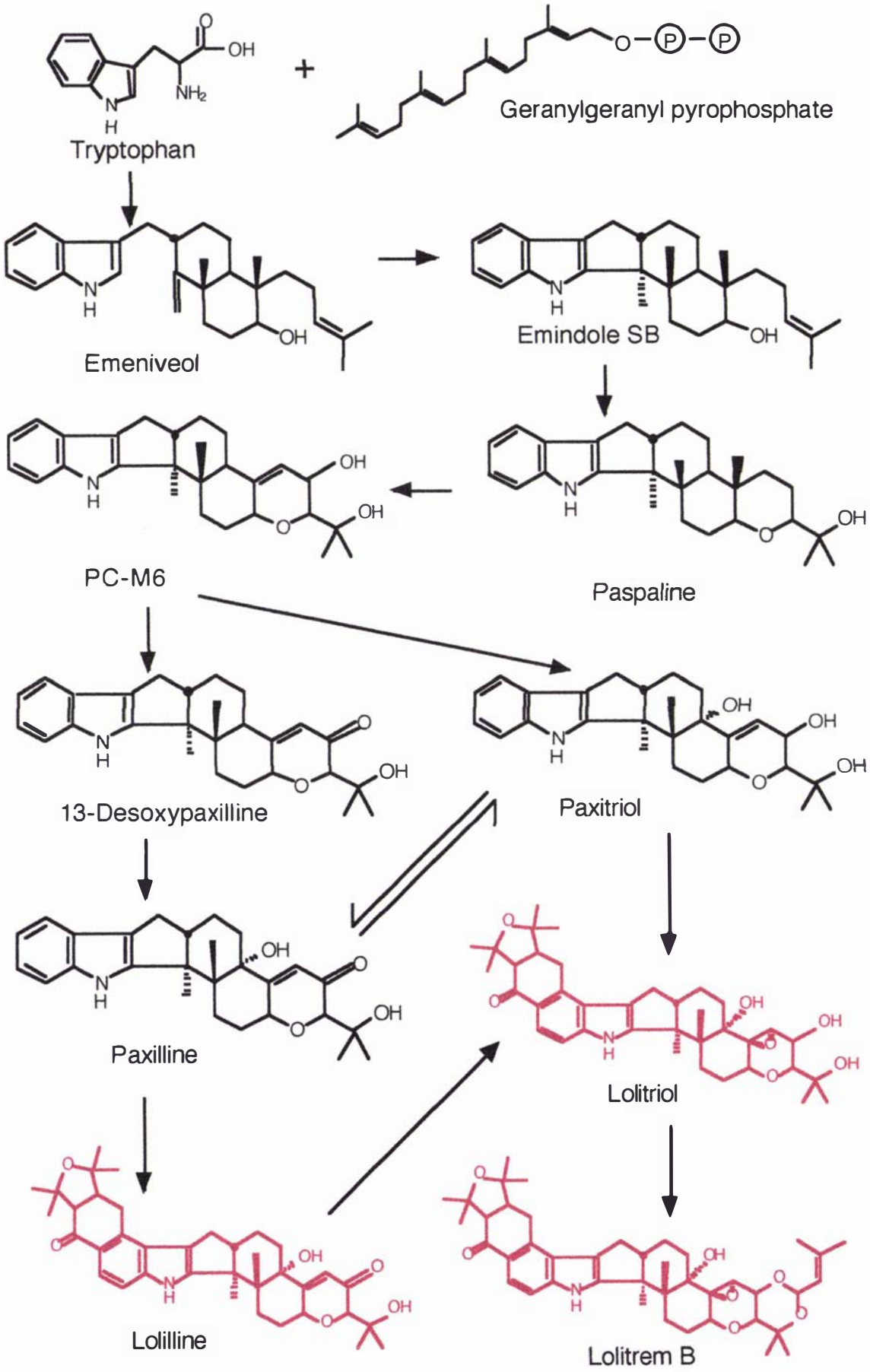
There are also examples of terpenes that are beneficial. The plant *Celastrus hindsii* produces a number of triterpenes. Of four isolated recently (celasdin-A, celasdin-C, celasdin-B, and maytenfolone-A), one, maytenfolone-A was considerably cytotoxic to hepatoma and nasopharynx carcinoma. Another, celasdin-B was found to inhibit HIV replication in H9 lymphocyte cells (Kuo & Kuo, 1997).

1.7.6 Paxilline and Lolitrem B

It is the alkaloid indole-diterpenoids, lolitrem B and a proposed precursor paxilline, produced by *N. lolii* and *P. paxilli* respectively that are the main focus of this research. However, other species also produce similar structures such as the endophytic *Phomopsis* which synthesises paspalitrem A and C (Bills *et al.*, 1992). Other tremorgenic mycotoxins include the penitrems and janthitrems from *Penicillium* spp. and the aflatrems from *Aspergillus* spp. (Cole *et al.*, 1974; Gallagher *et al.*, 1980). These lipophilic toxins, capable of crossing the blood brain barrier, are known as tremorgens, because they cause the neurological disorder ryegrass staggers in agricultural ruminants. When injected into the gastrointestinal smooth muscle of sheep, paxilline induced tremors which lasted for 1-2 h and lolitrem B induced tremors for over 24 h (Smith *et al.*, 1997). It is thought that the effect may be a result of toxin interactions with Ca^{2+}-activated K^+ (maxi-K) channels and neurotransmitter release at both central and peripheral levels (Knaus *et al.*, 1994). Binding of paxilline to maxi-K channels is reversible (Sanchez & McManus, 1996). There is also evidence that the tremorgenic mycotoxins affect γ-aminobutyric acid (GABA) receptors by binding close to the Cl^- channel (Gant *et al.*, 1987). Although the biosynthetic pathway is unknown, attempts to elucidate a potential pathway, by examining the chemistry required for the production of potential precursors through to the end product, lolitrem B, have provided vital information. Enzymatic functions predicted to be involved include; cyclases, oxygenases, P450 monooxygenases and dehydrogenases. The primary substrates of the

Figure 1.1 Proposed biosynthetic pathway of paxilline and lolitrem B

This figure is based on figures from the papers by Munday-Finch *et al.* (1996a) and Miles *et al.* (1996). Only the principal structures have been included for simplicity. Products that are synthesised in *Neotyphodium lolii* but not in *P. paxilli,* are shown in red.


Tryptophan
Geranylgeranyl pyrophosphate
Emeniveol
Emindole SB
PC-M6
Paspaline
13-Desoxypaxilline
Paxitriol
Paxilline
Lolitriol
Lolilline
Lolitrem B

lolitrem B biosynthetic pathway are tryptophan, which provides the indole moiety (Laws & Mantle, 1989) and mevalonic acid, which provides the C5 prenyl units (Acklin *et al.*, 1977). There are two pieces of evidence that support paxilline as the biological precursor of lolitrem B. Firstly, the spatial positioning of core structural atoms in paxilline are conserved with lolitrem B. Secondly, paxilline has been isolated from *N. lolii* grown in liquid culture (Weedon & Mantle, 1987). A more immediate precursor of lolitrem B, may be lolitriol, which differs from lolitrem B by only a single acetal moiety. Intermediates between paxilline and lolitriol, appear to be the diastereoisomeric allylic alcohols α- and β-paxitriol. Interestingly, although paxilline and lolitrem B are toxic tremorgens, the paxitriols and lolitriol are toxic, but lack tremorgenicity (Miles *et al.*, 1992). Precursors of paxilline are proposed to be paspaline, paspaline B and 13-desoxypaxilline (Munday-Finch *et al.*, 1996a). Recently a new indole-diterpenoid, called lolilline, because of its similarities to both paxilline and lolitrem B, has been isolated from *N. lolii* infected seeds of *Lolium perenne*. This proposed new intermediate was also found to be nontremorgenic (Munday-Finch *et al.*, 1997). The biosynthesis of both paxilline and lolitrem B forms an interrelated grid of compounds once the initial indole-diterpenoid structure has formed (Figure 1.1). In this way, isomers of the main structures can be formed, like the isomer of lolitrem B, lolitrem F (Munday-Finch *et al.*, 1996b).

1.8 3-Hydroxy-3-methylglutaryl Coenzyme A reductase

1.8.1 Isoprenoid biosynthesis

The enzyme 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR) produces mevalonate and is the rate-limiting step of many primary and secondary metabolic pathways. During the enzymatic reduction, HMG CoA is converted through the intermediates mevaldyl-CoA and mevaldehyde to mevalonate. Frimpong & Rodwell (1994) used these intermediates to determine the role of catalytically active residues His^{865}, Glu^{558} and Asp^{766} in Syrian hamster HMGR. The proposed reaction, including the intermediate substrates, is shown below.

$$\text{HMG CoA} \xrightarrow{\text{Asp}^{766}} [\text{mevaldyl-CoA} \xrightarrow{\text{Glu}^{558} + \text{His}^{865}} \text{mevaldehyde}] \xrightarrow{\text{Asp}^{766}} \text{mevalonate}$$

Further phosphorylation and decarboxylation converts mevalonate to isopentenyl pyrophosphate (IPP). IPP and Dimethylallylpyrophosphate (DMAPP), an isomer of IPP, are the starting point for isoprenoid biosynthesis (Chappell, 1995). These pathways and their end products in filamentous fungi are depicted in Figure 1.2. Synthesis of ergosterol (and cholesterol in mammalian cell membranes) involves the condensation of two, 15 carbon (C), FPP units to form the 30 C squalene (Brown & Goldstein, 1980; Lees *et al.*, 1995). Alternatively, the addition of a single IPP unit to FPP, by the enzyme GGPP synthase, results in the synthesis of GGPP, the starting substrate for diterpene biosynthesis. GGPP has an important primary metabolic function and it is the most common prenyl group added to prenylated proteins (Jiang *et al.*, 1995). Prenylated proteins have increased hydrophobicity and the ability to associate with lipid membranes (Parmryd & Dallner, 1996).

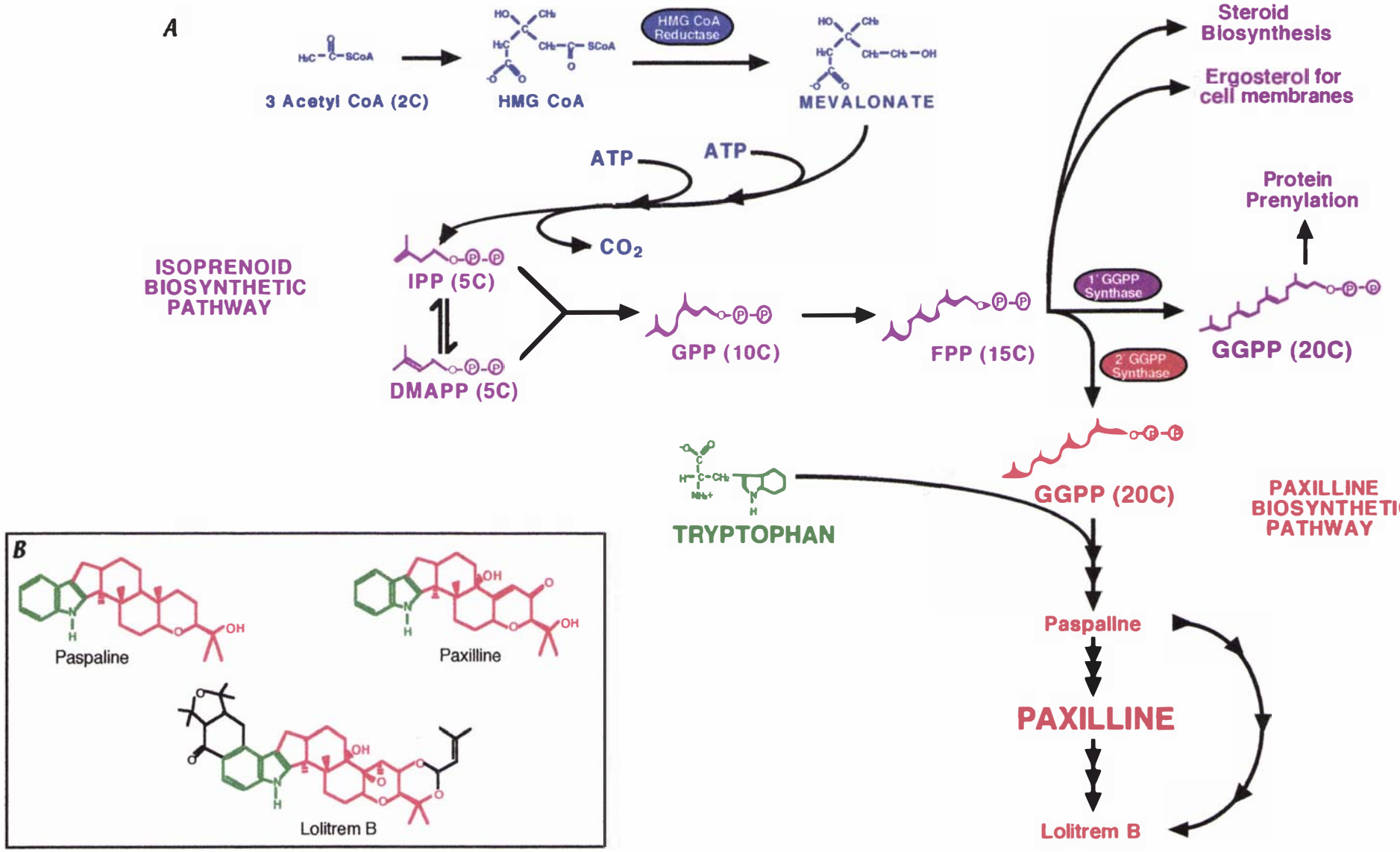
1.8.2 Regulation of HMGR

Internal synthesis of sterols, particularly cholesterol, has to be carefully balanced with the external cholesterol available. Although the majority of work has focused on cholesterol biosynthesis in mammals, levels of ergosterol production in fungi are presumably maintained by similar mechanisms. Cholesterol enters cells via Low Density Lipoproteins (LDLs), which are endocytosed after interaction with the LDL receptor (Stryer, 1995). Sterol homeostasis can be achieved by regulating the expression of cholesterogenic genes such as HMGR and LDL Receptors. Experiments to examine this phenomenon have made use of competitive inhibitors of HMGR, such as compactin and pravastatin (Brown & Goldstein, 1980; Imbault *et al.*, 1996) and showed that expression of these genes is repressed under conditions of excess sterols. Addition of excess LDL to fibroblast cell culture media represses HMGR activity by 98%. Total repression was never achieved, as other non-sterol products produced from mevalonate are also required. This type of repression was termed multivalent feedback regulation (Brown & Goldstein, 1980).

Figure 1.2 Isoprenoid biosynthesis

A) The interconnecting biosynthetic pathways required for paxilline biosynthesis include the primary metabolic pathways for mevalonate and isoprenoid biosynthesis and the secondary metabolic pathway specific for paxilline biosynthesis. The genes *hmg*, *ggs-1* and *paxG* in *P. paxilli* encode the enzymes HMG CoA reductase, 1' GGPP synthase and 2' GGPP synthase respectively.

B) The structure of 3 indole diterpenoids isolated from *N. lolii*. Lolitrem B is not synthesised by *P. paxilli*, but paspaline and paxilline are.


A
3 Acetyl CoA (2C)
HMG CoA
HMG CoA Reductase
MEVALONATE
ATP
ATP
CO2
ISOPRENOID BIOSYNTHETIC PATHWAY
IPP (5C)
DMAPP (5C)
GPP (10C)
FPP (15C)
Steroid Biosynthesis
Ergosterol for cell membranes
Protein Prenylation
1° GGPP Synthase
2° GGPP Synthase
GGPP (20C)
GGPP (20C)
TRYPTOPHAN
PAXILLINE BIOSYNTHETIC PATHWAY
Paspaline
PAXILLINE
Lolitrem B
B
Paspaline
Paxilline
Lolitrem B

1.8.2.1 Transcriptional control of sterol biosynthetic genes

Sterols affect transcription of cholesterogenic genes (Osborne *et al.*, 1985) through sterol response elements (SREs) in their promoters (Goldstein & Brown, 1990). Binding of the ubiquitous transcription factor, Nuclear Factor Y (NF-Y), is also required before increased transcription of cholesterogenic genes is seen (Jackson *et al.*, 1995). NF-Y, which binds to the inverted CCAAT box, may have a role in recruiting the sterol response element-binding proteins (SREBP) (Ericsson *et al.*, 1996b). In Syrian hamster, the gene for HMGR has numerous transcription start sites which lack TATA boxes and CAAT boxes, the core promoter elements found in most eukaryotic genes (Reynolds *et al.*, 1985).

1.8.2.2 Post-transcriptional control of sterol biosynthetic genes

In the presence of compactin, enzyme activity was increased in three ways. Firstly, increased transcription of the HMGR gene (called *hmg* by convention), secondly, increased translation and thirdly, decreased degradation (Goldstein & Brown, 1990). Analysis of a fusion protein of HMGR and green fluorescent protein (GFP), showed that HMGR is localised in the endoplasmic reticulum in *S. cerevisiae* (Hampton *et al.*, 1996b), as are the mammalian HMGR proteins (Liscum *et al.*, 1985). Overexpression of both *hmg* genes from *S. cerevisiae* resulted in the formation of a novel membrane structure, possibly to provide a matrix for the abundant protein to be inserted into (Wright *et al.*, 1988). The regulatory degradation of HMGR in response to sterols, involves the transmembrane domain (Luskey & Stevens, 1985). A truncated isoform of the protein expressed in Chinese hamster ovary cells was degraded five times slower than the membrane bound holoenzyme (Gil *et al.*, 1985). The enzyme is held in the endoplasmic reticulum membrane by 2-8 membrane spanning hydrophobic domains, with the number of membrane spans varying between species. In archaebacteria, there are no membrane spanning domains, plants have two domains, yeast and filamentous fungi have 7 putative domains (Dobson, 1997; Woitek *et al.*, 1997) and higher mammals have 7 domains (Hampton *et al.*, 1996b). Work by Roitelman *et al.* (1992) used a method that predicted 8 not 7 transmembrane domains in hamster HMGR. Replacement of the seventh transmembrane domain with a transmembrane span from

bacteriorhodopsin abolished the degradative response to sterols. HMGR found in sea urchin embryos is not degraded in the presence of excess sterols, however a mutant generated to contain the second transmembrane span from hamster HMGR, became sterol-responsive (Kumagai *et al.*, 1995). Expression of a mutant form of one of the *S. cerevisiae* HMGR genes, which lacked the transmembrane domain, caused an increase in squalene synthesis, but not of ergosterol, indicating that many levels of regulation exist in sterol biosynthetic pathways (Donald *et al.*, 1997; Polakowski *et al.*, 1998). Overexpression of both native and transgenic *Arabidopsis thaliana hmg* did not result in accumulation of isoprenoid products (Re *et al.*, 1995).

HMGR can also be reversibly inactivated by phosphorylation at a conserved serine residue in the C-terminal catalytic domain (Friesen & Rodwell, 1997; Kennelly & Rodwell, 1985). The response was observed when hormones involved in sterol regulation were added to rat hepatocytes (Ingebritsen *et al.*, 1979). The original report showed HMGR activity was decreased when treated with Mg^{2+} and ATP (Beg *et al.*, 1973). The inactivation was shown to be independent of the membrane-spanning domain and dependent on protein phosphatases for reversal (Kennelly & Rodwell, 1985). HMGR in rat hepatocytes was phosphorylated and inactivated in response to fructose. Studies with ^{32}P revealed that the protein was phosphorylated at the conserved Ser^{871} residue (Gillespie & Hardie, 1992). HMGR from *P. mevalonii* is not regulated by phosphorylation. Friesen & Rodwell (1997) created a site for AMP-dependent protein kinase inactivation by replacing Arg^{387} in *P. mevalonii* HMGR with a serine. The activity of the mutant enzyme could then be reversibly phosphorylated in a manner analogous to mammalian HMGR.

1.8.2.3 A mechanism for regulation by non-sterol end-products?

While regulation of mRNA transcription, protein inactivation and degradation appear to be sterol dependent, another post-transcriptional mechanism, involving heterogeneity in the 5' untranslated region (UTR) of *hmg*, could be a target of non-sterol end-product regulation (Goldstein & Brown, 1990). Sixteen different transcripts are produced from the *hmg* gene in Chinese hamster. These arise from a combination of multiple transcription start sites and alternative splicing of an intron in the 5' UTR. A cluster of

four transcription start sites, upstream of the most 5' intron splice site, initiate 70% of the transcripts. The remaining 30% of transcripts were initiated from 3 alternative transcription start sites closer to the translation start site (Reynolds *et al.*, 1985). However, two alternatively spliced products from the HMG CoA Synthase gene were found in equal abundance in numerous tissues from Chinese hamster and humans (Gil *et al.*, 1987).

1.9 Fungal endophytes

Neotyphodium lolii, an asexual filamentous fungus of the taxonomic grouping Ascomycetes, forms a mutualistic symbiosis with the perennial ryegrass *Lolium perenne,* a crop of major agricultural importance. From this association, the endophyte is provided with a nutrient supply and, in the case of this asexual endophyte, seed dissemination by maternal transmission. Benefits to the host include; drought tolerance, and resistance to fungal pathogens, nematodes, insects and mammalian herbivores (Ball *et al.*, 1995). There is a potential for these endophytes to be used as natural biocontrol agents against insect pests, if the problems of associated livestock toxicosis can be solved (Clay, 1989). *N. lolii* and *N. coenophialum*, which infects tall fescue (*Festuca arundinacea*) are anamorphs of the Clavicipitaceous choke pathogen *Epichloë* spp (Schardl & An, 1993). Strains of *Epichloë* can vary in their degree of pathogenicity, some will always form the sexual spore structure called a stroma which 'chokes' the ryegrass inflorescence, others remain asexual and are passed on by seed dissemination (Bucheli & Leuchtmann, 1996). *N. lolii* and *N. coenophialum* never produce spores while in symbiosis with the host plant (Siegel *et al.*, 1985). Another interesting feature of these fungal-plant symbioses, has been the formation of interspecific hybrids. An endophyte isolated from *L. perenne*, LpTG-2 (*L. perenne* Taxonomic grouping 2) was shown to be a hybrid derived from *Epichloë typhina* (isolate E8) and *N. lolii* (isolate Lp5) (Schardl *et al.*, 1994). The formation of interspecific hybrids may be a potential mechanism for the horizontal transfer of secondary metabolite biosynthetic gene clusters between asexual and sexual species.

1.9.1 Mycotoxins

Toxins produced by *N. lolii* include peramine, lolitrem B and ergovaline, whilst *N. coenophialum* produces peramine, ergovaline and loline (Siegel *et al.*, 1990). Peramine, a pyrrolopyrazine alkaloid isolated from ryegrass infected with *N. lolii,* was shown to deter feeding by adult Argentine stem weevil (*Listronotus bonariensis*) (Rowan & Gaynor, 1986). It has also proved to be a potent inhibitor of many other herbivorous insect species (Rowan & Latch, 1994). Unlike lolitrem B and the ergot alkaloids, peramine does not appear to affect mammals (Scott & Schardl, 1993; van Heeswijck & McDonald, 1992). Analysis of the distribution of peramine and lolitrem B within *L. perenne*, revealed that whilst lolitrem B concentrations are highest in the leaf blade, peramine was highest in the leaf sheath. Concentrations of peramine were highest in younger leaves, compared to lolitrem B, which increased with the age of leaves (Keogh *et al.*, 1996).

Ergovaline, an ergopeptine alkaloid, is produced via the same precursors as lolitrem B and paxilline. Mevalonate is converted to DMAPP through four enzymatic steps (Figure 1.2) and is then condensed with tryptophan by the enzyme DMAT synthase to produce the first product of ergovaline biosynthesis dimethylallyltryptophan (DMAT). Due to the common precursors of the lolitrem B and ergovaline biosynthetic pathways, there is potential for downstream effects if one or the other pathway becomes blocked. Ergovaline has also been shown to affect herbivorous insect feeding, particularly Argentine stem weevil (Rowan & Latch, 1994). However, it has also been correlated to the livestock disorder, fescue toxicosis, though no direct cause and effect has been established (van Heeswijck & McDonald, 1992).

Lolines pyrrolizidine alkaloids, are not produced by *N. lolii*, but they have been shown to deter feeding of an aphid species (*Rhopalosiphum padi*) (Siegel *et al.*, 1990). Until recently, lolines, like lolitrem B could only be isolated from the producing endophyte in planta (van Heeswijck & McDonald, 1992). However, lolines have since been isolated from cultures of *N. uncinatum* (Wilkinson *et al.*, 1997).

1.10 *Penicillium paxilli*

Penicillium paxilli is a filamentous fungi of the subdivision Ascomycotina and reproduces asexually by way of conidia (Kendrick, 1992). The tremorgenic indole-diterpenoid paxilline is the major secondary metabolite produced by *P. paxilli* (Cole *et al.*, 1974) and is also synthesised by the endophytic fungus *N. lolii* (Weedon & Mantle, 1987). Paxilline has been predicted as the precursor of lolitrem B. Due to the adverse effects of mammalian tremorgenic toxins present in agricultural grazing pastures, there has been interest in isolating and characterising the genes responsible for toxin biosynthesis from fungal endophytes. Isolation of lolitrem B biosynthetic genes from *N. lolii* would be difficult due to the inability of the fungus to produce sufficient lolitrem B in liquid culture together with a slow growth rate. *N. lolii* is only disseminated through the host seed and does not sporulate on solid media. *P. paxilli* produces large amounts of paxilline in liquid culture (Ibba *et al.*, 1987) and spores can be purified from solid media within 4-7 days. Therefore *P. paxilli* has been adopted as a more suitable organism for the isolation of the biosynthetic gene cluster responsible for paxilline biosynthesis.

1.10.1 *P. paxilli* paxilline negative mutants

A paxilline negative mutant of *P. paxilli* was first generated by heterologous plasmid tagging (Itoh *et al.*, 1994a). The mutant YI-20 was found to have a 120-150 kb deletion and partial translocation of chromosome Va (Young *et al.*, 1998). The left boundary of this deletion was isolated by plasmid rescue, however the right boundary has yet to be isolated. Using the sequence isolated from the site of pAN7-1 integration in YI-20, homologous plasmid integration was used to generate three further mutants. CY-2 and CY-102 were deleted in a similar fashion to YI-20, but without the associated chromosomal translocation. Both mutants were shown to be paxilline negative as determined by high performance liquid chromatography (HPLC) and competitive enzyme-linked immunosorbant assay (ELISA). A third mutant, CY-35, has a smaller deletion of 7.6 kb, and produces wild type levels of paxilline (Young *et al.*, 1998). The

chromosome walking technique was then used to isolate sequence from the CY-102 deletion.

An additional Pax$^-$ mutant was generated using the REMI technique (restriction enzyme-mediated integration), which uses a restriction enzyme, in conjunction with a linear plasmid to generate double stranded breaks in the DNA (Schiestl & Petes, 1991). After screening 1000 transformants by thin layer chromatography (TLC) for their ability to produce paxilline, a single Pax$^-$ mutant, LM-662, was identified. The sequences adjacent to the site of plasmid integration were isolated by plasmid rescue. However DNA sequencing of these regions failed to identify any ORFs that could logically be associated with the biosynthesis of paxilline. Southern analysis of this mutant revealed that the pAN7-1 tagged region was located away from the original deletion found in YI-20, CY-2 and CY-102. A second un-tagged deletion was detected in the same location as the original deletion mutants. This deletion resulted from the addition of the restriction enzyme *Hin*dIII, which caused a 22.3 kb section of DNA to be removed. Sequencing of this locus identified the first gene, *paxG*, a geranylgeranyl pyrophosphate synthase. 'Knockout' mutants, constructed in this laboratory, were generated by replacing the *paxG* gene with a hygromycin resistance gene (*hph*) (L. McMillan pers. comm.) When the *paxG*$^-$ mutants were screened with HPLC neither paxilline, or any of the predicted precursors (13-desoxypaxilline, paspaline or paspaline B) were produced. In addition to the replacement of *paxG* by a double cross-over recombination event, a small deletion mutant was also created that left *paxG* intact, but removed all or part of three additional genes located next to *paxG*. This mutant, called LM-G130 is also paxilline negative and lacks *paxM1* (a putative monooxygenase), *paxU1* (a putative cyclase with prenyl transferase motifs) and part of *paxP1* (a putative cytochrome P450 monooxygenase).

1.10.2 The paxilline biosynthetic gene cluster

Subsequent sequencing of this locus has revealed 17 genes, with putative functions associated with paxilline biosynthesis, in a 50 kb region of chromosome Va (Figure 1.3). They are; *paxG*, a GGPPS; *paxM1* and *pax M2*, two FAD-dependent monooxygenases; *paxP1* and *paxP2*, cytochrome P450 monooxygenases; *paxD*, a

Figure 1.3 *Paxilline biosynthetic gene cluster from P. paxilli*

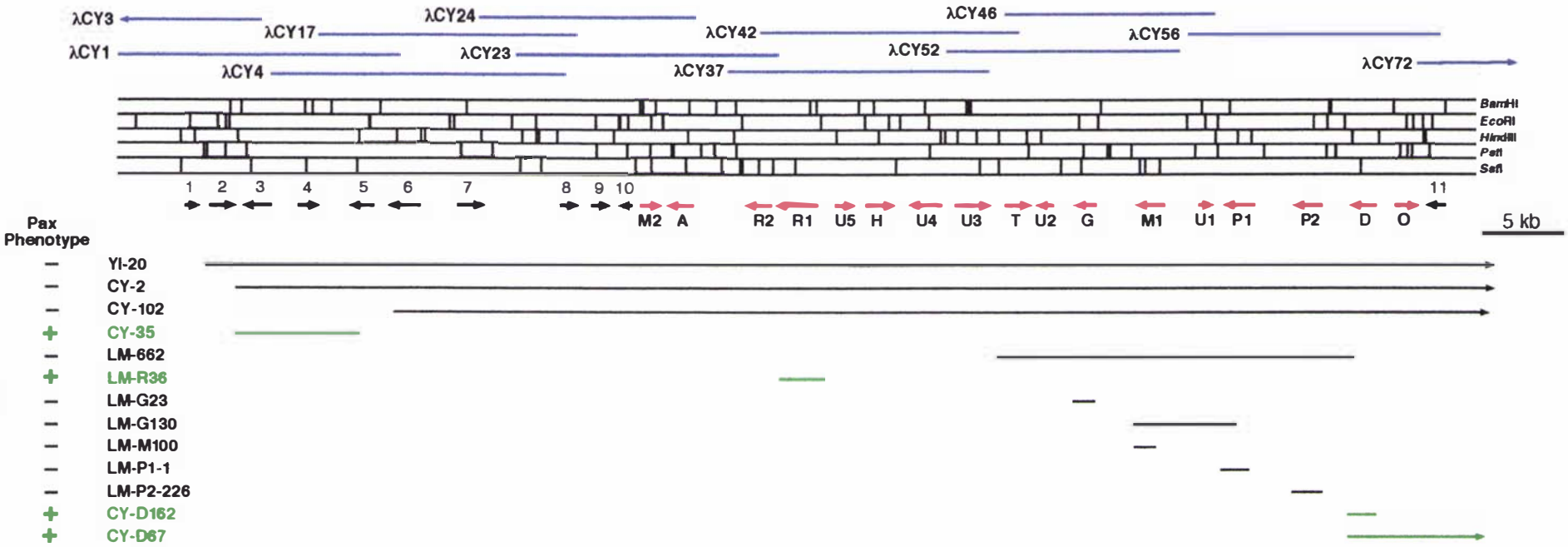
This figure contains information from the paper by Young *et al.* (2000) and is adapted from Figure 1 in the same paper.

A restriction map of the 50 kb paxilline biosynthetic gene cluster and flanking sequence is shown below the individual λ clones isolated during chromosome walking by Carolyn Young. The 17 cluster genes are shown with red arrows and genes not predicted to function in paxilline biosynthesis are shown as black arrows. The direction of each arrow indicates the orientation of individual genes within the cluster.

The cluster genes show homology to the following proteins M2) *paxM2*, a monooxygenase, A) *paxA*, a pH responsive protein, R2) *paxR2*, a $Zn(II)_2Cys_6$ binuclear cluster DNA-binding protein, R1) *paxR1*, a $Zn(II)_2Cys_6$ binuclear cluster DNA-binding protein, U5) *paxU5*, unknown function, H) *paxH*, a dehydrogenase, U4) *paxU4*, unknown function, U3) *paxU3*, unknown function, T) *paxT*, a transporter, U2) *paxU2*, unknown function, G) *paxG*, a geranylgeranyl pyrophosphate synthase, M1) *paxM1*, a monooxygenase, U1) *paxU1*, unknown possible prenyl transferase, P1) *paxP1*, a P450 monooxygenase, P2) *paxP2*, a P450 monooxygenase, D) *paxD*, a dimethylallyl-tryptophan synthase and O) *paxO*, an oxidoreductase.

Non-cluster genes show homology to the following proteins 1) *dec*, a phenylacrylic decarboxylase 2) *lip1*, a lipase, 3) *amy*, an α-amylase, 4) *gal*, a glucoamylase, 5) *trn*, an ABC transporter, 6) *kin*, a kinesin, 7) *adh*, an alcoholdehydrogenase, 8) *perm*, a permease, 9) *red*, a reductase, 10) *lip2*, a lipase and 11) *arb*, an arabinase.

Strains of *P. paxilli,* containing deletions within the paxilline biosynthetic cluster are shown, below the restriction map. Mutants created by Yasuo Itoh are labelled YI, mutants created by Carolyn Young are labelled CY and mutants created by Lisa McMillan are labelled LM. Mutants that produce paxilline are shown in green.


λCY3
λCY17
λCY1
λCY4
λCY24
λCY23
λCY37
λCY42
λCY46
λCY52
λCY56
λCY72
BamHI
EcoRI
HindIII
PstI
SalI
1 2 3 4 5 6 7 8 9 10 11
M2 A R2 R1 U5 H U4 U3 T U2 G M1 U1 P1 P2 D O
5 kb
Pax Phenotype
– YI-20
– CY-2
– CY-102
+ CY-35
– LM-662
+ LM-R36
– LM-G23
– LM-G130
– LM-M100
– LM-P1-1
– LM-P2-226
+ CY-D162
+ CY-D67

DMATS; *paxR1* and *paxR2*, with $Zn(II)_2Cys_6$ binuclear cluster DNA binding motifs; *paxH*, a dehydrogenase; *paxT*, a transporter protein; *paxA*, a pH-responsive protein; *paxO*, an oxidoreductase; *paxU1*, a possible cyclase with prenyl transferase motifs and four other genes, named *paxU2-5*, of unknown function. Gene replacement 'knockout' mutants of *paxD*, *paxR1* *paxM1*, *paxP1* and *paxP2* have been analysed as well. Paxilline is produced by replacement mutants of both *paxD* (Carolyn Young pers. comm.) and *paxR1* (Lisa McMillan pers. comm.). The mutants missing *paxP1* (created by Lisa McMillan) and *paxP2* (Carr, 1999) have been shown to accumulate paspaline and 13-desoxypaxilline respectively, but not paxilline (Lisa McMillan pers. comm.).

1.11 Analysis of Gene expression

A number of techniques exist for the analysis of gene expression. Levels of protein can be measured directly or as a result of gene fusions between a native promoter of interest and a reporter product, such as β-Glucuronidase (GUS) and Green fluorescent protein (GFP). This technique was used to examine expression of the *ver1* gene from *A. flavus*, involved in aflatoxin biosynthesis. Increased GUS activity was seen in concert with an increase in aflatoxin biosynthesis. Northern analysis showed a correlation between an increase in native *ver1* mRNA and GUS activity (Flaherty *et al.*, 1995). In filamentous fungi, the majority of regulation occurs at the level of transcription (Gurr *et al.*, 1987), therefore the direct measurement of steady state mRNA levels is a good measure of the rate of gene expression.

Traditionally, mRNA levels have been determined by Northern blotting. This technique, based in theory on the method of Southern (1975), separates either total RNA of purified mRNA by electrophoresis, before transferring them to a nylon membrane. Using a labelled gene specific probe, the mRNA of interest can be identified. Messenger RNA from different time points, developmental stages or tissues can then be compared.

With the development of the PCR, a number of techniques have evolved to quantify mRNA based on the amount of product amplified from cDNA. Total RNA or purified

mRNA from different time points, developmental stages or tissues is reverse transcribed into cDNA and a PCR performed with gene specific primers. Product yields are compared by the intensity of ethidium bromide fluorescence after electrophoretic separation. This technique, known as RT-PCR can be performed both qualitatively and quantitatively. An added benefit of the RT-PCR technique over Northern analysis, is that once cDNA has been synthesised, templates are no longer sensitive to RNase degradation.

Competitive RT-PCR uses known concentrations of a competitive template to titre the unknown concentration of the native mRNA species. Frequently a mutant form of the mRNA is constructed so that the amplified product is a different size from the native product (Riedy *et al.*, 1994). Simple PCR-based techniques exist for the construction of such RNA internal controls (Vanden Heuvel *et al.*, 1993). Alternatively gDNA can be amplified as a competitor, provided intron splicing has occurred between the two gene specific primers (Harting & Wiesner, 1997). Messenger RNA from another species has also been used as a successful competitor for quantification of mRNA in RT-PCR (Levesque *et al.*, 1994). With techniques such as these, it is possible to calculate the exact concentration of mRNA in a particular sample. This can be a powerful tool when studying subtle changes in the level of gene expression.

With both Northern analysis and RT-PCR techniques, the amount of total RNA added to each reaction needs to be standardised, so that the differences recorded can accurately reflect changes in the original sample. This is usually done by comparing the expression of the gene of interest to the gene expression of a constitutively expressed housekeeping gene. Frequently ribosomal RNA genes or α- and β-tubulin genes are used. Different methods for standardising competitive quantitative RT-PCR were examined in a paper by Haberhausen *et al.* (1998). They found that an internal control, where both the standard and test gene were amplified in a multiplex RT-PCR reaction, was the most reliable. However, this results in a very complex PCR reaction, where multiple templates and multiple primer combinations are required.

A simple qualitative RT-PCR technique can also be used to reveal qualitative patterns of expression, rather than quantitative data. If a single pool of cDNA is generated with random primers, subsequent PCR reactions can be performed with gene specific

primers, which include amplification of a control gene. In this way the steady state mRNA profile of a range of cDNA templates can be compared.

While the RT-PCR technique has greater sensitivity than Northern analysis, a method more sensitive than RT-PCR has also been devised. Femino *et al.* (1998) used fluorescent in situ hybridisation (FISH) to detect single RNA molecules from a single cell.

1.12 Objectives

The aim of this study has been to begin to understand how paxilline biosynthesis is regulated in *P. paxilli.* To achieve this, a gene from the primary isoprenoid biosynthetic pathway, specifically *hmg*, was isolated and characterised. Expression of this gene was then compared with the genes involved in the secondary metabolic biosynthesis of paxilline. The gene *tub-2* was also isolated to provide an independent control for these experiments. In order to identify reproducible patterns of expression, the physiological conditions for growth in liquid media were optimised. In the experimental procedure developed, the induction of paxilline biosynthesis and homogeneous loose hyphal morphology were observed in a reproducible manner. These conditions were then used together with a RT-PCR gene expression assay to determine patterns of gene expression during onset of paxilline biosynthesis.

2.1 Fungal and Bacterial strains, λ Clones and Plasmids

2.1.1 Stocks and strains used

All fungal and bacterial strains, λ clones and plasmids used are shown in Table 2.1.

2.1.2 Growth of cultures

Bacterial strains grown on solid media were streaked onto LB agar plates (section 2.2) with a sterile wire loop and grown at 37°C overnight. When required, antibiotic was added to a final concentration of 100 μg/mL for ampicillin or 10 μg/mL for tetracycline.

Bacterial cells grown in liquid media were transferred to LB media (section 2.2) from a single colony using a sterile wire loop. Cultures were grown overnight at 37°C and 220 rpm. When required, antibiotic was added to a final concentration of 100 μg/mL for ampicillin or 10 μg/mL for tetracycline.

P. paxilli strains grown on solid media were inoculated from a spore suspension (section 2.1.5). Spores were streaked onto solid media (Potato Dextrose (PD) or Czapex Dox (CD)) (section 2.2) with a sterile wire loop to produce individual colonies. PD plates were incubated at 22°C for 4-5 days until sporulation was seen. CDYE plates were used to determine if any yeast or bacterial contamination was present in large scale liquid cultures. Approximately 100 μL of culture was spread over the surface with a sterile glass spreader and the plates were incubated at 30°C for 24 h.

For isolation of DNA, fungi were grown in PD liquid media. Six 250 mL Erlenmeyer flasks containing 25 mL of PD media (section 2.2), were inoculated with 5 x 10^6 spores/mL from a fresh *P. paxilli* WT spore suspension (section 2.1.5). Cultures were grown at 22°C with shaking at 230 rpm for 3 days. The broth was decanted, the pellets washed with sterile Milli Q water and divided between two 15 mL falcon tubes. Samples were frozen at -20°C and freeze-dried overnight.

Table 2.1 Organisms, strains, λ clones and plasmids

Organism, Strain λ Clone or Plasmid	Characteristics	Reference
Organism		
Penicillium paxilli		
PN2013 WT	Pax^+, Brs^+, Hyg^S.	(Itoh *et al.*, 1994a)
Neotyphodium sp. (=LpTG-2)		
Lp1	*E. typhina-N. lolii* hybrid from *Lolium perenne.*	(Christensen *et al.*, 1993)
Neotyphodium sp. (=LpTG-1)		
Lp19	*N. lolii* from *L. perenne.*	(Christensen *et al.*, 1993)
Escherichia coli		
XL-1	*recA*1, *endA*1, *gyrA*46, *thi*, *hsdR*17, *supE*44, *posA*1, λ^-, *lac*$^-$, [F' *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15, Tn10(Tet^R)].	(Bullock *et al.*, 1987)
KW251	F^-, *supE44*, *galT22*, *metB1*, *hsdR2*, *mcrA* [*argA*81: Tn10] *recD*1014, Tet^R.	Promega Corp.
PN1194	contains plasmid pBT6.	
λ Clones		
λGEM®-11		Promega Corp.
λET1	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET2	λGEM®-11 clone containing *hmg* from *P. paxilli*	This study
λET4	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET5	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET6	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET10	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study

Organism, Strain λ Clone or Plasmid	Characteristics	Reference
λET11	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET12	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET13	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET15	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET17	λGEM®-11 clone containing *hmg* from *P. paxilli.*	This study
λET22	λGEM®-11 clone containing *tub-2* from *P. paxilli.*	This study
λET23	λGEM®-11 clone containing *tub-2* from *P. paxilli.*	This study
λET24	λGEM®-11 clone containing *tub-2* from *P. paxilli*	This study
λET27	λGEM®-11 clone containing *tub-2* from *P. paxilli.*	This study
λET29	λGEM®-11 clone containing *tub-2* from *P. paxilli.*	This study
Plasmids		
pUC118	Amp^R	(Vieira & Messing, 1987)
pGEM®-T	Amp^R	Promega corp.
pGEM®-T EASY	Amp^R	Promega corp.
pET1	pUC118 containing 3.8 kb *Sst*I fragment of λET2.	This study
pET2	pUC118 containing 5.2 kb *Sst*I fragment of λET2.	This study
pET3	pUC118 containing 3.8 kb *SstI* fragment of λET2 in a negative orientation.*	This study

Organism, Strain λ Clone or Plasmid	Characteristics	Reference
pET4	pUC118 containing 0.4 kb *Sst*I fragment of λET2.	This study
pET5	pUC118 containing 5.2 kb *Sst*I fragment of λET2 in a negative orientation.*	This study
pET6	pUC118 containing 2.4 kb *Pst*I-*Sst*I fragment of λET2.	This study
pET7	pUC118 containing 1.7 kb *Pst*I-*Sst*I fragment of λET2 in a negative orientation.*	This study
pET8	pUC118 containing 3.1 kb *Bam*HI-*Sst*I fragment of λET2.	This study
pET9	pUC118 containing 2.1 kb *Bam*HI-*Sst*I fragment of λET2 in a negative orientation.*	This study
pET10	pUC118 containing 2.8 kb *Pst*I-*Sst*I fragment of λET2 in a negative orientation.*	This study
pET11	pUC118 containing 3.8 kb *Sal*I-*Sst*I fragment of λET2 in a negative orientation.*	This study
pET12	pUC118 containing 2.5 kb *Sst*I fragment of λET22 in a negative orientation.*	This study
pET13	pUC118 containing 5.1 kb *Sst*I fragment of λET22.	This study
pET14	pUC118 containing 2.7 kb *Sst*I fragment of λET22.	This study
pET15	pUC118 containing 0.8 kb *Sst*I fragment of λET22.	This study
pET16	pUC118 containing 2.9 kb *Sst*I fragment of λET22.	This study
pET17	pUC118 containing 0.7 kb *Sst*I fragment of λET22.	This study
pET18	pUC118 containing 1.1 kb *Sst*I fragment of λET22.	This study
pET19	pGEM®-T containing *P. paxilli* RT-PCR product (ET22-ET9).	This study

Organism, Strain λ Clone or Plasmid	Characteristics	Reference
pET20	pGEM®-T containing *P. paxilli* RT-PCR product (Etub5-Etub6).	This study
pET21	pGEM®-T containing *P. paxilli* 5'RACE product (UAP-ET3).	This study
pET22	pGEM®-T containing *P. paxilli* RT-PCR product (ET12-ET15).	This study
pET23	pGEM®-T containing Lp19 RT-PCR product (hmgeco-hmgnco).	This study
pET24	pGEM®-T containing *P. paxilli* 3'RACE product (ET22-UAP).	This study
pBT6	Modified pUC12 containing a 3.1 kb *Hin*dIII fragment of the *N. crassa* β-Tubulin gene.	(Orbach *et al.*, 1986)

*NB: Fragments have the 3' end of the gene sequence adjacent to the forward primer annealing site in pUC118.

For growth experiments, fungi were grown in CDYE based media (section 2.2), under paxilline-inducing conditions. Large scale liquid cultures, from which numerous samples were removed, were grown in 2 L Erlenmeyer flasks containing 300 mL of media. Small scale cultures, from which only one or two samples were removed, were grown in 500 mL Erlenmeyer flasks, containing 100 mL of media. Cultures were inoculated from either a spore suspension (section 2.1.7) or a seed culture (section 2.1.8). All cultures were grown in a 28°C incubator with shaking at 350 rpm.

2.1.3 Glycerol stocks

Bacterial cultures (5 mL) were grown overnight at 37°C (section 2.1.2) and the following day harvested by centrifugation (10 min, 5 000 rpm) in a 15 mL falcon tube. The media was decanted off and the cells resuspended in 3 mL of sterile 50% (v/v) glycerol. Aliquots of 750 µL were transferred to four 1 mL Nunc cryotubes and the samples snap frozen in an ice-box. Two samples (Table 2.1) were stored at -70°C and the remaining two stored at -20°C.

2.1.4 λ Phage lysates

Lambda stocks were grown to confluent lysis (section 2.14.3), overlaid with 5 mL of SM buffer (section 2.3.8) and stored at 4°C overnight. The lysate was transferred to a sterile 15 mL falcon tube and approximately 100 µL of chloroform was added. The lysates were kept at 4°C for long term storage.

2.1.5 Spore suspensions

Glycerol stocks of *P. paxilli* (Table 2.1) stored at -20°C, were streaked for single colonies on PD agar (section 2.2) as described in section 2.1.2. When the cultures had sporulated, 6 mL of 0.01% (v/v) Tween 80 (BDH) was transferred to the surface of the plate, and spores collected using a glass spreader. The spore suspension was transferred to a sterile 1.5 mL Eppendorf tube and centrifuged in a microcentrifuge to pellet the

spores (13 000 rpm, 3 min). The detergent was decanted and the pellet resuspended in sterile Milli Q water. The concentration was determined using haemocytometer counting.

2.1.6 Glassware preparation

For large scale liquid cultures, Erlenmeyer flasks were siliconised to prevent excess build-up of mycelia around the rim of the culture vessel. The 2 L flasks were scrubbed clean, rinsed with 95% (v/v) ethanol and left to air dry at 37°C. When the glassware was completely dry, approximately 5 mL of Sigmacote® (Sigma) was poured into the vessel and coated over the entire inner surface. The excess was then drained off and the flasks left to dry in a fumehood. Siliconised glassware was reused several times before a new application of Sigmacote was needed. The required volume of test media was transferred to the clean flasks and a cheese-cloth/cotton wool bung placed in the neck of the vessel. The glassware and media was then sterilised by autoclaving for 15 min at 121°C with 15 psi. For smaller scale preparations the media and glassware was sterilised as above although the smaller flasks were not siliconised.

2.1.7 Spore suspension inoculation

Liquid *P. paxilli* cultures were inoculated directly from a spore suspension (section 2.1.5). Once the concentration had been determined, spore suspensions could be stored for up to 6 weeks at 4°C. Liquid cultures were inoculated to a final concentration of 1 x 10^6 spores/mL and grown as described in section 2.1.2.

2.1.8 Seed culture inoculation

Replica cultures of wild type *P. paxilli* were inoculated using a seed culture. A 1 L Erlenmeyer flask (section 2.1.6) containing 100 mL CDYE broth (section 2.2) was inoculated with a spore suspension (section 2.1.5). The culture was grown in a shaking incubator (350 rpm, 28°C) for 12 h. After this time, a small sample was removed and

examined microscopically for the presence of germinating spores. Replica cultures were inoculated with 8 mL of seed culture per 100 mL of media. Where the test media varied from the standard CDYE broth, the seed culture was pelleted by centrifugation (10 min, 3 100 rpm) and resuspended in an equal volume of sterile Milli Q water. The resuspension was then checked microscopically for the presence of germinating spores. A sample of the seed culture was plated on CDYE to check for the presence of any contaminants (section 2.1.2).

2.2 Media

All media and solutions were sterilised for 15 min at 121°C and 15 psi.

Czapek Dox salts

CD salts contain per 1 L: Dipotassium phosphate 1 g, magnesium sulfate 0.5 g, potassium chloride 0.5 g, ferrous sulfate 0.01 g, sodium nitrate 2 g and Milli Q water to 1 L.

Czapek Dox & yeast extract media

CDYE media contains per 1 L: Czapek Dox media (Oxoid) 33.4 g, yeast extract (Oxoid) 5 g and Milli Q water to 1 L. For solid media, 15 g/L agar (Davis) was added prior to sterilisation.

Luria-Bertani broth

LB media contains per 1 L: Sodium chloride 5 g, tryptone 10 g and yeast extract (Merck) 5 g and Milli Q water to 1 L. The pH was adjusted to 7-7.5 prior to sterilisation. For solid media, 15 g/L agar (Davis) or agarose (BDH) was added prior to sterilisation.

Potato dextrose media

PD media contains per 1 L: Potato dextrose media (Difco) 24 g and Milli Q water to 1 L. For solid media, 15 g/L agar (Davis) was added prior to sterilisation.

SOC media

SOC media contains per 1 L: Glucose 20 mM, KCl 2.5 mM, $MgCl_2$ 10 mM, $MgSO_4.7H_2O$ 10 mM, NaCl 10 mM, tryptone (Difco) 2% (w/v), yeast extract (Difco) 0.5% (w/v) and Milli Q water to 1 L.

Top agarose

Top agarose contains per 1 L: Tryptone (Difco) 10 g, NaCl 5 g, and agarose (BDH) 8 g.

2.3 Solutions

2.3.1 Stock solutions

Name	Concentration	pH
Ethanol	70% (v/v), 95% (v/v)	
Sodium Acetate	3 M	
Tween 80	0.01% (v/v)	
Sodium dodecyl sulfate (SDS)	10% (w/v)	
Tris-HCl	1 M	7.2, 7.5, 8.0
NA_2EDTA	250 mM	8.0
Glycerol	50% (v/v)	
Maltose	20% (w/v)	
$MgSO_4.7H_2O$	1 M	

2.3.2 Acrylamide mix

Acrylamide mix contains per 1 L: Urea 480 g, acrylamide 57 g and bis-acrylamide 3 g. These reagents were dissolved in 800 mL of Milli Q water and deionised by mixing with Amberlite MB-3 (Sigma). The solution was filtered through a sintered glass funnel and 10 mL of 10 x TBE sequencing buffer (section 2.3.10) added. Milli Q water was added to a final volume of 1 L.

2.3.3 10 x Denhardt's solution

Denhardt's solution (10 x) (Southern, 1975) contains per 1 L: 50 mL of 1 M HEPES (BRL) pH 7.0, 150 mL of 20 x SSC (section 2.3.9), 6 mL of phenol extracted herring sperm DNA (3 mg/mL, Sigma), 5 mL of 20% (w/v) sodium dodecyl sulfate (SDS), 2 g of Ficoll (Type 70, Sigma), 2 mL of *Escherichia coli* transfer RNA (10 mg/mL, Sigma), 2 g of Bovine Serum Albumin (BSA) (Sigma), 2 g of polyvinylpyrrolidone (PVP-10, Sigma), and Milli Q water to 1 L.

2.3.4 DNA extraction buffer

150 mM Na_2EDTA, 50 mM Tris-HCl pH 8.0, and 2% (w/v) Sodium Lauroyl Sarcosine (Byrd *et al.*, 1990).

2.3.5 DNase I buffer

100 mM sodium acetate and 5 mM $MgSO_4$ pH 5.0, in DEPC-treated water (section 2.17.2).

2.3.6 PEG solution

20% (w/v) PEG 6000 in SM buffer (section 2.3.8) without gelatin.

2.3.7 SDS loading dye

1% (w/v) SDS, 0.02% (w/v) bromophenol blue, 20% (w/v) sucrose and 5 mM Na_2EDTA (pH 8.0).

2.3.8 SM buffer

100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl (pH 7.5) and 0.01% (w/v) gelatin.

2.3.9 Southern blotting solutions (Southern 1975)

Solution 1: 0.25 M HCl.

Solution 2: 0.5 M NaOH and 0.5 M NaCl.

Solution 3: 2.0 M NaCl and 0.5 M Tris-HCl (pH 7.4).

SSC x 20: 5 M NaCl and 0.3 M sodium citrate.

SSC x 2: a 10 fold dilution of 20 x SSC.

2.3.10 Tris based buffers

STE buffer

100 mM NaCl, 10 mM Tris-HCl, and 1 mM Na_2EDTA (pH 8.0).

STET buffer

8% (w/v) Sucrose, 5% (v/v) Triton X-100, 50 mM Na_2EDTA (pH 8.0), and 50 mM Tris-HCl (pH 8.0).

1 x TAE

40 mM Tris, 20 mM Acetic acid and 2 mM Na_2EDTA (pH 8.5).

1 x TBE

89 mM Tris, 89 mM boric acid and 2.5 mM Na_2EDTA (pH 8.2).

10 x TBE for sequencing

1.3 M Tris-HCl, 4.4 M boric acid and 0.25 M Na_2EDTA.

TE (10:0.1)

10 mM Tris-HCl, 0.1 mM Na_2EDTA.

1 x TNE

10 mM Tris-HCl, 100 mM NaCl and 1 mM Na_2EDTA (pH 7.4).

2.3.11 Tris-equilibrated phenol

Tris-equilibrated phenol was supplied by Amersham and stored at 4°C. Prior to use, 0.47 g/ 500 mL of 8- Hydroxyquinone was added to keep the phenol reduced.

2.4 Enzymes and Biological Solutions

Ampicillin

An ampicillin stock solution was prepared with sterile Milli Q water to a final concentration of 100 mg/mL and stored at -20°C.

DNase (RNase free)

A stock solution of DNase I was prepared in 150 mM NaCl and 50% (v/v) glycerol to a final concentration of 10 mg/mL and stored at -20°C.

IPTG

A stock solution of isopropyl-β-D-galactoside (IPTG) was prepared in sterile Milli Q water to a final concentration of 20% (w/v) and stored at -20°C.

Lysozyme

A stock solution of Lysozyme was prepared in 10 mM Tris-HCl (pH 8.0) to a final concentration of 10 mg/mL and stored at -20°C.

RNase (DNase-free)

RNase A (Sigma) was prepared at a stock concentration of 10 mg/mL in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl. RNase was boiled for 10 min to inactivate any DNase.

Tetracycline

A tetracycline stock was prepared in methanol to a final concentration of 10 mg/mL, and stored at -20°C.

X-GAL

A stock solution of 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-GAL) was prepared in dimethylformamide to a final concentration of 2% (w/v) and stored at -20°C.

2.5 Isolation of DNA

2.5.1 Fungal DNA

Based on the method of Byrd *et al.* (1990), the freeze dried mycelia was weighed and 0.5-1 g ground to a fine powder under liquid nitrogen in a chilled mortar and pestle. The powdered mycelia was transferred to a fresh mortar at room temperature, resuspended in 10 mL extraction buffer (section 2.3.4) and Proteinase K (BRL) added to a final concentration of 2 mg/mL. The solution was transferred to a polypropylene tube and centrifuged at 2 000 x g (Sorval SS34, 4 000 rpm, 10 min, 4°C), to remove cellular debris. The cleared supernatant was transferred to a new tube and 1 volume of Tris-equilibrated phenol (section 2.3.11) added. The solution was mixed by inversion and separated by centrifugation (Sorval SS34, 13 000 rpm, 15 min, 4°C). Two volumes of chloroform/phenol (1:1) were added to the aqueous phase and mixed by inversion. The aqueous phase was separated as before and a final volume of chloroform added. After centrifugation, DNA was precipitated by the addition of one volume of isopropanol and the solution incubated on ice for 30 min. The DNA pellet was collected by centrifugation (Sorval SS34, 8 000 rpm, 10 min, 4°C), the supernatant removed and the pellet carefully washed with 1 volume of 70% (v/v) ethanol. If the pellet became dislodged a second centrifugation step was performed, before decanting off the 70% (v/v) ethanol. The DNA pellet was dried at 37°C and resuspended in 500 µL TE (section 2.3.10).

2.5.2 λ Phage DNA (medium scale)

Lambda clones (section 2.14) were diluted with SM buffer (section 2.3.8) in a sterile Eppendorf tube and plated (section 2.14.3) onto LB agarose plates (section 2.2). Plates were incubated at 37°C until they reached confluent lysis (~8h), overlaid with 5 mL of SM buffer (section 2.3.8) and stored at 4°C overnight. The lysate was transferred to a sterile 15 mL corex tube and the volume made up to 5 mL with SM buffer (section 2.3.8). KW251 cell debris was removed by centrifugation (Sorval SS34, 5 000 rpm, 10 min, 4°C), and the cleared supernatant transferred to a clean 15 mL corex tube. Contaminating *Escherichia coli* RNA and DNA was removed with the addition of RNase and DNase (section 2.4) to final concentrations of 1 μg/mL and the reaction incubated at 37°C for 30 min. To precipitate the phage, PEG solution (5 mL) (section 2.3.6) was added and the solution incubated on ice for 1 hr. Phage were harvested by centrifugation (Sorval SS34, 7 000 rpm, 20 min, 4°C), the pellet resuspended in 0.5 mL SM buffer (section 2.3.8) and transferred to a sterile Eppendorf tube. Phage coat proteins were dissolved by the addition of 5 μL 10% (w/v) SDS and 10 μL 250 mM Na_2EDTA (pH 8.0) and the solution incubated at 68°C for 15 min. To separate protein and DNA an equal volume of Tris-equilibrated phenol (2.3.11) was added and mixed thoroughly by vortexing. The aqueous phase was separated by centrifugation in a microcentrifuge (13 000 rpm, 5 min) and transferred to a fresh Eppendorf tube. Equal volumes of both Tris-equilibrated phenol (section 2.3.11) and chloroform were added and the aqueous phase separated by centrifugation as above. Finally, an equal volume of chloroform was added and the aqueous phase containing phage DNA was separated as above. Phage DNA was precipitated by adding an equal volume of isopropanol and the solution incubated at -20°C for 20 min. The DNA pellet was collected in a microcentrifuge (13 000 rpm, 10 min), and washed with 1 mL 70% (v/v) ethanol. After drying, DNA was resuspended in 50 μL TE (section 2.3.10) and 5 μL 200 μg/mL RNase (section 2.4) added. Uncut λ DNA (2 μL) was examined as in sections 2.10.1 and 2.10.3.

2.5.3 Plasmid DNA by rapid boil method

Based on method of Holmes & Quigley (1981). Plasmid containing colonies were grown in 3 mL of LB media (section 2.2) containing ampicillin (section 2.4) as described in section 2.1.2. Cells were harvested from 1.5 mL of culture in a microcentrifuge (13 000 rpm, 3 min) and resuspended in 350 µL of STET buffer (section 2.3.10). Lysozyme (25 µL) (section 2.4) was added and the mixture boiled for 40 sec. Precipitated material was pelleted in a microcentrifuge for 10 min at 13 000 rpm and the resulting gelatinous pellet was removed with a sterile toothpick. DNA was precipitated by adding of 300 µL of isopropanol and the solution incubated at -20°C for 20 min. The DNA was pelleted in a microcentrifuge (13 000 rpm, 5 min), the supernatant discarded and the pellet washed with 350 µL of 95% (v/v) ethanol. The pellet was reconstituted in a microcentrifuge (13 000 rpm, 2 min) and allowed to dry at 37°C for approximately 15 min. DNA was resuspended in 50 µL of TE (section 2.3.10) and stored at 4°C until required. Plasmid DNA was examined by restriction enzyme digestion (section 2.7) and mini-gel electrophoresis (section 2.10.1 and 2.10.3).

2.5.4 DNA from agarose gel slices

SeaPlaque agarose gel slices containing DNA were excised under long wave UV with a sterile scalpel blade. The gel slices were placed in a sterile 1.5 mL Eppendorf tube and melted at 65°C. Tris-equilibrated phenol (section 2.3.11) (500 µL) was added, mixed thoroughly for 15 sec and the mixture left to incubate at -20°C for two and a half hours. The aqueous phase was separated in a microcentrifuge (13 000 rpm, 10 min) and transferred to two sterile 1.5 mL Eppendorf tubes. One volume of Tris-equilibrated phenol:chloroform was added, mixed by vortexing and the aqueous phase separated in a microcentrifuge (13 000 rpm, 3 min). Traces of phenol were removed with the addition of one volume of chloroform and centrifugation as above. The aqueous phases of both tubes were then pooled into a single sterile 1.5 mL Eppendorf tube and DNA precipitated by the addition of 1/10 x volume 3 M sodium acetate and 2 volumes of isopropanol. The reaction was incubated overnight at -20°C, and the DNA pelleted in a microcentrifuge (13 000 rpm, 15 min). The supernatant was decanted off and the pellet washed with 700 µL of 95% (v/v) ethanol, then centrifuged (13 000 rpm, 2 min). The

clean pellet was left to dry briefly at 37°C, resuspended in 20 μL of sterile Milli Q water and stored at 4°C.

2.5.5 Plasmid DNA by Alkaline lysis-based miniprep kits

A number of small scale plasmid isolation kits have been used in the process of this work including the Quantum Prep® Plasmid Miniprep kit (Biorad), the QIAprep Spin Miniprep kit (Qiagen) and the CONCERT™ Rapid Plasmid Miniprep System (BRL). In all cases the general protocol was based on the basic alkaline lysis technique. Bacterial cultures were grown overnight in 5 mL of LB media (section 2.2) containing ampicillin (section 2.4) as described in section 2.1.2. Cells were harvested from 3 mL of culture and resuspended according to the protocol outlined in the particular kit being used.

2.6 Quantification of Nucleic Acids

There are a number of methods available for the quantification of nucleic acids. Genomic DNA or DNA isolated from agarose gel was frequently compared to an appropriate DNA mass standard by agarose gel electrophoresis (section 2.10.1) and ethidium bromide staining (section 2.10.3). Plasmid DNA, and samples where only a small amount of material was available, were determined fluorometrically using Hoechst dye. UV spectrophotometry was used to determine the concentration and quality of RNA and DNA.

2.6.1 Intensity of ethidium bromide fluorescence

Genomic DNA was quantified by running on a 0.7% (w/v) agarose gel (section 2.10.1) alongside λ concentration standards (range 10-200 ng). Smaller DNA fragments isolated from agarose gels were run alongside the Low DNA Mass™ ladder (BRL) (range 5-100 ng) on appropriate % (w/v) agarose gel. DNA was visualised with ethidium bromide (section 2.10.3).

2.6.2 Fluorometeric determination

Using the Hoefer Scientific TKO 100 fluorometer, extremely small quantities of DNA can be quantified. The standards and samples were quantified in 1 x TNE buffer (section 2.3.10) containing 0.1 μg/mL Hoechst 33258 dye. A Calf thymus DNA (100 μg/mL) standard was used to calibrate the machine.

2.6.3 UV spectrophotometric determination

Nucleic acid concentrations were determined on a Shimadzu UV spectrophotometer by readings at A_{260}. Samples were diluted so that absorbency readings fell between the range of 0.1 to 1.0. Sample purity was determined by calculating the ratio between A_{260} and A_{280}.

2.7 Restriction Endonuclease Digestion of DNA

Restriction endonucleases (RE) were used with the specific buffer recommended by the manufacturer. Incubations were performed in a 37°C water bath for 1-2 h. After this time a small sample was run on an agarose gel (section 2.10.1 and 2.10.3) to determine if digestion was complete. For incomplete digestions, additional enzyme was added and the digestion continued for another hour. Digestion was terminated with the addition of 1/5 volume SDS loading dye (section 2.3.7). In cases where two RE were used, the enzyme requiring the lower salt buffer was used first. The salt concentration was then altered by the addition of NaCl and the second digestion performed.

2.8 Purification of DNA with Phenol/Chloroform

DNA was routinely purified by phenol/chloroform extraction in equal volumes of Tris-equilibrated phenol (section 2.3.11) and chloroform. Reagents were mixed by vortexing for at least 10 seconds. The aqueous phase containing the DNA was separated in a microcentrifuge (13 000 rpm, 2 min). The clear upper phase was transferred to a new

Eppendorf tube and an equal volume of chloroform added. The aqueous phase was again separated as above and transferred to a new Eppendorf tube.

2.9 Precipitation of DNA with Ethanol or Isopropanol

DNA in solution was precipitated with either 95% (v/v) ethanol or isopropanol. Precipitation with ethanol required the addition of 1/10 volume 3 M sodium acetate and two and a half volumes of 95% (v/v) ethanol. Precipitation with isopropanol required the addition of 0.6 volumes of isopropanol. The reactions were then incubated either on ice or at -20°C for approximately 30 min. The precipitated DNA was pelleted by centrifugation in a microcentrifuge (13 000 rpm, 5 min) and the supernatant removed. The pellet was washed gently with an equal volume of 75% (v/v) ethanol then left to air dry for approximately 15 min. Samples were then resuspended in either sterile Milli Q water or TE (section 2.3.10).

2.10 Agarose Gel Electrophoresis

2.10.1 Mini-gel

For most DNA separations, gels were prepared and run in 1 x TBE buffer (section 2.3.10), with the concentration of agarose appropriate for the size of fragments to be separated (Table 2.2). Molecular biology grade agarose (BRL) was melted using a microwave then allowed to cool to ~50°C before pouring into the mini-gel apparatus. For separation of low molecular weight bands, such as PCR products, NuSieve agarose (FMC) was used. When DNA was purified from agarose gel slices, the gels were prepared and run in 1 x TAE (section 2.3.10) and a low melting temperature grade agarose such as SeaPlaque agarose (FMC) was used. Gels were run between 60-100 volts. In general, 10 μL samples, containing 1/5 volume SDS loading dye (section 2.3.7), were transferred into each well and run alongside 5 μL of the appropriate size ladder.

Table 2.2 Separation of DNA by agarose gel electrophoresis

Agarose % (w/v)	Separation	DNA ladder
0.7	2 kb-25 kb	1 kb ladder (BRL), λ/*Hin*dIII
1.0	5 kb-10 kb	1 kb ladder (BRL), λ/*Hin*dIII
2.0	0.3 kb-1.5 kb	1 kb plus Ladder™ (BRL), 100 bp ladder (BRL), (BioLabs)*
3.0	0.1 kb-1 kb	1 kb plus Ladder™ (BRL), 100 bp ladder (BRL), (BioLabs)*

*NB: 100 bp ladder from two different suppliers were used. The ladder from BRL has the 600 bp band of brightest intensity. The ladder from BioLabs has the 500 bp band of brightest intensity.

2.10.2 Submarine gel

A 150 mL 0.7% (w/v) agarose gel prepared in 1 x TBE buffer (section 2.3.10) was poured into the apparatus with the required number of wells. DNA samples containing 1/5 x volume SDS dye were loaded into wells alongside 25 μL of the appropriate ladder. Electrophoresis was performed overnight at 30 volts, and DNA visualised as in section 2.10.3. To aid the sizing of DNA fragments, the gel was photographed next to a fluorescent 15 cm ruler.

2.10.3 Staining DNA with ethidium bromide

DNA separated by agarose gel electrophoresis was visualised by staining the gel in a 2 μg/mL ethidium bromide solution for 15 min. Excess ethidium bromide was removed by destaining in RO water for 15 min and the gel exposed to short wave UV transillumination.

2.11 Polymerase Chain Reaction (PCR)

2.11.1 Primer design

Degenerate primers HMG1 and HMG3 (Dobson, 1997), designed to conserved regions of the 3' end of *hmg* genes, contained a number of Inosine residues. Gene specific primers were designed to have a GC content of between 40-60% and the 3' position was always a G or C nucleotide. Primers were ordered from either Life Technologies or Sigma Genosys (Table 2.3).

2.11.2 Protocol for routine PCR

For routine PCR, the following protocol was used. A cocktail was prepared containing (n+1) x [1 x Taq DNA polymerase Buffer (Roche), 50 μM dNTPs , 200 nM Primer 1, 200 nM Primer 2, 0.5 U Taq DNA polymerase (Roche) and sterile Milli Q water to a final volume of 20 μL]. The reaction mix was (20 μL) was aliquoted into n x 0.2 mL PCR tubes and stored on ice. To one of these tubes 5 μL of sterile water was added, to serve as a negative control. Where possible a positive control was included as well. The DNA template was diluted to a suitable concentration and 5 μL added to the remaining tubes. Reactions were placed in a Corbett thermocycler, preset to 94°C, and the following PCR programme performed; 1 cycle of 2 min at 94°C, 25 cycles of 45 sec at 94°C, 45 sec at 60°C and 90 sec at 72°C, 1 cycle of 5 min at 72°C. Reactions were terminated by the addition of 1/5 volume of SDS loading dye (section 2.3.7) and separated by mini-gel electrophoresis (section 2.10.1 and 2.10.3). The primers used are listed in Table 2.2.

2.11.3 Bacterial colony PCR

Where specific primers were available, transformed bacterial clones (section 2.15) could be identified directly using PCR. A routine PCR cocktail (section 2.11.2) was prepared to a final volume of 25 μL. Primer 1 and Primer 2 were specific for the DNA insert. Using a sterile P10 pipette tip a small amount of the ampicillin resistant colonies

Table 2.3 Primers

NAME	SIZE (nt)	SEQUENCE	ORGANISM	GENE	APPLICATION
M13Forward	17	GTT TTC CCA GTC ACG AC			Seq
M13Reverse	17	CAG GAA ACA GCT ATG AC			Seq
AUAP	20	GGC CAC GCG TCG ACT AGT AC			5'RACE/cloning
AAP	36	GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG			5'RACE
UP	23	GAG AGA ATT CGG ATC CTC TAG AG			3'RACE/cloning
Oligo dT	43	GAG AGA ATT CGG ATC CTC TAG AGT TTT TTT TTT TTT TTT TTT T			3'RACE
ET1	20	TGA ATC AAG TTC CAG TCT CC	*P. paxilli*	*hmg*	Seq
ET2	20	GTG GGA GAT TGT TTC TCA AG	*P. paxilli*	*hmg*	Seq/RT-PCR
ET3	20	GAA TTA CCG CCG TGT TGC CG	*P. paxilli*	*hmg*	Seq/RT-PCR/cloning
ET4Bam	25	CTG GAT CCT CGA GTG CAG TAA GTT C	*P. paxilli*	*hmg*	Seq/RT-PCR
ET5	20	AAC ATC GTC GAT CAC AGG AC	*P. paxilli*	*hmg*	RT-PCR/cloning
ET6	20	TTG CTG CCT CTG TCC ATC AG	*P. paxilli*	*hmg*	RT-PCR
ET7	18	CAG AGT AGG GAA GGT CAC	*P. paxilli*	*hmg*	Seq
ET8	20	TCC TTG CGG ACC GTC ATA AC	*P. paxilli*	*hmg*	RT-PCR
ET9	18	GCG CAG AAT GGT GAA TTG	*P. paxilli*	*hmg*	Seq/RTR-PCR/cloning
ET10	20	AAG ATG CTT GGA CCG AAC AG	*P. paxilli*	*hmg*	RT-PCR/cloning
ET11	18	TGC TCG TTT GGT TCG AAC	*P. paxilli*	*hmg*	Seq
ET12	20	AGT CGG CAA TCG CGT GTT TC	*P. paxilli*	*hmg*	RT-PCR/cloning
ET13	18	ACG TTG CTG GAC TTG ATC	*P. paxilli*	*hmg*	Seq
ET14	18	CAG CAG TTC TGC TTC CTC	*P. paxilli*	*hmg*	Seq

NAME	SIZE (nt)	SEQUENCE	ORGANISM	GENE	APPLICATION
ET15	18	GAG CAG TAT CCA ACA GAC	*P. paxilli*	*hmg*	Seq/RT-PCR/cloning
ET16	18	CAA CGC TGT GAT GAT TTC	*P. paxilli*	*hmg*	Seq
ET18	18	AGT ACA AGC TGG AGT ACC	*P. paxilli*	*hmg*	Seq
ET20	18	TCT TGT TCA CGG TGC TTG	*P. paxilli*	*hmg*	Seq/RT-PCR/cloning
ET22	18	TTG GTC GAG CTC AAT GTG	*P. paxilli*	*hmg*	Seq/RT-PCR/cloning
ET24	20	CTC CAA TTC ATC TCT CGT CG	*P. paxilli*	*hmg*	RT-PCR
HMG1	17	ATG GGI ATG AAI ATG AT	Degenerate	*hmg*	Gene isolation/Seq
HMG3	23	ACI TTT TGI GCI GGI TCT TGI CC	Degenerate	*hmg*	Gene isolation/Seq
hmgeco	25	CTG CAG AAT TCA ATT CAG GGT CCA G	Lp19	*hmg*	RT-PCR/cloning
hmg2eco	25	ATT CGG AAT TCG TAC AAG CCT TTG G	Lp19	*hmg*	RT-PCR
hmgnco	25	AGA TAC CAT GGC GAC TTG ATC GGA A	Lp19	*hmg*	Seq/RT-PCR/cloning
hmg29	21	GAC GTG GTC AAA TCC GTG TTG	Lp19	*hmg*	RT-PCR
hmg30	20	GTT GGA TGT GAT CCT GCG TG	Lp19	*hmg*	RT-PCR
Etub1	20	GCC GAG AAC TAA CAA AGA TC	*P. paxilli*	*tub-2*	Seq
Etub2	20	AGG CGT TGG CAT TAC GGC TG	*P. paxilli*	*tub-2*	Seq/RT-PCR
Etub3	20	CCG ACG AGA CCT TCT GTA TC	*P. paxilli*	*tub-2*	Seq/RT-PCR
Etub4	18	CTG TCC ATC ACC GTC AAG	*P. paxilli*	*tub-2*	Seq
Etub5	18	ATA GGT TCA CCT CCA GAC	*P. paxilli*	*tub-2*	Seq/RT-PCR/cloning
Etub6	18	GGG ATA GAG AAT GTA GGC	*P. paxilli*	*tub-2*	Seq/RT-PCR/cloning
CYP1	20	GCT TGC CTC GAG CCT TCT TC	*P. paxilli*	*paxP1*	RT-PCR
CYP6	20	GAC AAG GGA CAT ATC TTG AC	*P. paxilli*	*paxU1*	RT-PCR

NAME	SIZE (nt)	SEQUENCE	ORGANISM	GENE	APPLICATION
CYP8	20	TCT GCA CAC TGA GAC AAT TG	*P. paxilli*	*paxP1*	RT-PCR
dmat4	20	TTT CGA CAC TAT GTT GAC CG	*P. paxilli*	*paxD*	RT-PCR
dmat7	20	TCC TTA ACT CTC GTA TAC GG	*P. paxilli*	*arb*	RT-PCR
dmat10	20	TAC CAA AGA ACA TCG GAC AG	*P. paxilli*	*arb*	RT-PCR
dmat15	20	GCC CCT TGT ATG AGA AAG AG	*P. paxilli*	*paxD*	RT-PCR
ggpps4	20	ATC TAG CAG TGA CCT TGT AA	*P. paxilli*	*paxG*	RT-PCR
ggpps9	20	TTT CTT CCA GTC CCA TTT CT	*P. paxilli*	*paxG*	RT-PCR
ggpps36	18	GTA TTC GTC TTC GGT GGG	*P. paxilli*	*ggs-1*	RT-PCR
ggpps40	18	GCC CAT ATG ACT ATA TGC	*P. paxilli*	*ggs-1*	RT-PCR
mono3	20	CTG CGC CGA CAA GAA GAT CC	*P. paxilli*	*paxM1*	RT-PCR
mono4	20	AGC ATA TCG AAC CGC TAA GC	*P. paxilli*	*paxM1*	RT-PCR
paxP2p1	18	GCC GAG ACA GAC TTT CTG	*P. paxilli*	*paxP2*	RT-PCR
pax3	20	ATC GAA GCC TGC TGC TAC GG	*P. paxilli*	*lip1*	RT-PCR
pax5	20	TTG CGA GAG AGA CTC CGC TG	*P. paxilli*	*dec*	PCR
pax6	20	CGG AGA TGT ACT AGG AAT AC	*P. paxilli*	*dec*	PCR
pax7	20	CAA TGG CAT CAT CGG TGT CC	*P. paxilli*	*amy*	RT-PCR
pax8	20	ACA TCC ATA ACA GTC GAC TC	*P. paxilli*	*amy*	RT-PCR
pax9	20	ATC CAT ACC AGG ACA GGT AG	*P. paxilli*	*lip1*	RT-PCR
pax39	20	GCC CAC CAA ATT CTC TTC CA	*P. paxilli*	*paxR1*	RT-PCR
pax40	20	TTC ATC GGA GTC AGC CTT TG	*P. paxilli*	*paxR1*	RT-PCR
pax58	20	TGC TTG AAA CAA TGG ATG TC	*P. paxilli*	*paxU1*	RT-PCR
pax60	20	GAA TTG ACT ATT CCT GAA GG	*P. paxilli*	*paxP2*	RT-PCR

NAME	SIZE (nt)	SEQUENCE	ORGANISM	GENE	APPLICATION
pax69	20	ACG ATT CTT CCC AGT TGG AC	*P. paxilli*	*paxO*	RT-PCR
pax70	20	GCC ATC AGT GAC ACA TAA TG	*P. paxilli*	*paxO*	RT-PCR
pax81	20	GAG GAG TTC CGC AAC TAC AC	*P. paxilli*	*paxA*	RT-PCR
pax84	20	AGT AGC CTT GGC CAA CCA	*P. paxilli*	*paxA*	RT-PCR
pax112	18	TTC AGT CGA CTC AGT ACC	*P. paxilli*	*paxH*	RT-PCR
pax113	18	CTA TAT CGT TCT CGC GAC	*P. paxilli*	*paxH*	RT-PCR
pax114	18	TTG TTG GAG CCA TCT TGG	*P. paxilli*	*paxT*	RT-PCR
pax115	18	AAG ATG CGC CAC CAG TAC	*P. paxilli*	*paxT*	RT-PCR
pax133	17	GAT AGG TGG AAA GCG TC	*P. paxilli*	*paxM2*	RT-PCR
pax134	18	AAT GAG TCT TGG ACT CCG	*P. paxilli*	*lip2*	RT-PCR
pax135	18	CGT ATA CGA GCC TGC ATC	*P. paxilli*	*lip2*	RT-PCR
pax138	18	ATC GCC ATT GGC ATC GAG	*P. paxilli*	*paxM2*	RT-PCR

were transferred to each reaction tube. A water control was left without any template, and a positive control containing 2 µL of gDNA (0.5 ng/µL) was included. Tubes were placed in a Corbett thermocycler preset to 95°C and the following PCR programme performed; 1 cycle of 3 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 60°C and 90 sec at 72°C, and 1 cycle of 5 min at 72 °C. PCR products were visualised as in section 2.11.2.

2.11.4 Gel stab PCR

When multiple PCR products were produced or a single product was weakly amplified, a second round of amplification could be performed on a small sample of DNA isolated directly from the agarose gel. Under long wave UV transillumination, a disposable P20 pipette tip was used to remove a small piece of gel from each product and transfer it to an Eppendorf containing 100 µL of sterile Milli Q water. The agarose plug was melted at 65°C. Second round PCR amplification was performed with serial dilutions (10-, 10^2-, and 10^3-fold) of the isolated plug solution from each product. The new reactions were separated on an appropriate agarose gel (section 2.10.1 and 2.10.3). Purified products could then be isolated directly from the agarose gel (section 2.5.4) or cleaned using a PCR purification kit (section 2.11.5).

2.11.5 Purification of PCR products

When PCR reactions yielded a single product, they were purified with the CONCERT™ Rapid PCR purification system (BRL). This was done according to the instructions provided with the kit, except for step 5 (DNA elution), where DNA was eluted with 50 µL warm sterile Milli Q water instead of TE.

2.12 DNA Sequencing

2.12.1 Cycle sequencing with the AmpliCycle™ kit

Sequencing was performed with the AmpliCycle™ sequencing kit (Perkin-Elmer) which uses the Sanger dideoxy chain termination method (Sanger *et al.*, 1977). The following, 30 μL cocktail was prepared; (1-3 μg template DNA, 3.2 pM of appropriate primer, 4 μL 10 x Cycling mix, 1 μL [α-^{33}P] dATP (Amersham) and sterile Milli Q water). Four 0.2 mL PCR tubes were prepared for individual cocktail reactions, each containing 2 μL of one of the four chain terminating dideoxy nucleotides (ddATP, ddGTP, ddTTP, or ddCTP). Then 6 μL of cocktail reaction was added to each of the four tubes. Reactions were placed in a Corbett thermocycler preset to 95°C and the following PCR programme performed; 1 cycle of 1 min at 95°C, 25 cycles of 30 sec at 95°C, 30 sec at 48°C, and 1 min at 72°C, and 1 cycle of 1 min at 72°C. Reactions were terminated with the addition of 4 μL of stop solution and stored at -20°C until required.

2.12.2 Denaturing polyacrylamide gel electrophoresis

Polyacrylamide gels were poured with 60 mL of acrylamide mix (section 2.3.2) to which 36 μL of TEMED and 360 μL of 10% (w/v) ammonium persulfate had been added. The gel apparatus was assembled in 1 x sequencing TBE buffer (section 2.3.10) and run for 15 min at 1500 Volts, prior to sample loading. The radioactively labelled sequencing reactions (section 2.12.1) were denatured for 2 min at 75°C, and 3 μL loaded into each well. The gel was run at 1500 volts, limited by approximately 65 watts, with duplicate samples being run through either one (short run) or three (long run) dye fronts. The gel was transferred to blotting paper, dried for 1 hr at 80°C and exposed to x-ray film for several days. The order of bands revealed was manually recorded.

2.12.3 Automated sequencing

Automated sequencing using the Sanger dideoxy chain termination method (Sanger *et al.*, 1977) was carried out by the MuSeq DNA analysis facility at Massey University, according to the recommendations of the facility operator Lorraine Berry. Sequencing reactions incorporating ABI PRISM™ dye or ABI PRISM™ BigDye™ chemistry (PE Applied Biosystems, Foster City, Calif.) were separated on either an ABI 373 or 377 DNA Sequencer (Perkin-Elmer Technologies), and viewed using the Edit View programme (Version 1.0.1, Perkin-Elmer Technologies).

2.13 Southern Blotting and Hybridisation

2.13.1 Capillary transfer of DNA to nylon membrane

DNA was transferred from agarose gel to a nylon membrane using the Southern protocol (Southern, 1975). The submarine gel (section 2.10.2) was carefully washed in solution 1 for 15 min, solution 2 for 30 min, solution 3 for 30 min, and finally for 2 min in 2 x SSC (section 2.3.9). Two sheets of Whatman 3MM paper soaked in 20 x SSC were placed in the blotting apparatus, to act as a wick between two reservoirs of blotting buffer (20 x SSC). A sheet of glad-wrap plastic film, with a hole cut in it slightly smaller than the dimensions of the gel, was positioned between the gel and the wick, to ensure uni-directional capillary transfer through the gel. A pre-soaked sheet of Hybond™-N+ Nylon membrane (Amersham), cut to the appropriate size, was place on the gel, followed by two sheets of wet Whatman 3MM paper, two sheets of dry Whatman 3MM paper and a stack of paper towels. Finally, a weight was added to the top and the apparatus left overnight. The membrane was washed in 2 x SSC for 5 min and baked at 80°C in a vacuum oven for 2 h to fix the DNA to the membrane.

2.13.2 Labelling DNA with [α-^{32}P] dCTP

Purified DNA (20 ng) was made up to a final volume of 11 µL with sterile Milli Q water in a 1.5 mL Eppendorf tube. Using a 22 gauge syringe needle, a hole was pierced

in the top of the tube and the DNA denatured by boiling for 3 min. The single stranded DNA was stored on ice, following the addition of 4 μL of High prime reagent (Roche). Radioisotope (5 μL of [α-^{32}P] dCTP (Amersham)) was added and the mixture incubated for 1 hr at 37°C. A Probequant micro column G-50 (Amersham) was prepared by placing it on top of a 1.5 mL Eppendorf tube and spinning in a microcentrifuge for 1 min at 2 000 rpm. The compacted column was transferred to a new Eppendorf tube and the probe mixture transferred to the top of the column. Unincorporated dNTPs were separated from the labelled probe by centrifugation at 2 000 rpm for 2 min. Labelled probe was collected in the new Eppendorf, made up to a final volume of 50 μL with STE buffer (section 2.3.10) and stored at -20°C.

2.13.3 Hybridisation of [α-^{32}P] dCTP-labelled probe

Nylon membranes were pre-hybridised in 25 mL of 10 x Denhardt's solution (section 2.3.3) at 65°C for 2 h. Labelled probe (section 2.13.2) was removed from -20°C and denatured by boiling for 3 min. The single stranded DNA probe was stored on ice before being transferred to the hybridisation tube. Labelled probe homologous to λ DNA was also added when λ/*Hin*dIII ladder had been included on the submarine gel. The membranes were incubated overnight at 65°C with gentle shaking in a Bachofer rotating oven. The following morning, excess probe was removed by washing once in 2 x SSC (section 2.3.9) for 5 min, four times in 2 x SSC for 10 min, and once in 0.5 x SSC for 10 min. The washed membranes were positioned between Glad-wrap plastic film, and exposed to x-ray film (100NIF, Fuji) for several days at -70°C. Film was developed manually according to manufacturers instructions.

2.13.4 Stripping labelled probe from nylon membrane

After the exposed x-ray film had been developed, labelled probe was stripped from the nylon membranes by repeated washes in boiling 0.1% (w/v) SDS that was allowed to cool to room temperature. The washed membranes were then re-exposed to x-ray film to ensure the complete removal of the labelled probe.

2.14 Library Screening

2.14.1 Titre of a λGEM®-11 genomic DNA library

An Eppendorf containing an aliquot of a λGEM®-11 *P. paxilli* genomic DNA library (Itoh & Scott, 1994b) was removed from -70°C storage and a drop of chloroform added. A set of serial dilutions (10^2 - 10^8) were made with SM Buffer (section 2.3.8), and 2 x 100 µL of each transferred to duplicate sterile Eppendorf tubes. The library was plated as described in section 2.14.3. Plaque forming units were counted and the library titre calculated.

2.14.2 Calculating library concentration required for isolating clone

To maximise the probability of isolating a gene of interest, the following equation was used.

$$N = \ln(1-P) / \ln[1-(I/G)]$$

Where N = the number of plaques needed to screen, I = Insert size (15 kb), G = Genome size (30 000 kb), and P = Probability of success (0.999).

N is equal to 13 812 plaques. Maximum density of plaques per plate is 3 000, therefore 5 plates containing 3 000 plaques per plate should be sufficient. To ensure success, 10 plates, each containing approximately 3 000 plaques, were screened in the first round.

2.14.3 Library plating

Two sterile 20 mL tubes containing 5 mL of LB media (section 2.2), 0.2% (w/v) Maltose, and 10 mM $MgSO_4$, were inoculated with KW251 cells (Table 2.1) and grown overnight (section 2.1.2). The appropriate dilution of library in a final volume of 100 µL was transferred to a sterile 1.5 mL Eppendorf tube. To each tube, 100 µL of the KW251 culture was added. One tube, containing 100 µL of KW251 cells only, was also included as a control, and all tubes were incubated at 37°C for 30 min. The library dilution and KW251 cells were transferred to sterile 5 mL tubes pre-equilibrated at 50°C

and 3 mL of Top agarose (section 2.2) containing 10 mM $MgSO_4$ was added with gentle vortexing. The top agarose was quickly transferred to LB agar plates (section 2.2) and incubated for approximately 6-8 hours at 37°C. Plates were then stored at 4°C until the following morning.

2.14.4 Transfer of plaques to nylon membranes

Plaques were transferred to Hybond™-N+ circular membranes (Amersham). Where duplicate lifts were performed, the first membrane was overlaid for 1 min and the second membrane for 2 min. For single lifts, the membrane was overlaid for 1 min. The membranes were left to air dry, then washed in solution 2 for 2 min, in solution 3 for 5 min and finally in 2 x SSC (section 2.3.9) for 2 min. DNA was permanently fixed to the membrane by baking for 2 h at 80°C in a vacuum oven.

2.14.5 Hybridisation of [α-^{32}P] dCTP-labelled probe

Membranes were pre-hybridised in approximately 25 mL of 10 x Denhardt's solution (section 2.3.3) at 65°C for 2 h. The radio-labelled probe (section 2.13.2) was hybridised as described in section 2.13.3 in a shaking water bath. After the final wash in 0.5 x SSC, the membranes were carefully orientated between two layers of plastic wrap and exposed to x-ray film (100NIF, Fuji) at -70°C. Phosphorescent Tracker Tape™ (Amersham) was used to orientate the films for future reference.

2.14.6 Isolation of positive clones

After developing the exposed x-ray film, plaques that appeared on duplicate membranes were isolated by aligning the appropriate plate to the x-ray film. An approximate 5 mm plug was removed and transferred to a sterile Eppendorf containing 500 µL SM buffer (section 2.3.8) and a few drops of chloroform.

2.14.7 First round library screening

The λGEM®-11 *P. paxilli* genomic DNA library was diluted to the required concentration [3 μL library and 97 μL SM Buffer (section 2.3.8)] and plated as described in section 2.14.3. Plaques were transferred to duplicate nylon membranes (section 2.14.4), hybridised to the gene specific probe (section 2.14.5) and positive clones isolated (section 2.14.6).

2.14.8 Second and third round library screening

A 10^2-, 10^3-, and 10^4-fold dilution of the isolated clones were prepared with SM buffer (section 2.3.8) in sterile Eppendorf tubes, and plated as described in section 2.14.3. Duplicate lifts were performed for second round screening and single lifts for third round screening (section 2.14.6). Lifts were taken from whichever dilution plate had produced between 100 and 300 plaques in second round or 50 and 200 plaques in third round. Membranes were hybridised with the labelled probe as in section 2.14.5. After development of the exposed x-ray film, two positive plaques were isolated from each clone as described in section 2.14.6.

2.15 Sub-Cloning

2.15.1 Ligation into CAP'd pUC118

DNA to be inserted was purified prior to ligation. Lambda DNA was digested with *Sst*I as described in section 2.7 and the DNA purified by phenol/chloroform (section 2.8) and ethanol precipitation (section 2.9). The ligation reactions contained; 2 μL of 10 x Ligase buffer (BioLabs), 1.5 μL of CAP'd *Sst*I digested pUC118, *Sst*I digested λ clone in a 2-3 molar excess of insert:vector, 0.1 μL of T4 DNA Ligase (BioLabs) and sterile Milli Q water to a final volume of 20 μL. The ligation mixes, including a vector only control, were incubated overnight at 4°C. A sample (3 μL) was removed before the addition of enzyme and after the incubation period to confirm ligation was successful. The samples were run on a 0.7% (w/v) agarose gel (section 2.10.1 and 2.10.3).

2.15.2 Ligation into pGEM®-T and pGEM®-T Easy

Purified PCR products (section 2.11.5) were cloned into either the pGEM®-T or the pGEM®-T Easy vector from Promega. The ligation reactions were set up according to the recommendations provided and contained; 5 μL of ligation buffer x 2, 0.5 μL of pGEM®-T or pGEM®-T Easy vector, 3.5 μL of purified PCR product and 1 μL of T4 Ligase. The reactions were incubated at 4°C overnight.

2.15.3 Electrocompetent cells

Escherichia coli strain XL-1 (Table 2.1) was grown in 5 mL LB media (section 2.2) with tetracycline as described in section 2.1.2. The 5 mL *E. coli* culture was sub-cultured into 500 mL LB media (section 2.2) containing tetracycline. The cultures were grown with vigorous shaking (300 rpm) at 37°C to mid-log phase (optical density of 0.5-1.0 at 600nm), which usually occurred after 3 h growth. The cells were chilled on ice for 20 min before being pelleted by centrifugation at 4 000 x g (Sorval GSA, 10 min, 5 000 rpm, 4°C). After the supernatant was decanted, the cells were resuspended in 1 L of chilled water. The cells were harvested as above and resuspended in 500 mL of chilled water. The cells were harvested a third and fourth time as above and resuspended in firstly, 20 mL of chilled 10% (v/v) glycerol and finally 4 mL of chilled 10% (v/v) glycerol. Aliquots of 40 μL were transferred to sterile 1.5 mL Eppendorf tubes, and stored at -70°C.

2.15.4 Electroporation

Based on the method of Dower *et al.* (1988). Individual Eppendorf tubes containing 40 μL of electrocompetent XL-1 cells (section 2.15.3) were removed from -70°C and 2 μL of ligation mixture (section 2.15.1 and 2.15.2) added. The two controls for electroporation transformation were 2 μL of pUC118 (20 ng/μL) added to 40 μL XL-1 cells and a tube containing 40 μL of XL-1 cells only. The transformation mixes were transferred to chilled electroporation cuvettes (Bio-Rad) and placed in the electroporation bracket. The samples were pulsed with 2.5kV, 25μFD and 200Ω in a

Bio-Rad Gene pulser, and 1 mL of SOC media (section 2.2) added. The transformation mixes were transferred to a new sterile 1.5 mL Eppendorf tube and incubated for 1 hr at 37°C.

2.15.5 Selection of transformants

Transformation mixes (100 µL and 50 µL) were plated onto LB agar plates (section 2.2), containing ampicillin and 40 µL of both IPTG and X-GAL (section 2.4.). Colonies containing recombinant pUC118, pGEM®-T or pGEM®-T EASY were identified as ampicillin resistant white colonies. Serial dilutions of the pUC118 only transformation mix were also transferred to the selection media, as was 100 µL of the XL-1 cells only transformation mix. Serial dilutions of the XL-1 cells only transformation mix were then plated onto LB agar plates (section 2.2) to determine the concentration of viable cells. All plates were incubated overnight (section 2.1.2).

2.15.6 Drop-outs and mapping

Where DNA inserts were particularly large, further sequence information could be obtained be removing restriction enzyme fragments from the insert and religating novel sequence next to the vector's forward primer site. Restriction enzyme sites in the pUC118 multiple cloning cassette include; *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Xma*I, *Bam*HI, *Sal*I, *Acc*I, *Hin*cII, *Pst*I, *Sph*I and *Hin*dIII. Once successful sub-clones containing the gene of interest had been identified by automated sequencing (section 2.12.3) with the universal forward and reverse primers (Table 2.2), these restriction enzymes were used to map the insert (section 2.7, 2.10.1 and 2.10.3). Enzymes that cut once within the insert and once in the vector were used to remove a section of insert DNA. The shortened vector was then religated back together (section 2.15.1). Although blue/white selection was no longer possible, ampicillin resistant sub-clones were selected and screened by the rapid boil method (section 2.5.3) until the required clones were identified.

2.16 Paxilline Analysis

2.16.1 Sampling liquid cultures for analysis of paxilline

For High Performance Liquid Chromatography (HPLC) analysis, a 5 mL sample was removed from fungal liquid cultures (section 2.1.2) and transferred to a pre-weighed 15 mL falcon tube. The mycelia were pelleted by centrifugation (10 min 3 100 rpm), and following removal of the excess broth, snap frozen in liquid nitrogen and stored at -70°C. For Thin Layer Chromatography (TLC), a 10 mL sample was removed except where the biomass was extremely low. In this case the entire 100 mL biomass was harvested and stored as above.

Broth removed from mycelia samples isolated for extraction of paxilline and RNA (section 2.17.1) was collected in a sterile 15 mL falcon tube, snap frozen in liquid nitrogen and stored at -70°C.

2.16.2 Isolation of paxilline from freeze-dried samples

Freeze dried mycelia was weighed to determine the biomass per sample. Paxilline was extracted into chloroform:methanol (2:1) using a Virtis homogeniser. Samples were homogenised in chloroform and then methanol was added to a final volume of 1.2 mL chloroform:methanol (2:1) per 1 mL of culture removed. Samples were shaken gently for 2 h and the mycelia debris pelleted by centrifugation (10 min, 3 100 rpm). Finally, 1.5 mL was removed and evaporated overnight in a fumehood.

Samples to be analysed by HPLC were filtered to remove any remaining mycelia debris capable of blocking the HPLC column. A Waterman Sep-Pak® silica cartridge was pre-wet with 3 mL of Dichloromethane (DCM). Dried paxilline samples (1.5 mL) extracted from mycelia were resuspended in 1 mL of DCM and transferred to the column. The column was washed with 1 mL of DCM:Acetonitrile (ACN) (80:20), then the sample collected in 3 mL of ACN:DCM (80:20). Cartridges were reused after inverting the column and flushing through with 4 mL of DCM:ACN (80:20) up to a maximum of four times.

2.16.3 HPLC

Standards and samples (10 μL) were separated with a Shimadzu LC-4A HPLC in normal phase [DCM:ACN (80:20) containing 0.4% (v/v) Methanol] using a Zorbax-SIL Silica column (4.6 mm x 25 cm) at a rate of 1.2 mL/min. UV absorbency at 230 nm was determined with a Shimadzu SPD-2AS Spectrophotometric Detector. Several paxilline standards of 25 μg/mL were run as an internal standard for each set of samples. Peak areas were calculated by the Shimadzu C-R3A Chromatopac printer, and using peak areas from the paxilline standards, an average paxilline content/peak area unit was calculated (Appendix 3.0).

2.16.4 TLC

The 1.5 mL dried samples extracted from mycelia (section 2.16.2) were resuspended in 50 μL of chloroform:methanol (2:1) and spotted onto a Polygram Silica gel TLC plate (Macherey-Nagel) at 1 cm intervals. The plate was run through two solvent fronts in chloroform:acetone (10:1). When the plate was dry, indole-diterpene moieties were identified by spraying with alcoholic Erhlich's reagent (2% (w/v) p-dimethyl-aminobenzaldehyde in 12% (v/v) HCl and 50% (v/v) ethanol) and heated to 120°C for 5-7 min. After cooling the plates were laminated and stored away from light to prevent fading.

2.17 Isolation of RNA

2.17.1 Sampling liquid cultures for isolation of RNA

Two samples of 5 mL were removed from fungal liquid cultures (section 2.1.2) and strained through sterile mira-cloth. The mycelia was washed with sterile Milli Q water and transferred to sterile aluminium foil packets. The mycelia were snap frozen in liquid nitrogen and stored at -70°C.

2.17.2 RNase-free solutions and equipment

All solutions except those containing Tris, EDTA and MOPS were made with DEPC-treated water. Milli Q water (1 L) was treated with diethyl pyrocarbonate (DEPC) to a final concentration of 0.01% (v/v). The water was incubated at 37°C overnight then autoclaved twice for 15 min at 121°C with 15 psi. All solutions were stored in prepared glassware.

Where possible glassware was soaked overnight in a chromic acid bath and baked for 2 h at 180°C. Lids for Schott bottles were autoclaved for 15 min at 121°C with 15 psi in a solution of DEPC-treated water. Any equipment unable to be acid-washed and oven-baked, was soaked overnight in 0.3% (v/v) H_2O_2, rinsed in 95% (v/v) ethanol and left to air-dry in a fume hood. The working area was scrubbed with Virkon and rinsed with 95% (v/v) ethanol and allowed to air-dry. All Eppendorf tubes, PCR tubes and 1-5 mL pipette tips were autoclaved for 15 min at 121°C with 15 psi and stored in RNase-free glassware. Aerosol Resistant, RNase-free, DNase-free pipette tips (10 µL, 20 µL, 200 µL, and 1000 µL) were used at all times. Latex gloves were used when handling any equipment.

2.17.3 Isolation of total RNA with TRIzol® reagent

Frozen mycelia in foil packets was removed from the -70°C freezer and transported in liquid nitrogen. Mycelia (1.25 g) was removed, re-wrapped in tin foil and emersed in liquid nitrogen. The remaining mycelia were returned to -70°C freezer. Mycelia were ground to a fine powder under liquid nitrogen in a mortar and pestle. Powdered mycelia was then mixed to a paste with 10 mL of TRIzol® reagent (BRL) and transferred to an 30 mL tube. The tube was sealed with parafilm and aluminium foil and left to stand at room temperature for 5 min. The homogenate was centrifuged at 12 000 x g (Sorval SS34, 10 min, 9 000 rpm, 4°C) and the cleared supernatant transferred to a fresh 30 mL tube. Chloroform (2 mL) was added and the tube covered with parafilm and aluminium foil. Samples were shaken vigorously for 15 sec, then incubated at room temperature for 3 min. The aqueous phase was separated by centrifugation (Sorval SS34, 15 min, 9 000 rpm, 4°C) and transferred to a fresh tube. Total RNA was precipitated by adding

5 mL of isopropanol and incubating the solution at room temperature for 10 min. RNA was pelleted by centrifugation (Sorval SS34, 10 min, 9 000 rpm, 4°C) and washed with 10 mL of 75% (v/v) ethanol. The pellet was reconstituted by centrifugation at 7,500 x g (Sorval SS34, 5 min, 6 700 rpm, 4°C) and air-dried in a fumehood for approximately 15 min before resuspending in 200-500 µL of DEPC-treated water (section 2.17.2). Samples were quantified spectrophotometrically (section 2.6.3), and stored at -70°C.

2.17.4 DNase I treatment

Based on method described by Bradshaw & Pillar (1992). Reactions were performed in 15 mL tubes (section 2.17.2). Total RNA, equivalent to 120 µg was combined with 60 U DNase I (Roche), 80 U RNase Inhibitor (Roche), 500 µM 1,4-dithiothreitol (DTT) (Roche) and 1 x DNase I Buffer (section 2.3.5) to a final volume of 2.4 mL. The mixture was incubated at 37°C for 30 min, at which time another 60 U of DNase I was added and the incubation continued for a further hour. The sample was re-extracted in 2 volumes of chloroform and Tris-equilibrated phenol (section 2.3.11) with gentle vortexing. The aqueous phase was separated by centrifugation (Sorval SS34, 10 min, 11 500 rpm, 4°C) and transferred to a clean corex tube. Two volumes of chloroform were added, mixed briefly by vortexing and the aqueous phase separated as above. RNA was precipitated with the addition of 240 µL 3 M sodium acetate and 6 mL of 95% (v/v) ethanol. The precipitation was incubated on ice for 30 min, and the RNA pelleted by centrifugation (Sorval SS34, 15 min, 9 000 rpm, 4°C). The resulting pellet was carefully washed with 6 mL of 75% (v/v) ethanol and air dried at room temperature for approximately 15 min, before resuspending in 100 µL of DEPC-treated Milli Q water (section 2.17.2). The concentration of RNA was determined spectrophotometrically (section 2.6.3). Samples were stored at -70°C.

2.18 Reverse Transcription-PCR

2.18.1 Synthesis of cDNA with Expand™ reverse transcriptase

DNase I treated RNA (1 μg) and 0.6 μL of random hexamer primer (3 μg/μL) (BRL) were combined with DEPC-treated water (section 2.17.2) to a final volume of 11.95 μL in sterile 0.2 mL PCR tubes. The primers and template were denatured at 90°C for 5 min, cooled on ice, then briefly centrifuged in a microcentrifuge to collect the reagents to the bottom of the tube. A reaction cocktail containing n x (1x Expand buffer (Roche), 10 mM (DTT), 50 μM dNTPs, and 10 U RNase Inhibitor (Roche)) was prepared and 7.05 μL added to each reaction tube. Duplicate reactions for each RNA sample and a water only control were prepared. Expand™ Reverse Transcriptase (50 U) (Roche) was added to the water control and one of the RNA reactions. To the other RNA reaction, 1 μL of DEPC-treated water (section 2.17.2) was added to confirm the absence of any contaminating DNA. The reactions were gently mixed, centrifuged to collect all reagents in the bottom of the tube, and incubated at room temperature for 10 min to allow primer annealing. The cDNA synthesis was performed at 42°C for 45 min in a Corbett thermocycler. Reactions were collected by centrifugation, diluted 10-, 10^2- and 10^3-fold with sterile Milli Q water and stored at -20°C.

2.18.2 PCR amplification of cDNA

A routine PCR cocktail (section 2.11.2) was prepared to a final volume of 20 μL. The 10-fold dilutions of cDNA, the undiluted 'no reverse transcriptase' controls, undiluted 'no RNA' control, genomic DNA (0.5 ng/μL) and water controls (all 5 μL) were added to the reaction tubes. In a Corbett thermocycler preset to 94°C, the following PCR programme was performed; 1 cycle of 2 min at 94°C, 30 cycles of 45 sec at 94°C, 45 sec at 60°C, and 1 min at 72°C, and 1 cycle of 5 min at 72°C. For the initial control PCR to confirm cDNA synthesis and absence of DNA contamination, primers Etub2 and Etub3 (Table 2.2) for the *tub-2* gene were used. These primers flank a 62 bp intron, so cDNA PCR products are 62 bp smaller than gDNA PCR products, and distinguishable by mini-gel electrophoresis (section 2.10.1 and 2.10.3).

2.19 RACE

Rapid Amplification of cDNA Ends (RACE) was used to determine the position of transcription initiation and termination sites, after an initial estimation was made with RT-PCR. The general strategy is outlined in Figure 3.14. The initial steps (section 2.19.1) of the RACE were conducted under RNase free conditions as described in section 2.17.2.

2.19.1 Synthesis of cDNA with C. therm. reverse transcriptase

DNase I treated RNA (section 2.17.4) was removed from -70°C. Duplicate reactions containing, 500 ng RNA, 1pM gene specific primer for 5' RACE or oligo dT primer for 3' RACE and DEPC-treated water (section 2.17.2) to a final volume of 11.3 µL, were set up in 0.2 mL PCR tubes. A tube containing no RNA was included as a control. The reactions were incubated at 70°C for 10 min then stored on ice. A cocktail containing n x (1 x *C. therm* Reverse Transcriptase buffer, 5 mM DTT, 100 µM dNTPs and 3% (w/v) dimethylsulfoxide (DMSO)) was transferred to each of the tubes in 7.2 µL aliquots. To one of the RNA reactions and the water control, 1.5 mL of *C. therm* Reverse Transcriptase (Roche) was added. DEPC-treated water (section 2.17.2) was used to bring the remaining control reactions to a final volume of 20 µL. The reaction was incubated for 30 min at 60°C in a Corbett thermocycler. The reverse transcriptase was then heat inactivated at 95°C for 2 min.

2.19.2 Degradation of RNA

The RACE reactions were collected by centrifugation and placed at 37°C. To all tubes except no RNA control, 1 µL of RNase mix (Roche), containing both RNase H and RNase A, was added, gently mixed and incubated at 37°C for 30 min. The reactions were again collected by centrifugation and a 1 µL sample (sample A) removed and diluted 500-fold with TE (section 2.3.10). The remaining reaction was purified using the Life Technologies CONCERT™ PCR clean-up kit (BRL) (section 2.11.5) and

resuspended in 50 μL of warm sterile Milli Q water. A 5 μL sample (sample B) was removed and diluted 10^2-fold with TE (section 2.3.10).

2.19.3 Addition of 3' terminal dCTP with terminal transferase

For 5' RACE, an extra step is required to enable the AAP primer to bind to the 3' end of newly synthesised cDNA. Duplicate reactions containing; 10 μL of purified cDNA, 1 x Tailing buffer, 100 μM dCTP, and 0.75 mM $CoCl_2$ were prepared in 0.2 mL PCR tubes. To one set of reactions 50 U of Terminal transferase was added. To the remaining control tubes, sterile Milli Q water was added to a final volume of 20 μL. The reactions were incubated at 37°C for 20 min, then the enzyme was heat inactivated at 65°C for 10 min.

2.19.4 Second strand synthesis

Two sets of nested primers combined with the AUAP and UP primers for 5' RACE and the UP primer for 3' RACE were used to purify the RACE products further (Figure 3.14). Routine PCR reactions (section 2.11.2) were performed and the products separated by mini-gel electrophoresis (section 2.10.1 and 2.10.3).

2.19.5 Isolation and characterisation of RACE products

Bands were purified as described in section 2.11.5 and the second round PCR amplification reaction from the gel stab cleaned using the CONCERT™ PCR clean up kit (BRL) (section 2.11.5). The products were cloned using the Promega pGEM®-T system (section 2.15.2) and sequenced (section 2.12.3).

3.1 Isolation and Characterisation of *hmg* from *P. paxilli*

3.1.1 Isolation of a unique *hmg* with degenerate primers

Optimal conditions for amplifying *P. paxilli* genomic DNA (gDNA) were determined with primers pax5 and pax6 (Table 2.3) using a routine PCR protocol (section 2.11.2). These homologous primers were designed to sequence a specific site in the *P. paxilli* genome. The plasmid pAN7-1, was heterologously integrated into this site during the generation of paxilline negative mutants (Itoh *et al.*, 1994a). This locus is approximately 25 kb away from the left boundary of the paxilline biosynthetic cluster. When used in combination, pax5 and pax6 amplify a 451 bp product, encompassing part of an ORF with similarity to a phenylacrylic acid decarboxylase (Young *et al.*, 1998). The product yield from the range of DNA concentrations used, is shown in Figure 3.1 A. Lane 5, containing 5 ng of template gDNA, is the optimal DNA concentration.

The same primers were used to determine the optimal concentration of Mg^{2+} in the reaction buffer. A routine PCR cocktail was prepared (section 2.11.2), with a Roche Mg^{2+}-free buffer replacing the standard buffer. The concentration of Mg^{2+} in individual reaction tubes was adjusted by addition of $MgCl_2$. Within the range of concentrations tested, the product yield was unaffected by Mg^{2+} (Figure 3.1 B). The standard buffer (containing 1.5 mM Mg^{2+}), supplied with Roche Taq DNA polymerase, was used in all subsequent reactions.

Degenerate primers, designed to conserved regions of *hmg* genes, were used to isolate a unique *hmg* gene from *P. paxilli*.. The optimal temperature for annealing degenerate primers HMG1 and HMG3 (Table 2.3) to the *P. paxilli* gDNA was determined using a routine PCR reaction (section 2.11.2). Annealing temperatures from 42°C to 46°C were trialed and the optimal result, primer annealing at 44°C, is shown in Figure 3.1 C. Previously, a unique *hmg* was isolated from *N. lolii* Lp19 using the same primers (Dobson, 1997) and a single specific product is also amplified from *Saccharomyces cerevisiae* with HMG1 and HMG3. Therefore both species were used as controls to identify a product of the expected size from *P. paxilli* gDNA. For comparison, *N. lolii* Lp1 gDNA was also included. Genomic DNA from the four species was amplified with primers pax5 and pax6 as a positive control, as amplification with the homologous

Figure 3.1 Optimisation of PCR conditions

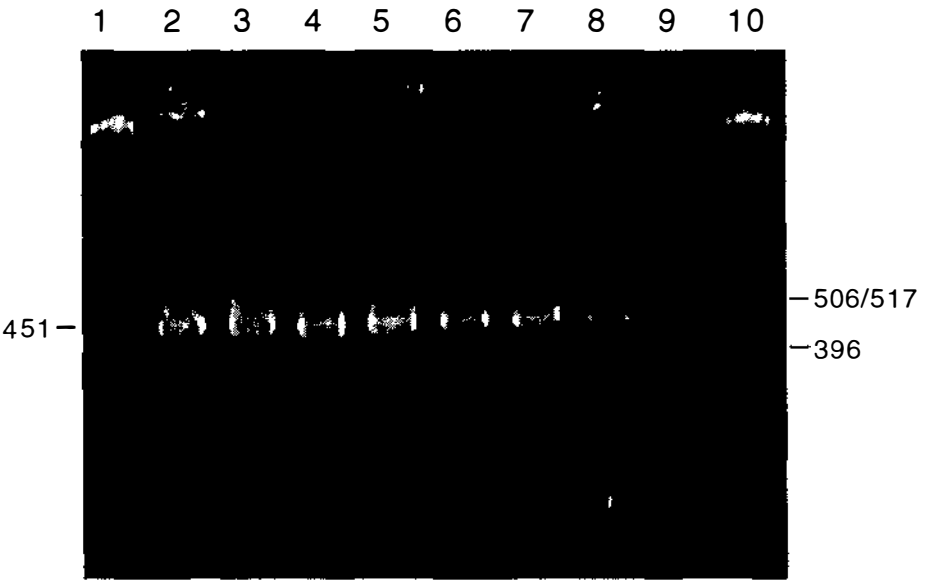
A) Effect of DNA concentration on product yield

A PCR was performed (section 2.11.3) using decreasing amount of template *P. paxilli* genomic DNA (gDNA). Samples were separated on a 3% NuSieve gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 10, 1kb ladder (BRL). Lanes 2-9, gDNA 100 ng, 25 ng, 10 ng, 5 ng, 1 ng, 0.1 ng, 0.01 ng, and water only. Sizes shown are in bp.

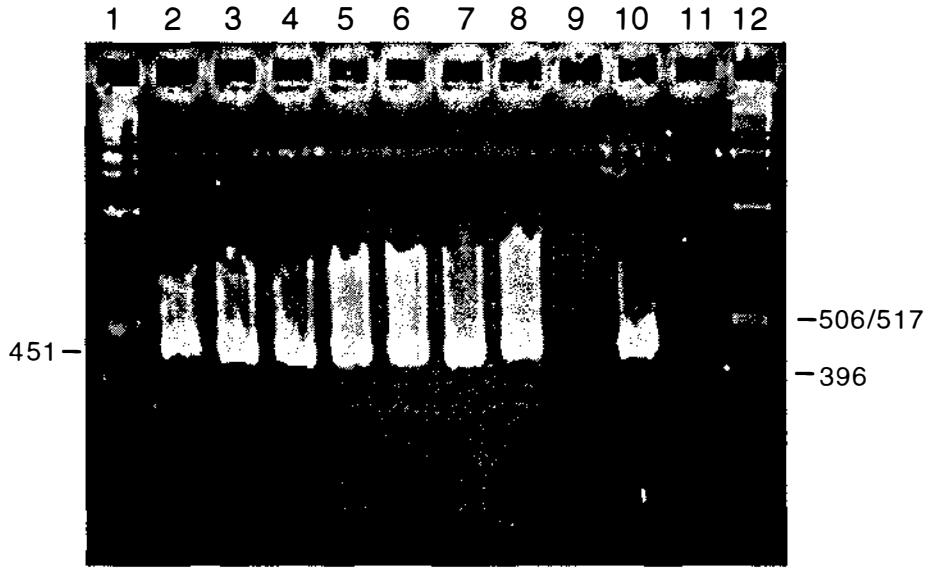
B) Effect of Mg^{2+} concentration on product yield

A PCR was performed (section 2.11.3) with each reaction contained either an increasing concentration of $MgCl_2$ in a Mg^{2+}-Free buffer (Roche), or a set concentration of Mg^{2+} in the standard Taq Polymerase buffer (Roche). *P. paxilli* gDNA (5 ng) was used in all samples were separated on a 3% NuSieve gel (section 2.10.1) as follows. Lanes 1 and 12, 1kb ladder (BRL). Lanes 2-8, Roche Mg^{2+}-free buffer containing $MgCl_2$ at 1 mM, 1.25 mM, 1.5 mM, 1.75 mM, 2 mM, 2.5 mM, and 5 mM. Lane 9, water control with 1.5mM $MgCl_2$. Lanes 10-11, Roche standard buffer (1.5 mM Mg^{2+}) with 5 ng gDNA, and water only. Sizes shown are in bp.

A


1 2 3 4 5 6 7 8 9 10
451
506/517
396

B


1 2 3 4 5 6 7 8 9 10 11 12
451
506/517
396

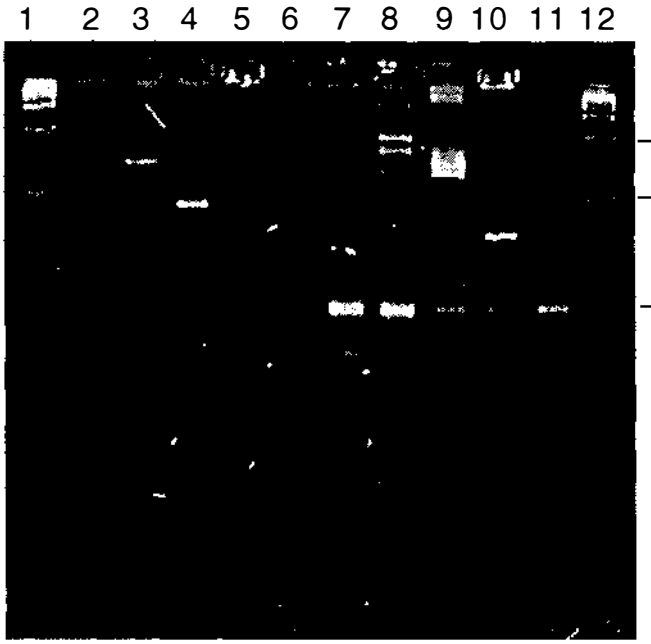
C) Product yield at 44°C annealing temperature

A PCR was performed (section 2.11.3) at 44°C annealing temperature, with 5 ng of gDNA from various species. Samples were separated on a 3% NuSieve gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 12, 1kb ladder (BRL). Lanes 2-6, gDNA from Lp1, Lp19, *P. paxilli*, *S. cerevisiae*, and water control amplified with pax5 and pax6. Lanes 7-11, gDNA from Lp1, Lp19, *P. paxilli*, *S. cerevisiae*, and water control amplified with HMG1 and HMG3. Sizes shown are in bp.

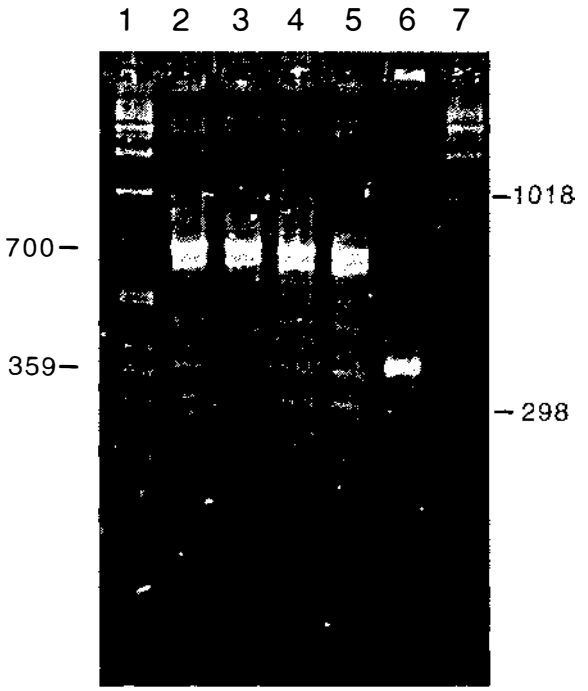
D) Optimal PCR for purification of products

A PCR was performed (section 2.11.2) at 44°C annealing temperature. Samples were separated on a 3% NuSieve gel (section 210.1) as follows. Lanes 1 and 7, 1kb ladder (BRL). Lanes 2-5, *P. paxilli* gDNA amplified with HMG1 and HMG3, Lane 6, *S. cerevisiae* amplified with HMG1 and HMG3. The 359 bp and 700 bp product were isolated and sequenced. Sizes shown are in bp.

C



D



primers would confirm a successful PCR. Primers pax5 and pax6 were not expected to amplify a product from *S. cerevisiae.* A product amplified from *N. lolii* Lp1 and *N. lolii* Lp19 gDNA, with pax5 and pax6, can be seen in lanes 2 and 3. The second set of reactions was amplified with HMG1 and HMG3 (lanes 7-11). A product of 359 bp is amplified from *N. lolii* Lp19, *P. paxilli* and *S. cerevisiae.* Lane 9, containing *P. paxilli* template, shows two strong products and several medium to faint products, including the 359 bp product. A product of 200 bp is present in lanes 7 -11, including the water control. This product was not seen in any subsequent reactions (Figure 3.1 D). At 44°C, the 359 bp product from *P. paxilli* is at its greatest intensity. A large scale PCR using all the optimal conditions and *S. cerevisiae* as a control was performed (Figure 3.1 D) to enable the 700 bp product and the 359 bp products to be purified from the gel (section 2.11.4). Both products were isolated (section 2.5.4) and sequenced to determine if a unique *hmg* gene was present.

3.1.2 Sequencing the 700 bp and 359 bp products from *P. paxilli*

The 700 bp and 359 bp products isolated from *P. paxilli* gDNA with the degenerate primers HMG1 and HMG3, were sequenced (section 2.12.1 and 2.12.2) using HMG1 and HMG3 as primers. The smaller product was sequenced in both directions (Appendix A.2.1) with sufficient results to produce a consensus overlap (Figure 3.2). A BLAST search (Altschul *et al.*, 1990) of the GenBank protein database was performed at the NCBI website. This confirmed that a unique *hmg* had been isolated, and that the novel sequence most closely resembled 3-hydroxy-3-methylglutaryl Coenzyme A reductase from *Gibberella fujikuroi* (E value of 1×10^{-28}). The 359 bp *hmg* product was then labelled with [α-^{32}P] and used to probe both a *P. paxilli* genomic Southern blot and a *P. paxilli* genomic library.

The 700 bp product was also sequenced in both directions with primers HMG1 and HMG3, however due to the size of the template, there was no double stranded overlap (Appendix A.2.2). A BLAST search (Altschul *et al.*, 1990) of the GenBank database was performed with the two sequences isolated from the 700 bp product. No significant homology was found for either sequence.

Figure 3.2 Consensus sequence of the 359 bp product

Consensus sequence for 359 bp product amplified with HMG1 and HMG3 and sequenced with the same primers (section 2.12.1 and 2.12.2). The raw data from which this consensus was assembled is shown in Appendix A 2.1. A *Sst*I site is underlined. This consensus sequence was used to search the Genbank protein database at the NCBI website. This product was labelled with[α-^{32}P] dCTP and used to probe a *P. paxilli* genomic Southern blot and a *P. paxilli* genomic library.

```
    ATGAAnATGATCTCCAAnnGCTGTGAAAAGGCTnTTGATGTCATGGTCCAAGTGAATGCG
  1          +          +          +          +          +          + 60

    GTTTTGACGACATGTCTATCATTTCGCTCTCTGGTAATTTCTGTACCGACAAGAAGTCTG
 61          +          +          +          +          +          + 120

    CGGCTATCAACTGGACTGATGGTCGTGGAAAGTCCGTCGTGGCTGAAGCGATTATTCCTG
121          +          +          +          +          +          + 180

    GTGATGTCGTTAAGAGTGTCCTGAAGAGTGATGTCAACGCCTTGGTCGAGCTCAATGTGA
181          +          +          +          +          +          + 240

    GCAAGAACTTGATCGGAAGTGCCATGGCTGGCAGCTTGGGCTGGCTTCAACGCCACGCTC
241          +          +          +          +          +          + 300

    GAACATTGTCnCGCATCTTCCTGGCCACTGGCCAAGA
301          +          +          +                                337
```

3.1.3 Confirmation the unique *hmg* exists in the *P. paxilli* genome

A unique *hmg* had been isolated using PCR, from *P. paxilli* with the degenerate primers. Southern analysis was performed on genomic DNA (section 2.5.1) to confirm the origin of the unique product and to determine the size and number of fragments homologous to the 359 bp *hmg* probe in the *P. paxilli* genome. DNA was digested with a range of restriction endonucleases (section 2.7 and 2.10.2), transferred to Nylon membrane (section 2.13) (Figure 3.3 A), and probed with the 359 bp *hmg* product. The autoradiograph (Figure 3.3 B) confirms a single copy of the unique *hmg* is present in the *P. paxilli* genome.

Table 3.1 Size of *P. paxilli* gDNA bands hybridising to the 359 bp *hmg* probe

Size of Bands (kb)						
Restriction enzyme						
*Bam*HI	*Bgl*I	*Eco*RI	*Hin*dIII	*Pst*I	*Sal*I	*Sst*I
2.2	6.2	+23.1	19.1	4.9	15.6	13.9
					5.5	5.6

Two bands are seen in lanes digested with *Sal*I (Figure 3.3 B, lane 7) and *Sst*I (lane 8). In the case of the *Sst*I digestion, sequencing later revealed a *Sst*I site between primers HMG1 and HMG3 (Figure 3.2). The smaller band seen in the *Sal*I digestion could result from a cryptic *Sal*I site that is cut rarely to produce a faint 5.2 kb band, although there are no sequences similar to the *Sal*I recognition site in this area. It remains unexplained at this time.

Figure 3.3 Southern analysis of hmg in the P. paxilli genome

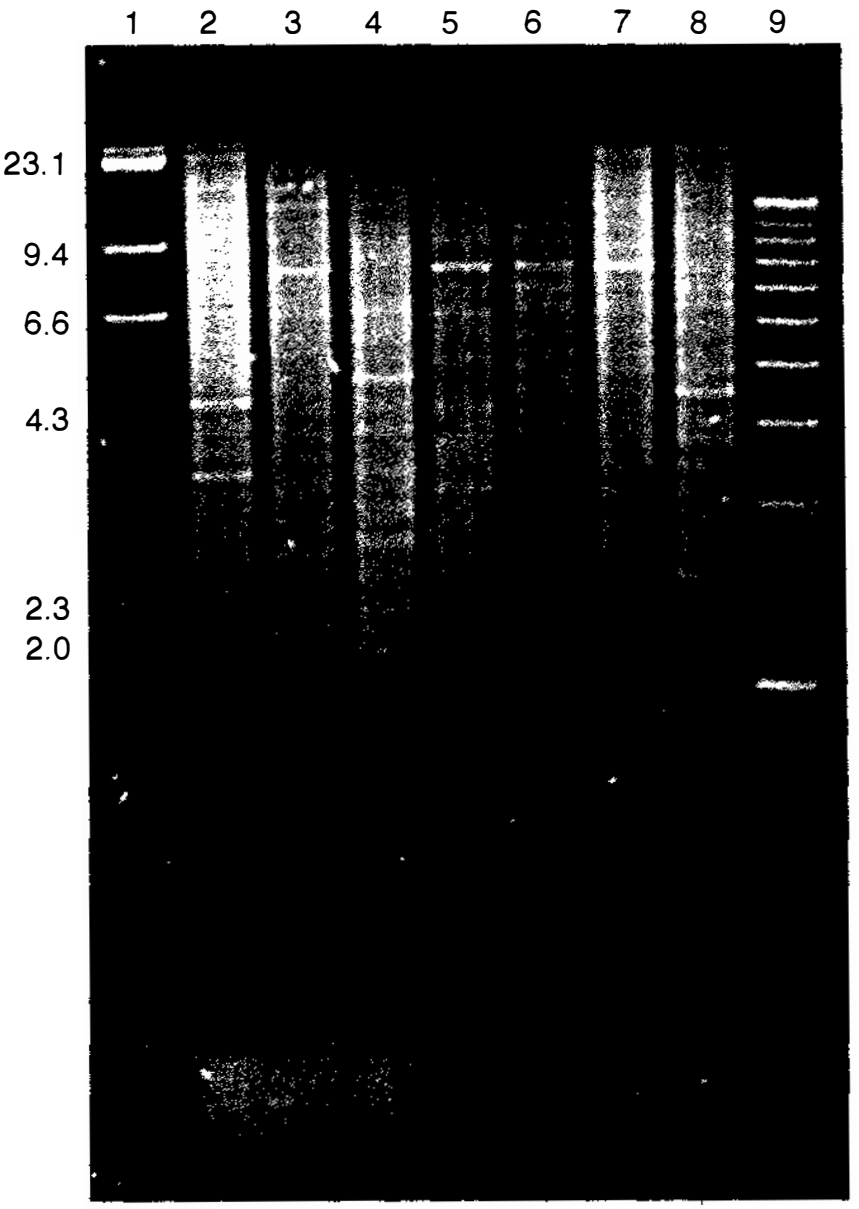
A) Restriction endonuclease digestion of P. paxilli gDNA

P. paxilli genomic DNA (2 µg) (section 2.5.1) was digested with restriction endonucleases (section 2.7). Each reaction was separated on a 0.7% agarose gel (section 2.10.2 and 2.10.3) as follows. Lane 1, λ/*Hin*dIII. Lanes 2-8, *P. paxilli* gDNA cut with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Sst*I. Lane 9, 1kb ladder (BRL). Sizes shown are in kb.

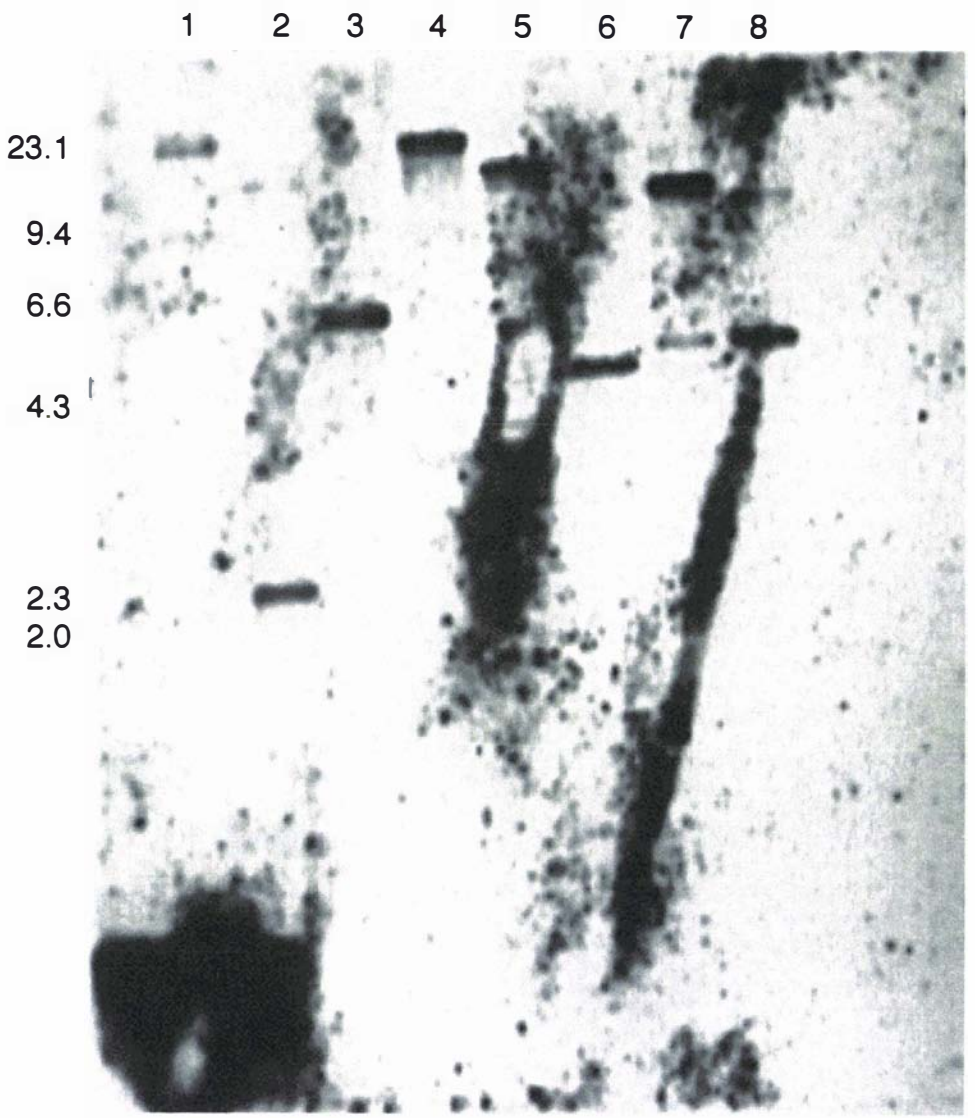
B) Confirmation that the 359 bp product is part of the P. paxilli genome

Autoradiograph showing hybridisation of the 359 bp product to the *P. paxilli* genome. Ethidium bromide stained gel seen in Figure 3.3 A was Southern blotted and probed with the [^{32}P]-labelled 359 bp *hmg* product (section 2.13). Sizes shown are in kb.

A



B



3.1.4 Library screening

A λGEM®-11 *P. paxilli* genomic DNA library (Itoh & Scott, 1994b) was titred (section 2.14.1) and found to contain 1.11 x 10^6 pfu/mL. After first round plating and hybridisation with the 359 bp *hmg* product, 17 positive clones were observed on duplicate membranes. The 12 clones with the brightest signals (λET1, λET2, λET3, λET4, λET5, λET6, λET10, λET11, λET12, λET13, λET15 and λET17) were selected for second round screening (section 2.14.8). One of these clones, λET3, failed to hybridise to the *hmg* probe. However, all other clones hybridised and duplicates of these were carried through to third round hybridisation (section 2.14.8). All clones were positive after third round screening and were grown to confluent lysis for DNA isolation (section 2.5.2).

3.1.5 Physical mapping of λ clones

Following a medium scale λ DNA prep, DNA was digested with *Sst*I (section 2.7) and separated on a 0.7% (w/v) agarose gel (section 2.10.2 and 2.10.3) (Figure 3.4 A). DNA was transferred to Nylon membrane and hybridised to the 359 bp *hmg* probe (section 2.13) (Figure 3.4 B). All clones contain the unique *hmg* fragment, the size and hybridisation patterns of which are represented in Table 3.2. A *Sst*I restriction map based on these sizes was produced (Figure 3.5).

3.1.6 Sub-cloning λET2 into a sequencing vector

Clone λET2 was chosen for sub-cloning and sequencing, as it was the most central of the clones with respect to probe hybridisation. DNA from this clone was digested with *Sst*I, before being purified with phenol/chloroform (section 2.8) and precipitated with ethanol (section 2.9). The purified DNA was ligated into pUC118 and transformed into *E. coli* XL-1 cells (section 2.15). Plasmids containing *Sst*I fragments of λET2 were purified (section 2.5.5) and sequenced with the forward and reverse universal primers. Some of the larger fragments were further reduced in size by the removal of restriction

Figure 3.4 Southern analysis of hmg λ Clones

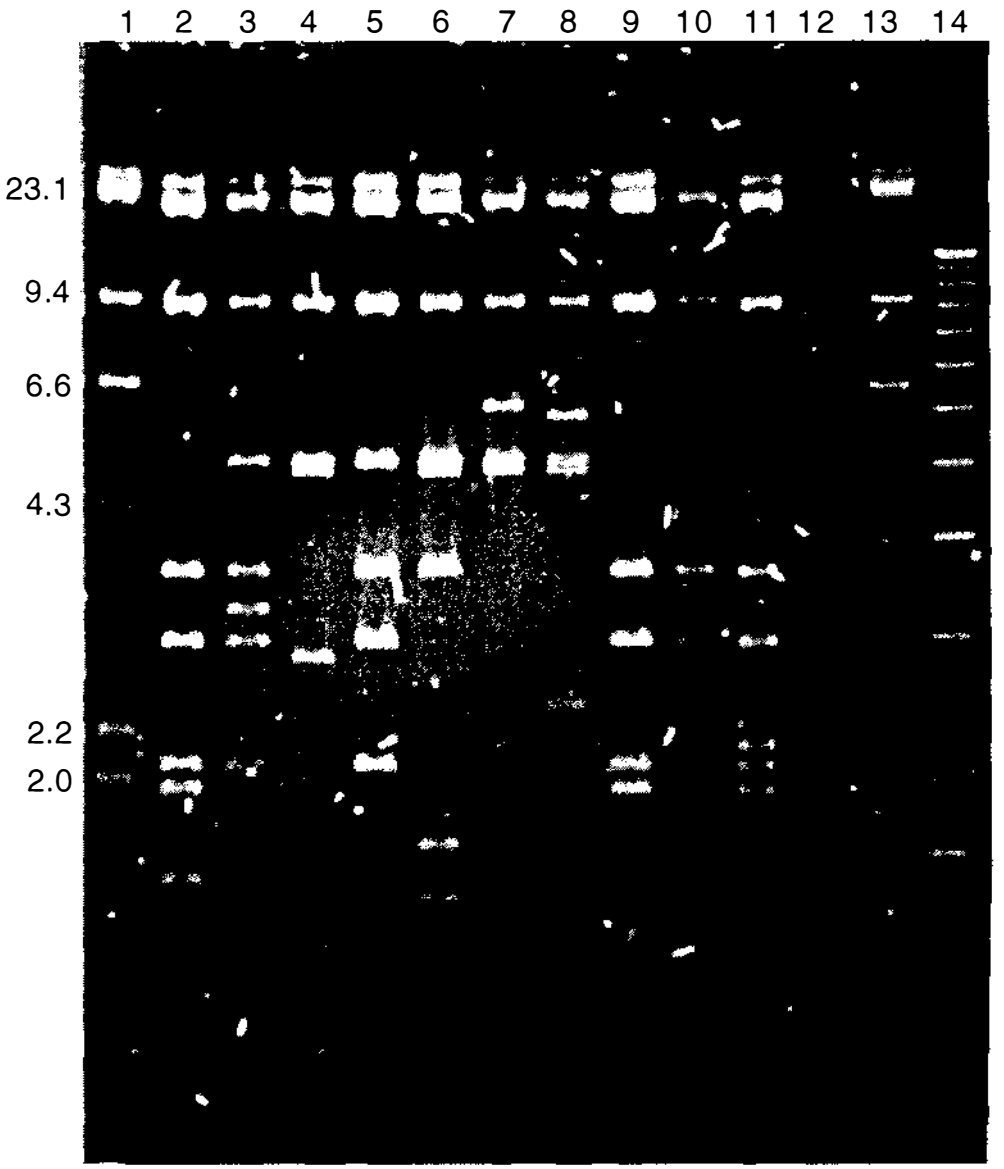
A) Restriction endonuclease digestion of hmg λclones, cut with SstI

DNA (~500 ng) from medium scale λ preps (section 2.5.2) was digested with *Sst*I (section 2.7) and run on a 0.7% agarose gel (section 2.10.2 and 2.10.3) as follows. Lane 1, λ/*Hin*dIII. Lanes 2-12, *Sst*I digestion of λET1, λET2, λET4, λET5, λET6, λE10, λET11, λET12, λET13, λET15, and λET17. Lane 13, 1kb ladder (BRL). Sizes shown are in kb.

B) Mapping the position of hmg on λclones

Autoradiograph showing hybridisation of the 359 bp product to the digested λ clones. Ethidium bromide stained gel seen if Figure 3.4 A was Southern blotted and probed with [^{32}P]-labelled 359 bp *hmg* product (section 2.13). Sizes shown are in kb.

A



B

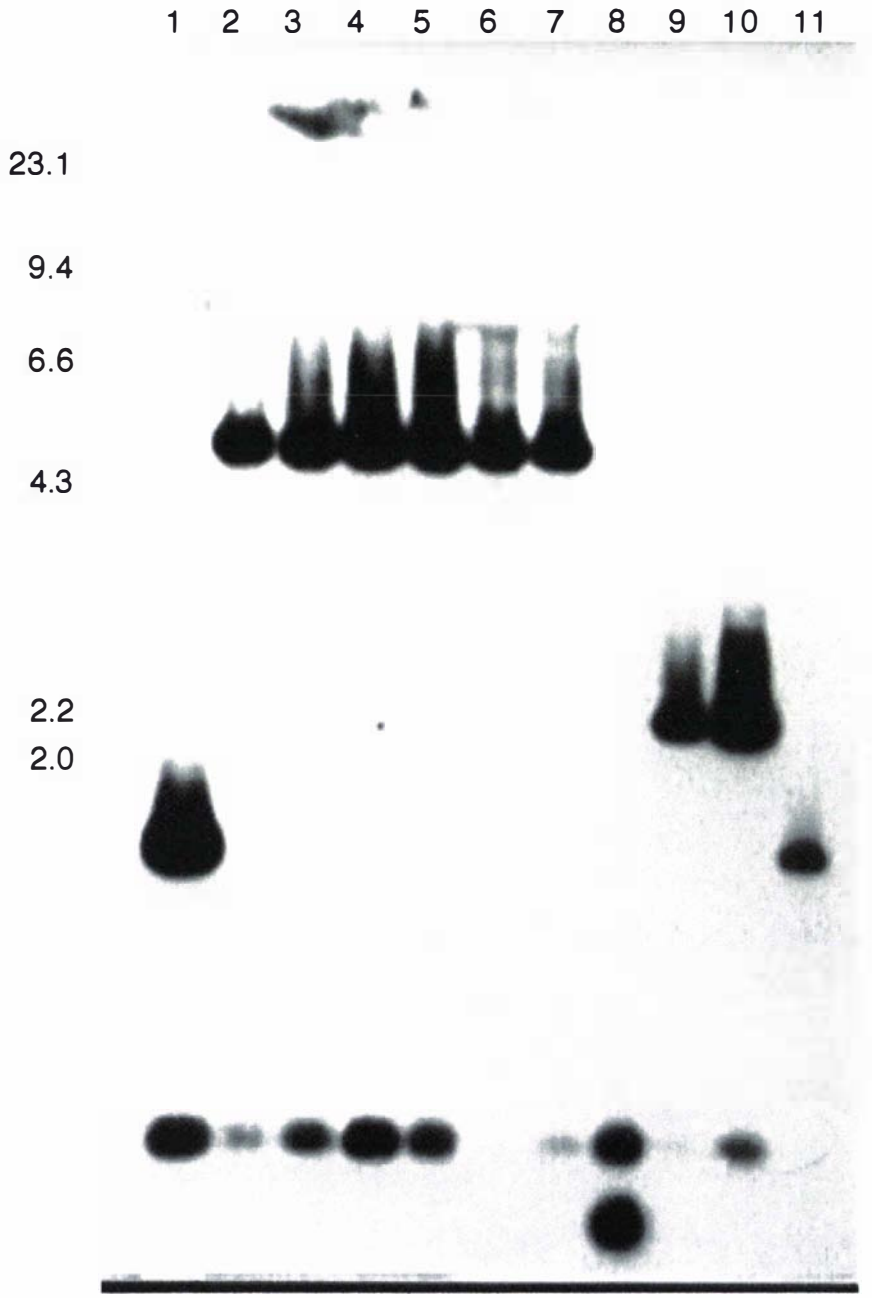
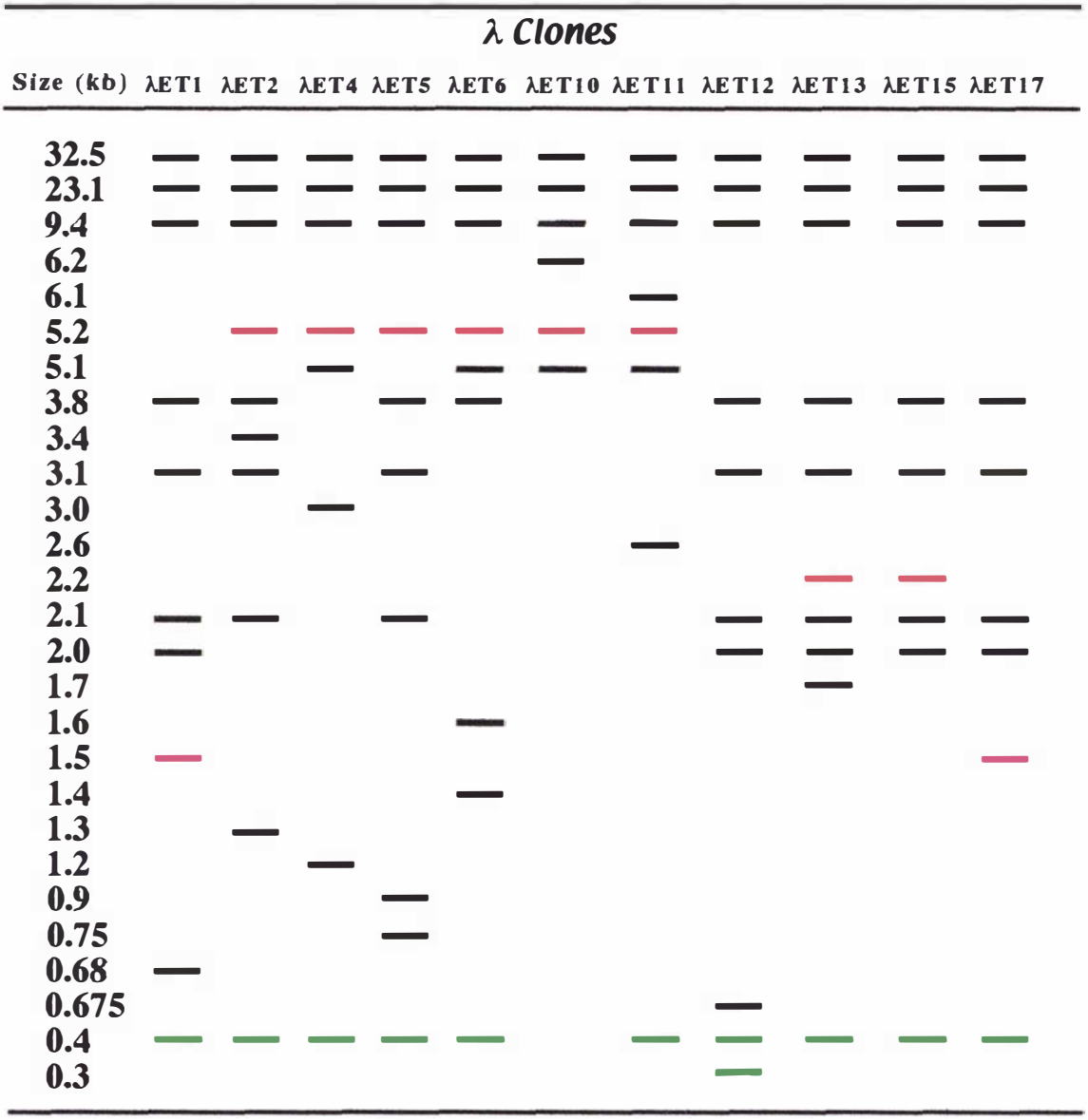


Table 3.2 *Size and hybridisation pattern of SstI digests of hmg λ clones probed with the 359bp product .*

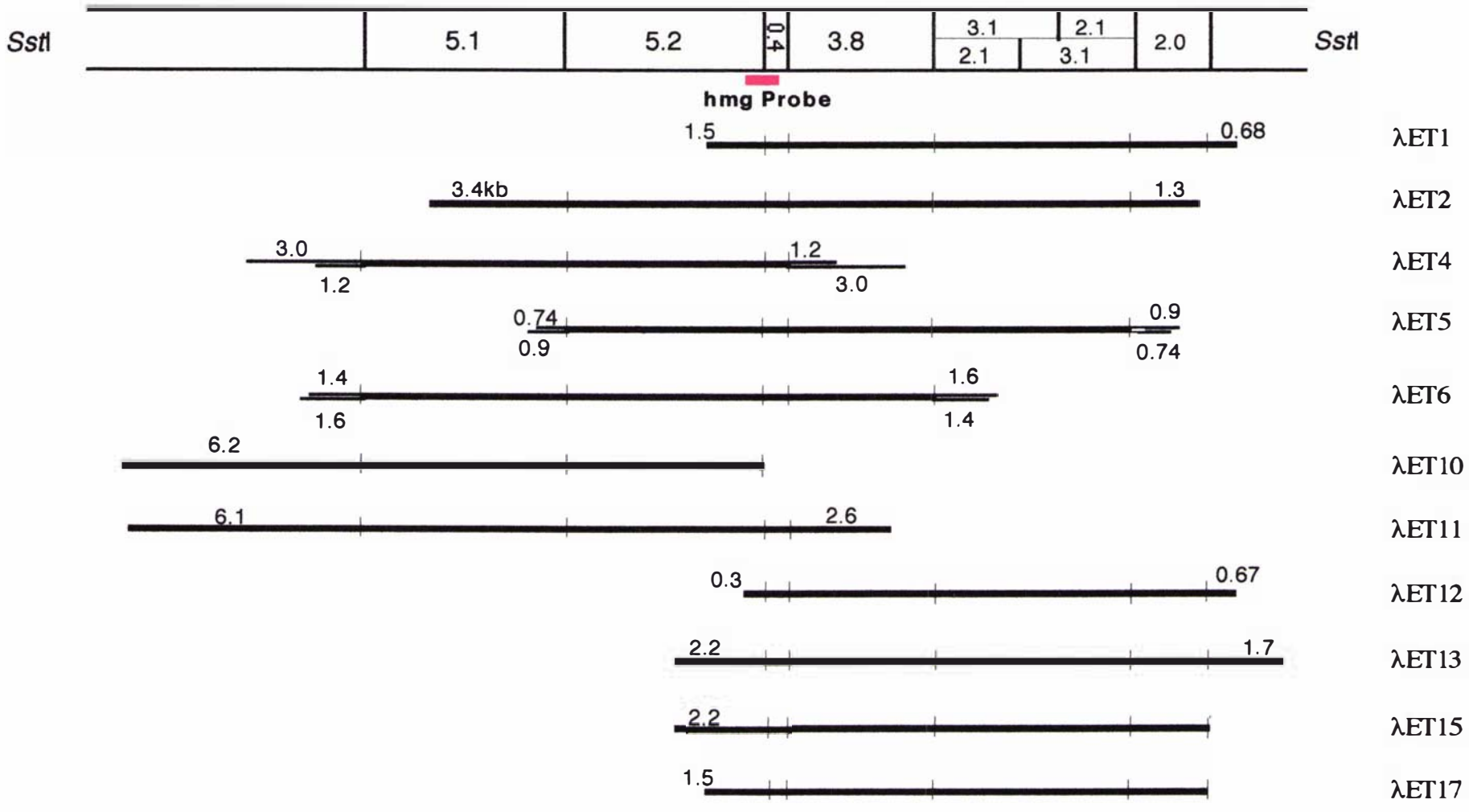


*Band stains with ethidium bromide and ^{32}P probe

*Band labels with ^{32}P probe only

Figure 3.5 SstI Restriction map of hmg λ Clones.

All sizes shown are in kb



fragments between the multiple cloning cassette and unique sites within the fragment (section 2.15.6). The new sequence repositioned next to the multiple cloning cassette was then isolated with the forward primer. All sub-clones used for sequencing are listed in Table 2.1. The restriction map of the *P. paxilli hmg* locus and relative position of the sub-clones used for sequencing are shown in Figure 3.6 A. Restriction fragments match fragment sizes seen in the genomic digest of *P. paxilli* DNA (Table 3.1).

3.1.7 Sequencing λET2 sub-clones

The pUC118 sub-clones, containing fragments of *P. paxilli hmg,* were sequenced at the MuSeq DNA Analysis facility (IMBS, Massey University, NZ) (section 2.12.3). As much sequence as possible was determined using the forward and reverse universal primers, but specific primers (Table 2.3) needed to be designed (section 2.11.1) to complete the double stranded sequence of this gene. Junctions between individual fragments were sequenced directly from clones λET2 and λET4 with the appropriate primers. Individual sequences were viewed using the Edit View programme (Version 1.0.1, Perkin Elmer), and assembled into a contig using both the GCG package and Sequencher™ software. The templates used to assemble the *hmg* contig, are displayed in Appendix A.2.3 with the Sequencher™ software. Sequencing analysis and deduced polypeptide sequence revealed a 1130 amino acid protein with three open reading frames (ORF) separated by two introns (introns 2 and 3) (section 3.1.8). The first ORF extends from +1 to +336 bp, ORF 2 from +408 to +3127 bp, and ORF 3 from +3196 to +3529 bp (Figure 3.6 B). A third intron (intron 1) was identified in the 5' untranslated region (UTR) (section 3.1.8). The position of transcription initiation and termination was determined with RACE (section 3.1.10 and 3.1.11). The translation start and stop sites were determined by the FRAMES programme of the GCG package, and the sequence surrounding the translation start site (Ballance, 1986; Kozak, 1984). The deduced polypeptide sequence was separated into three domains by comparison to existing *hmg* sequences. The transmembrane domain (residues 1 to 633) was analysed by the TMPRED programme at the ISREC Bioinformatics group EMB.net website and was predicted to contain 7 membrane-spanning regions. The linker domain (residues 634 to 683) has low conservation, which is seen in Figure 3.8, a comparison of HMGR proteins using the PILEUP programme from the GCG package. The catalytic domain

Figure 3.6 Physical map of the P. paxilli hmg locus

A) Restriction map of the P. paxilli hmg locus and position of subclones used for sequencing

Determined by restriction mapping fragments from λET2. Sizes shown are in kb.

B) Structure of hmg gene

Shows transcription start and stop sites, position of introns and primers used to determine sequence of the gene.

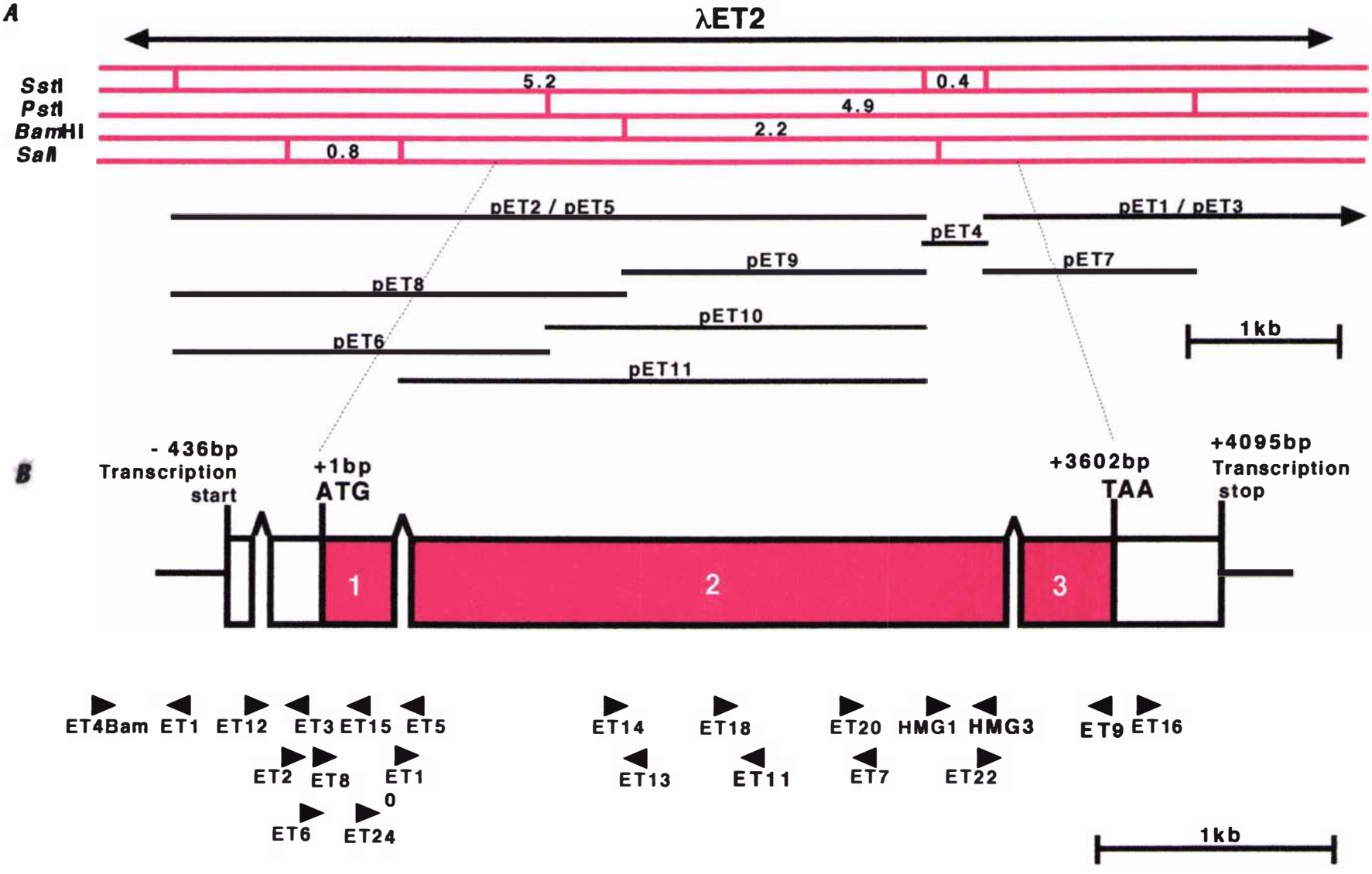
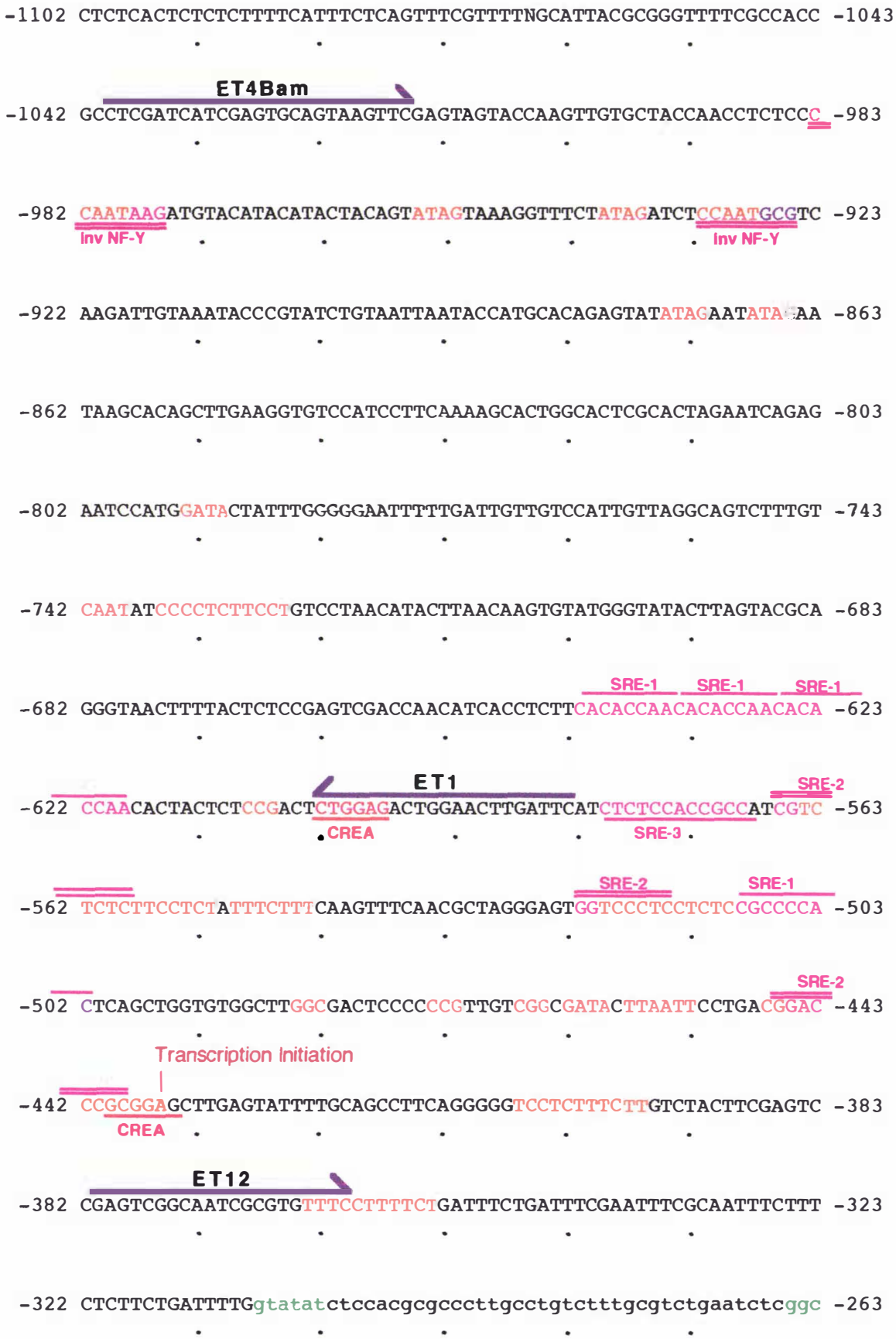

A
λET2
SstI
PstI
BamHI
SalI
5.2
0.4
4.9
2.2
0.8
pET2 / pET5
pET1 / pET3
pET4
pET9
pET7
pET8
pET10
pET6
pET11
1kb
B
- 436bp
Transcription
start
+1bp
ATG
+3602bp
TAA
+4095bp
Transcription
stop
1
2
3
ET4Bam
ET1
ET12
ET3
ET15
ET5
ET2
ET8
ET10
ET6
ET24
ET14
ET18
ET20
HMG1
HMG3
ET9
ET16
ET13
ET11
ET7
ET22
1kb

Figure 3.7 *DNA sequence and deduced amino acid sequence of P. paxilli hmg*

Sequence of *P. paxilli hmg* was determined as described in section 3.1.7. The 5220 bp of sequence isolated is shown here, including 1102 bp of 5' UTR (-1102 to -1), 3532 bp of coding sequence (+1 to +3532), and 586 bp of 3' UTR (+3533 to 4118). The predicted 1130 amino acids are shown beneath their respective codons. Introns are shown in lower case, with 5' and 3' splice junctions, and the lariat consensus sequence highlighted in green. Where present, intronic polypyrimidine tracts are highlighted in green as well.. The transcription start and stop sites, determined by RACE, are highlighted in red. The translation start and stop codons are highlighted in blue, with the Kozak consensus sequence surrounding the translation start codon highlighted in light blue. A putative polyadenylation signal is shown as purple. Core promoter elements, TATA-like boxes, CAAT boxes, CT-rich tracts, GATA sites and GC triplets, are highlighted in orange. CREA sites are underlined in red. Putative elements for sterol regulation of transcription are shown as pink. SRE-1 sites have a single overline, SRE-2 sites have a double overline, SRE-3 sites have a single underline and NF-Y sites have a double underline. Primers used for sequencing and gene analysis are shown as purple lines. Amino acids forming membrane spanning tracts are boxed and the entire transmembrane domain spans +1 to +1970 bp. The catalytic domain, separated by a 50 amino acid linker from the transmembrane domain, starts at +2121 bp and continues to the termination codon (+ 3532 bp). Residues involved in substrate binding are underlined. Conserved catalytic residues Glu^{793}, Asp^{1003}, and His^{1099} are shown in **bold**. A serine residue (Ser^{1105}), thought to be involved in regulatory phosphorylation, is also shown in **bold**.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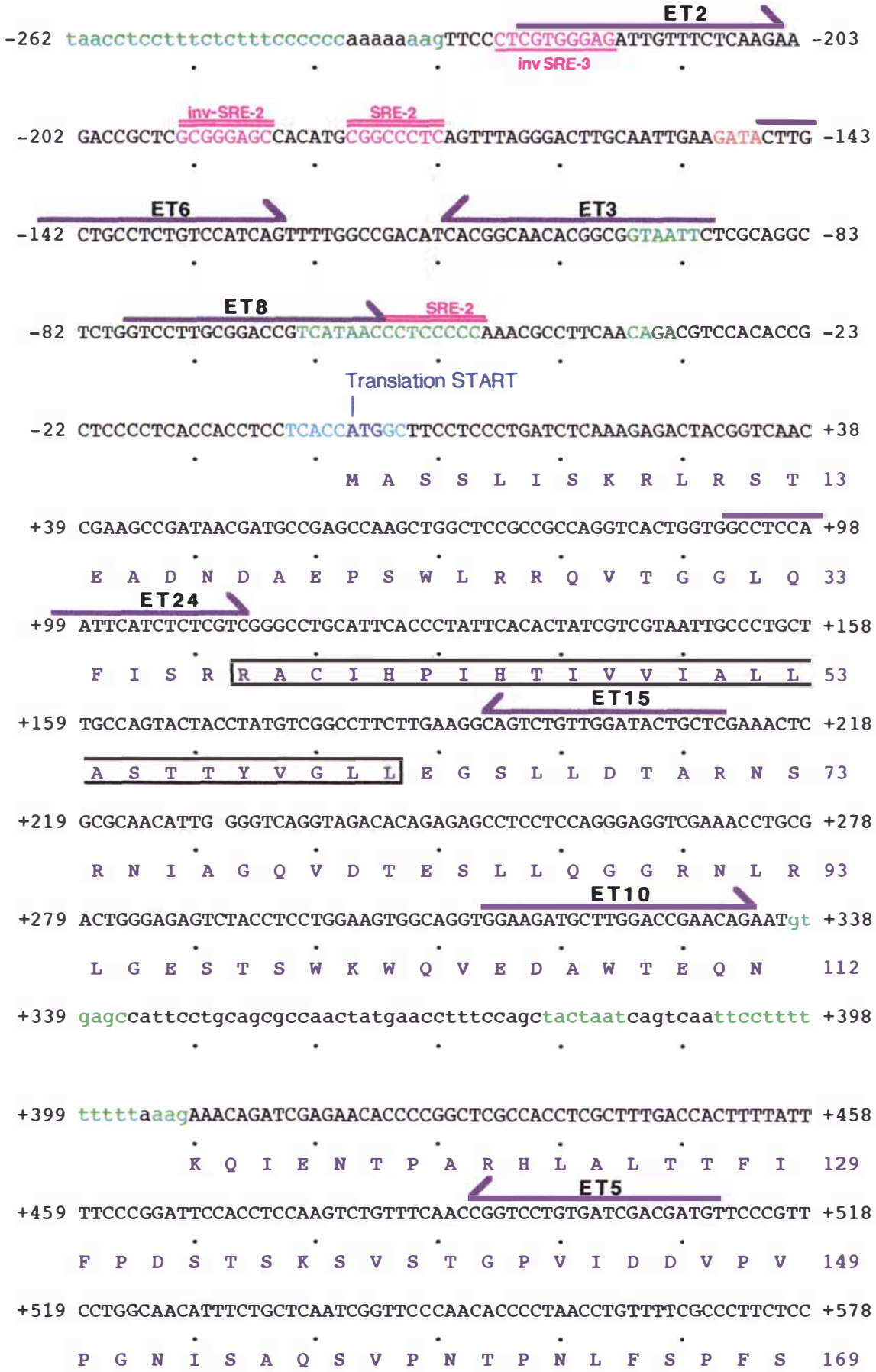
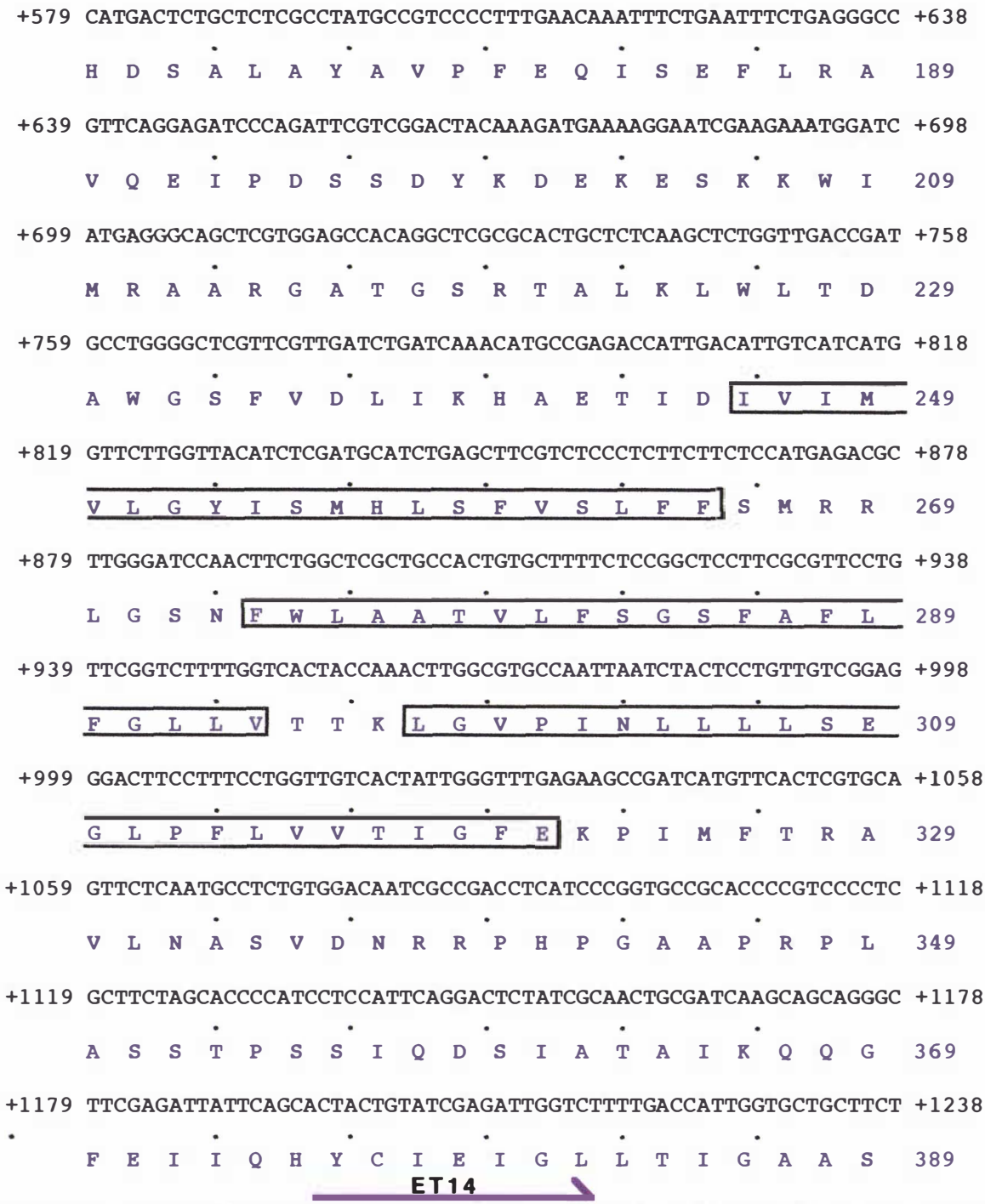
ET4Bam
-1042 GCCTCGATCATCGAGTGCAGTAAGTTCGAGTAGTACCAAGTTGTGCTACCAACCTCTCCC -983
-982 CAATAAGATGTACATACATACTACAGTATAGTAAAGGTTTCTATAGATCTCCAATGCGTC -923
Inv NF-Y
Inv NF-Y
-922 AAGATTGTAAATACCCGTATCTGTAATTAATACCATGCACAGAGTATATAGAATATAAA -863
-862 TAAGCACAGCTTGAAGGTGTCCATCCTTCAAAAGCACTGGCACTCGCACTAGAATCAGAG -803
-802 AATCCATGGATACTATTTGGGGGAATTTTTGATTGTTGTCCATTGTTAGGCAGTCTTTGT -743
-742 CAATATCCCCTCTTCCTGTCCTAACATACTTAACAAGTGTATGGGTATACTTAGTACGCA -683
SRE-1
SRE-1
SRE-1
-682 GGGTAACTTTTACTCTCCGAGTCGACCAACATCACCTCTTCACACCAACACACCAACACA -623
ET1
SRE-2
-622 CCAACACTACTCTCCGACTCTGGAGACTGGAACTTGATTCATCTCTCCACCGCCATCGTC -563
CREA
SRE-3
SRE-2
SRE-1
-562 TCTCTTCCTCTATTTCTTTCAAGTTTCAACGCTAGGGAGTGGTCCCTCCTCTCCGCCCCA -503
SRE-2
-502 CTCAGCTGGTGTGGCTTGGCGACTCCCCCCGTTGTCGGCGATACTTAATTCCTGACGGAC -443
Transcription Initiation
-442 CCGCGGAGCTTGAGTATTTTGCAGCCTTCAGGGGGTCCTCTTTCTTGTCTACTTCGAGTC -383
CREA
ET12
-382 CGAGTCGGCAATCGCGTGTTTCCTTTTCTGATTTCTGATTTCGAATTTCGCAATTTCTTT -323
-322 CTCTTCTGATTTTGgtatatctccacgcgcccttgcctgtctttgcgtctgaatctcggc -263


ET2
inv SRE-3
inv-SRE-2
SRE-2
ET6
ET3
ET8
SRE-2
Translation START
ET24
ET15
ET10
ET5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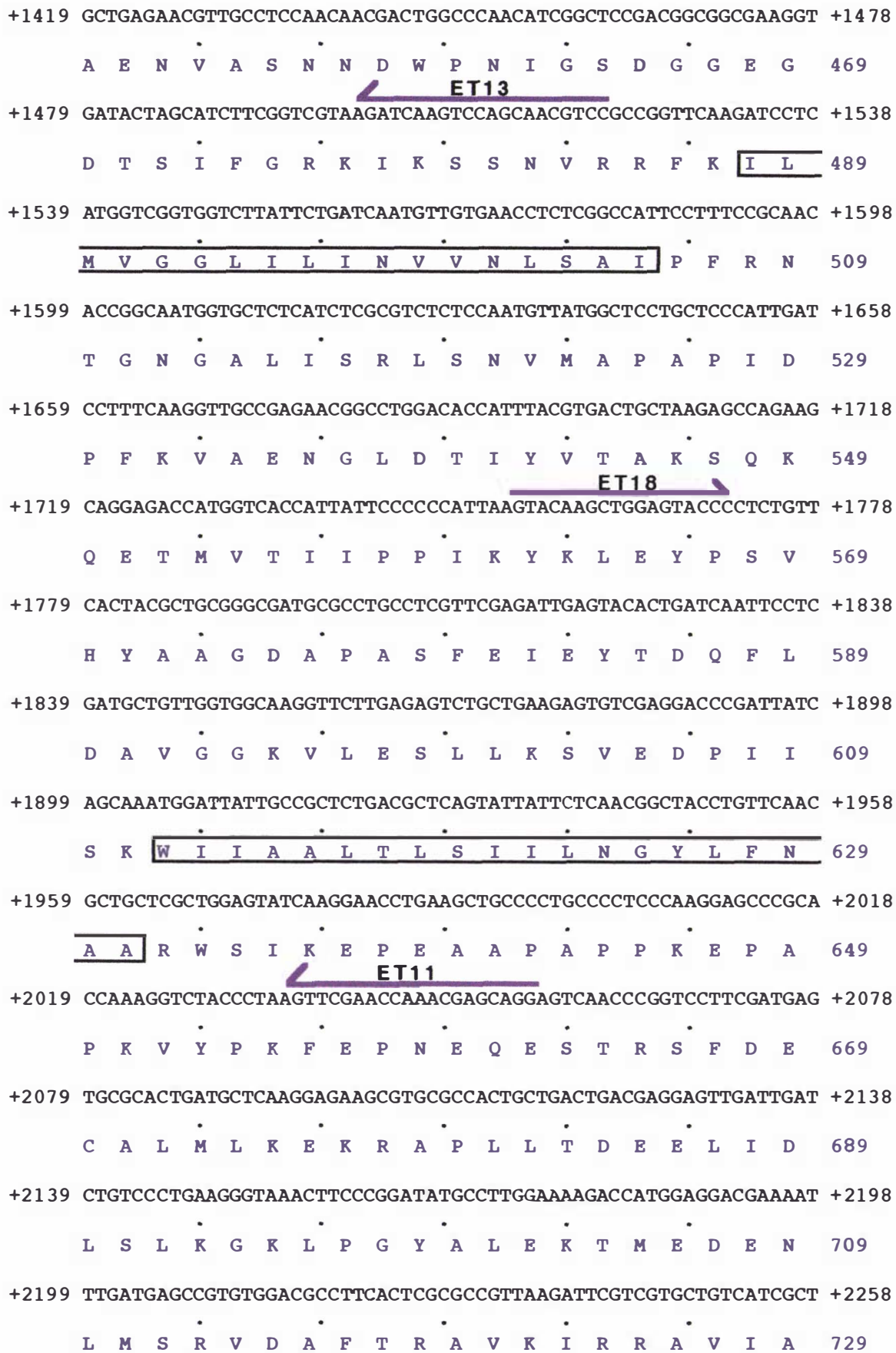
+639 GTTCAGGAGATCCCAGATTCGTCGGACTACAAAGATGAAAAGGAATCGAAGAAATGGATC +698
V Q E I P D S S D Y K D E K E S K K W I 209
+699 ATGAGGGCAGCTCGTGGAGCCACAGGCTCGCGCACTGCTCTCAAGCTCTGGTTGACCGAT +758
M R A A R G A T G S R T A L K L W L T D 229
+759 GCCTGGGGCTCGTTCGTTGATCTGATCAAACATGCCGAGACCATTGACATTGTCATCATG +818
A W G S F V D L I K H A E T I D I V I M 249
+819 GTTCTTGGTTACATCTCGATGCATCTGAGCTTCGTCTCCCTCTTCTTCTCCATGAGACGC +878
V L G Y I S M H L S F V S L F F S M R R 269
+879 TTGGGATCCAACTTCTGGCTCGCTGCCACTGTGCTTTTCTCCGGCTCCTTCGCGTTCCTG +938
L G S N F W L A A T V L F S G S F A F L 289
+939 TTCGGTCTTTTGGTCACTACCAAACTTGGCGTGCCAATTAATCTACTCCTGTTGTCGGAG +998
F G L L V T T K L G V P I N L L L L S E 309
+999 GGACTTCCTTTCCTGGTTGTCACTATTGGGTTTGAGAAGCCGATCATGTTCACTCGTGCA +1058
G L P F L V V T I G F E K P I M F T R A 329
+1059 GTTCTCAATGCCTCTGTGGACAATCGCCGACCTCATCCCGGTGCCGCACCCCGTCCCCTC +1118
V L N A S V D N R R P H P G A A P R P L 349
+1119 GCTTCTAGCACCCCATCCTCCATTCAGGACTCTATCGCAACTGCGATCAAGCAGCAGGGC +1178
A S S T P S S I Q D S I A T A I K Q Q G 369
+1179 TTCGAGATTATTCAGCACTACTGTATCGAGATTGGTCTTTTGACCATTGGTGCTGCTTCT +1238
F E I I Q H Y C I E I G L L T I G A A S 389
ET14
+1239 GGTGTTCAGGGCGGTCTTCAGCAGTTCTGCTTCCTCGCGGCCTGGATTTTGTTCTTTGAC +1298
G V Q G G L Q Q F C F L A A W I L F F D 409
+1299 TGTGTGCTCCTGTTCACCTTCTACACCACCATTCTCTGCATCAAGCTCGAGATTACACGT +1358
C V L L F T F Y T T I L C I K L E I T R 429
+1359 ATCAAGCGTCATGTTGCGCTGCGCAAGGCCCTTGAAGAGGATGGCATCACCCACAGCGTG +1418
I K R H V A L R K A L E E D G I T H S V 449


+1419 GCTGAGAACGTTGCCTCCAACAACGACTGGCCCAACATCGGCTCCGACGGCGGCGAAGGT +1478
A E N V A S N N D W P N I G S D G G E G 469
ET13
+1479 GATACTAGCATCTTCGGTCGTAAGATCAAGTCCAGCAACGTCCGCCGGTTCAAGATCCTC +1538
D T S I F G R K I K S S N V R R F K I L 489
+1539 ATGGTCGGTGGTCTTATTCTGATCAATGTTGTGAACCTCTCGGCCATTCCTTTCCGCAAC +1598
M V G G L I L I N V V N L S A I P F R N 509
+1599 ACCGGCAATGGTGCTCTCATCTCGCGTCTCTCCAATGTTATGGCTCCTGCTCCCATTGAT +1658
T G N G A L I S R L S N V M A P A P I D 529
+1659 CCTTTCAAGGTTGCCGAGAACGGCCTGGACACCATTTACGTGACTGCTAAGAGCCAGAAG +1718
P F K V A E N G L D T I Y V T A K S Q K 549
ET18
+1719 CAGGAGACCATGGTCACCATTATTCCCCCCATTAAGTACAAGCTGGAGTACCCCTCTGTT +1778
Q E T M V T I I P P I K Y K L E Y P S V 569
+1779 CACTACGCTGCGGGCGATGCGCCTGCCTCGTTCGAGATTGAGTACACTGATCAATTCCTC +1838
H Y A A G D A P A S F E I E Y T D Q F L 589
+1839 GATGCTGTTGGTGGCAAGGTTCTTGAGAGTCTGCTGAAGAGTGTCGAGGACCCGATTATC +1898
D A V G G K V L E S L L K S V E D P I I 609
+1899 AGCAAATGGATTATTGCCGCTCTGACGCTCAGTATTATTCTCAACGGCTACCTGTTCAAC +1958
S K W I I A A L T L S I I L N G Y L F N 629
+1959 GCTGCTCGCTGGAGTATCAAGGAACCTGAAGCTGCCCCTGCCCCTCCCAAGGAGCCCGCA +2018
A A R W S I K E P E A A P A P P K E P A 649
ET11
+2019 CCAAAGGTCTACCCTAAGTTCGAACCAAACGAGCAGGAGTCAACCCGGTCCTTCGATGAG +2078
P K V Y P K F E P N E Q E S T R S F D E 669
+2079 TGCGCACTGATGCTCAAGGAGAAGCGTGCGCCACTGCTGACTGACGAGGAGTTGATTGAT +2138
C A L M L K E K R A P L L T D E E L I D 689
+2139 CTGTCCCTGAAGGGTAAACTTCCCGGATATGCCTTGGAAAAGACCATGGAGGACGAAAAT +2198
L S L K G K L P G Y A L E K T M E D E N 709
+2199 TTGATGAGCCGTGTGGACGCCTTCACTCGCGCCGTTAAGATTCGTCGTGCTGTCATCGCT +2258
L M S R V D A F T R A V K I R R A V I A 729

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+2259 CGTACTGCGGCTACTGCCGAAACCACTGGTTCCCTGGAGGCATCTAAACTGCCCTACAAG +2318
      R  T  A  A  T  A  E  T  T  G  S  L  E  A  S  K  L  P  Y  K   749
                    ET20 >
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      H  Y  N  Y  G  L  V  H  G  A  C  C  E  N  V  I  G  Y  L  P   769
+2379 TTGCCTCTTGGTGTTGCTGGACCAATTAAGATTGACGGCCAAAGCTATTTCATCCCCATG +2438
      L  P  L  G  V  A  G  P  I  K  I  D  G  Q  S  Y  F  I  P  M   789
+2439 GCCACCACTGAGGGTGTTCTTGTTGCCAGTACCAGCCGTGGTTCCAAGGCCATCAACGCT +2498
      A  T  T  E  G  V  L  V  A  S  T  S  R  G  S  K  A  I  N  A   809
+2499 GGTGGTGGTGCCGTGACCGTTCTCACTGGTGACGGTATGACCCGTGGACCTTGCGTGACC +2558
      G  G  G  A  V  T  V  L  T  G  D  G  M  T  R  G  P  C  V  T   829
                                                   < ET7
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      F  P  T  L  A  R  A  A  A  A  K  V  W  I  D  S  E  E  G  R   849
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      S  I  I  T  A  A  F  N  S  T  S  R  F  A  R  L  Q  T  L  K   869
+2679 ACTGCGCTCGCTGGAACCTATCTCTACATTCGATTCAAGACTACTACCGGTGACGCTATG +2738
      T  A  L  A  G  T  Y  L  Y  I  R  F  K  T  T  T  G  D  A  M   889
          HMG1 >
+2739 GGTATGAACATGATCTCCAAGGGCTGTGAAAAGGCTCTTGATGTCATGTCCAAGGAATGC +2798
      G  M  N  M  I  S  K  G  C  E  K  A  L  D  V  M  S  K  E  C   909
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      G  F  D  D  M  S  I  I  S  L  S  G  N  F  C  T  D  K  K  S   929
+2859 GCGGCTATCAACTGGACTGATGGTCGTGGAAAGTCCGTCGTGGCTGAAGCGATTATTCCT +2918
      A  A  I  N  W  T  D  G  R  G  K  S  V  V  A  E  A  I  I  P   949
                                                ET22 >
+2919 GGTGATGTCGTTAAGAGTGTCCTGAAGAGTGATGTCAACGCCTTGGTCGAGCTCAATGTG +2978
      G  D  V  V  K  S  V  L  K  S  D  V  N  A  L  V  E  L  N  V   969
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      S  K  N  L  I  G  S  A  M  A  G  S  L  G  G  F  N  A  H  A   989
                                                < HMG3
+3039 TCGAACATTGTCTCCGCCATCTTCCTGGCCACTGGACAGGATCCTGCCCAGAACGTCGAG +3098
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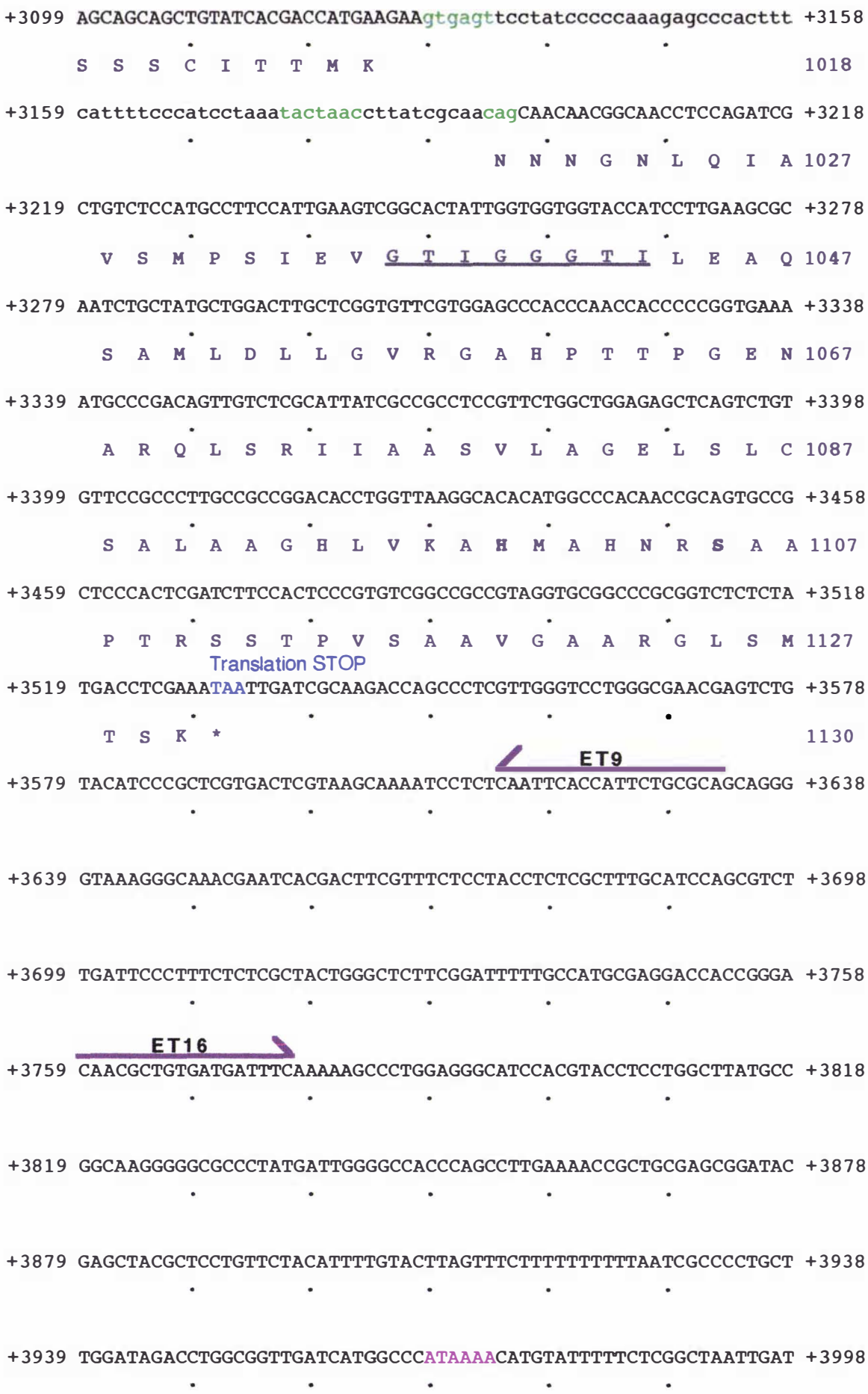
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N N N G N L Q I A 1027
+3219 CTGTCTCCATGCCTTCCATTGAAGTCGGCACTATTGGTGGTGGTACCATCCTTGAAGCGC +3278
V S M P S I E V G T I G G G T I L E A Q 1047
+3279 AATCTGCTATGCTGGACTTGCTCGGTGTTCGTGGAGCCCACCCAACCACCCCCGGTGAAA +3338
S A M L D L L G V R G A H P T T P G E N 1067
+3339 ATGCCCGACAGTTGTCTCGCATTATCGCCGCCTCCGTTCTGGCTGGAGAGCTCAGTCTGT +3398
A R Q L S R I I A A S V L A G E L S L C 1087
+3399 GTTCCGCCCTTGCCGCCGGACACCTGGTTAAGGCACACATGGCCCACAACCGCAGTGCCG +3458
S A L A A G H L V K A H M A H N R S A A 1107
+3459 CTCCCACTCGATCTTCCACTCCCGTGTCGGCCGCCGTAGGTGCGGCCCGCGGTCTCTCTA +3518
P T R S S T P V S A A V G A A R G L S M 1127
Translation STOP
+3519 TGACCTCGAAATAATTGATCGCAAGACCAGCCCTCGTTGGGTCCTGGGCGAACGAGTCTG +3578
T S K * 1130
ET9
+3579 TACATCCCGCTCGTGACTCGTAAGCAAAATCCTCTCAATTCACCATTCTGCGCAGCAGGG +3638
+3639 GTAAAGGGCAAACGAATCACGACTTCGTTTCTCCTACCTCTCGCTTTGCATCCAGCGTCT +3698
+3699 TGATTCCCTTTCTCTCGCTACTGGGCTCTTCGGATTTTTGCCATGCGAGGACCACCGGGA +3758
ET16
+3759 CAACGCTGTGATGATTTCAAAAAGCCCTGGAGGGCATCCACGTACCTCCTGGCTTATGCC +3818
+3819 GGCAAGGGGGCGCCCTATGATTGGGGCCACCCAGCCTTGAAAACCGCTGCGAGCGGATAC +3878
+3879 GAGCTACGCTCCTGTTCTACATTTTGTACTTAGTTTCTTTTTTTTTTAATCGCCCCTGCT +3938
+3939 TGGATAGACCTGGCGGTTGATCATGGCCCATAAAACATGTATTTTTCTCGGCTAATTGAT +3998

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+3999 TGGTTTTCCGCCCTCGAATAGCAGTCAAGCTAATGGTTTGGTGCACATTTAGATGAGTGT +4058
                                    Transcription Stop
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+4059 ATTTCATATCAATTAGTCAAGTTAGCAAACAGGTACTATGCTTTCGCAGGGCTGCCTCCA +4118
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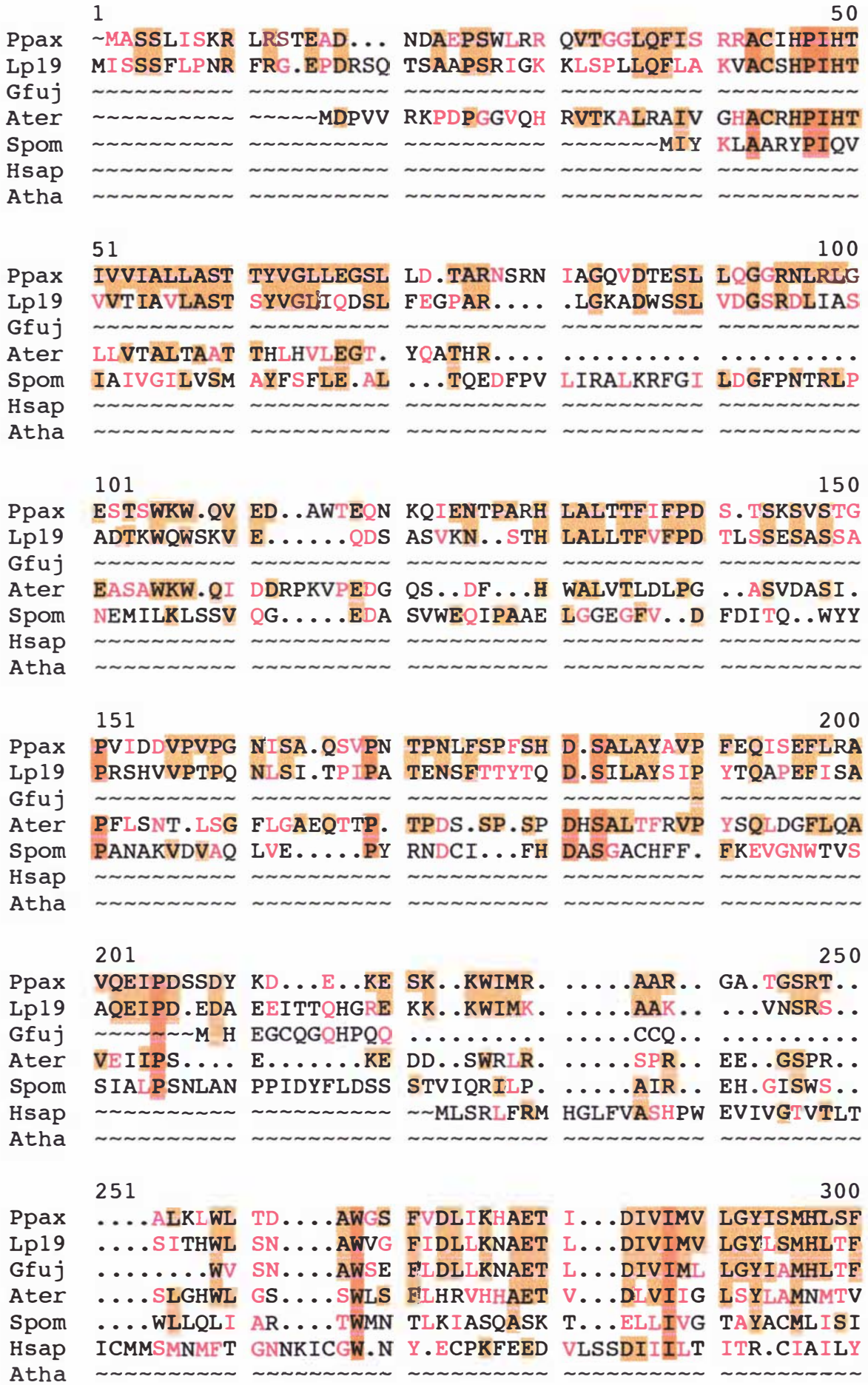
Table 3.3 Codon bias in *P. paxilli hmg*

Amino Acid	Codon	Number	Frequency	Amino Acid	Codon	Number	Frequency
Gly	GGG	1	0.01	Trp	TGG	14	1.00
	GGA	16	0.19	STOP	TGA	0	0.00
	GGT	44	0.51	Cys	TGT	7	0.50
	GGC	25	0.29		TGC	7	0.50
Glu	GAG	36	0.62	STOP	TAG	0	0.00
	GAA	22	0.38	STOP	TAA	1	1.00
Asp	GAT	23	0.50	Tyr	TAT	7	0.33
	GAC	23	0.50		TAC	14	0.67
Val	GTG	15	0.21	Leu	TTG	18	0.16
	GTA	3	0.04		TTA	0	0.00
	GTT	28	0.39	Phe	TTT	7	0.16
	GTC	26	0.36		TTC	38	0.84
Ala	GCG	14	0.12	Ser	TCG	18	0.17
	GCA	8	0.07		TCA	4	0.04
	GCT	43	0.36		TCT	19	0.18
	GCC	55	0.46		TCC	31	0.30
Arg	AGG	2	0.04	Arg	CGG	5	0.10
	AGA	2	0.04		CGA	7	0.14
Ser	AGT	13	0.13		CGT	19	0.37
	AGC	18	0.17		CGC	16	0.31
Lys	AAG	47	0.84	Gln	CAG	22	0.79
	AAA	9	0.16		CAA	6	0.21
Asn	AAT	12	0.26	His	CAT	5	0.28
	AAC	35	0.74		CAC	13	0.72
Met	ATG	25	1.00	Leu	CTG	36	0.32
Ile	ATA	0	0.00		CTA	2	0.02
	ATT	46	0.54		CTT	20	0.18
	ATC	39	0.46		CTC	35	0.32
Thr	ACG	2	0.03	Pro	CCG	4	0.07
	ACA	4	0.05		CCA	9	0.17
	ACT	32	0.42		CCT	20	0.37
	ACC	38	0.50		CCC	21	0.39

Third position nucleotide G (259) 23%, A (93) 8%, T (345) 31%, C (434) 38%.

Figure 3.8 *Alignment of hmg polypeptide sequences*

Protein sequences were aligned with the PILEUP programme from the GCG package and are coloured with respect to similarity and identity with the *P. paxilli* HMGR sequence. Amino acids conserved in all species present are also highlighted. Abbreviations for the species names are; Ppax, *Penicillium paxilli*; Lp19, *Neotyphodium lolii* isolate Lp19; Gfuj, *Gibberella fujikuroi*; Ater, *Aspergillus terreus*; Spom, *Schizosaccharomyces pombe*; Hsap, *Homo sapiens*; Atha, *Arabidopsis thaliana.*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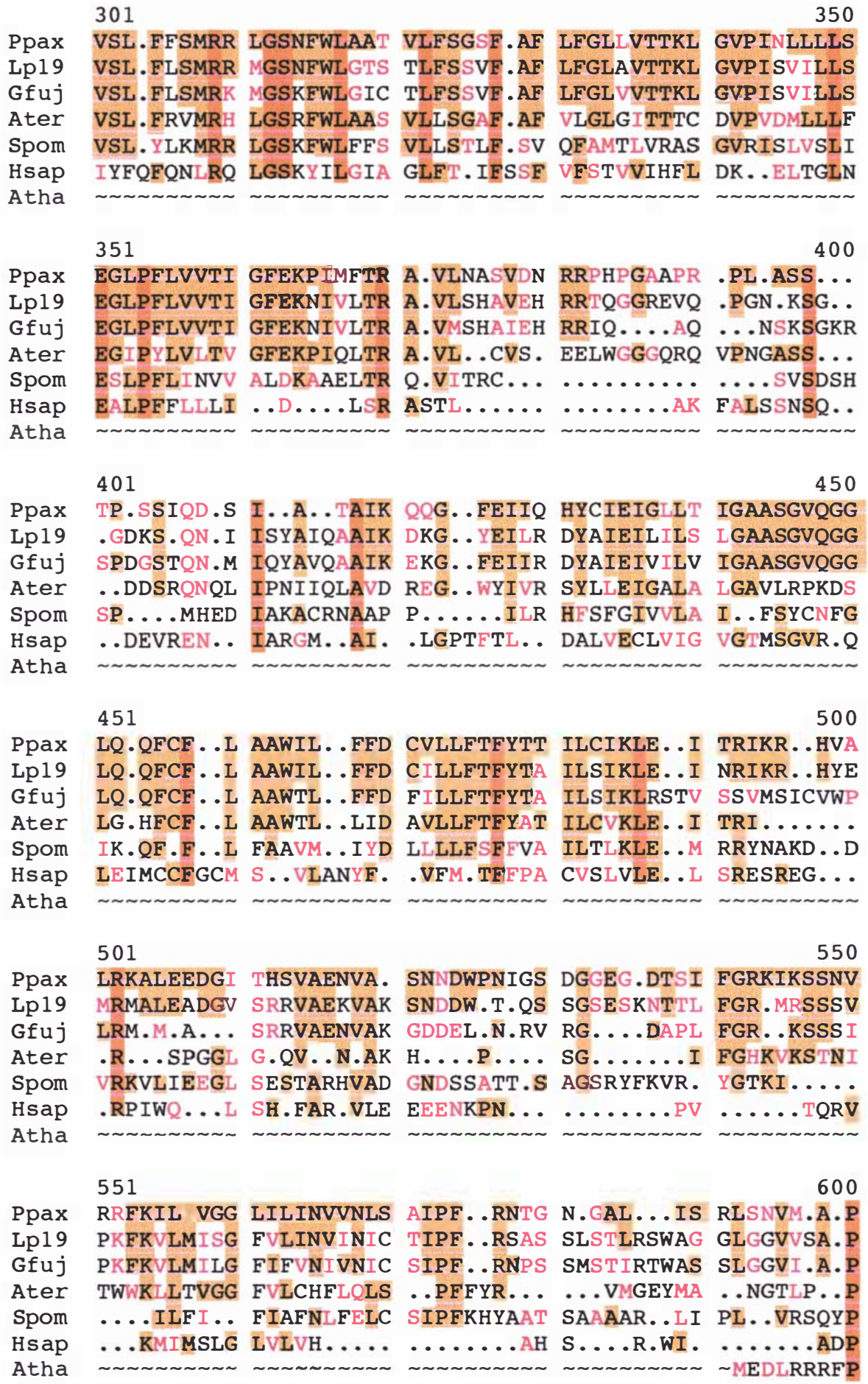
Ppax ~MASSLISKR LRSTEAD... NDAEPSWLRR QVTGGLQFIS RRACIHPIHT
Lp19 MISSSFLPNR FRG.EPDRSQ TSAAPSRIGK KLSPLLQFLA KVACSHPIHT
Gfuj ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Ater ~~~~~~~~~~ ~~~~~MDPVV RKPDPGGVQH RVTKALRAIV GHACRHPIHT
Spom ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MIY KLAARYPIQV
Hsap ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
51 100
Ppax IVVIALLAST TYVGLLEGSL LD.TARNSRN IAGQVDTESL LQGGRNLRLG
Lp19 VVTIAVLAST SYVGLIQDSL FEGPAR.... .LGKADWSSL VDGSRDLIAS
Gfuj ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Ater LLVTALTAAT THLHVLEGT. YQATHR.... ....
Spom IAIVGILVSM AYFSFLE.AL ...TQEDFPV LIRALKRFGI LDGFPNTRLP
Hsap ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
101 150
Ppax ESTSWKW.QV ED..AWTEQN KQIENTPARH LALTTFIFPD S.TSKSVSTG
Lp19 ADTKWQWSKV E......QDS ASVKN..STH LALLTFVFPD TLSSESASSA
Gfuj ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Ater EASAWKW.QI DDRPKVPEDG QS..DF...H WALVTLDLPG ..ASVDASI.
Spom NEMILKLSSV QG.....EDA SVWEQIPAAE LGGEGFV..D FDITQ..WYY
Hsap ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
151 200
Ppax PVIDDVPVPG NISA.QSVPN TPNLFSPFSH D.SALAYAVP FEQISEFLRA
Lp19 PRSHVVPTPQ NLSI.TPIPA TENSFTTYTQ D.SILAYSIP YTQAPEFISA
Gfuj ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Ater PFLSNT.LSG FLGAEQTTP. TPDS.SP.SP DHSALTFRVP YSQLDGFLQA
Spom PANAKVDVAQ LVE.....PY RNDCI...FH DASGACHFF. FKEVGNWTVS
Hsap ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
201 250
Ppax VQEIPDSSDY KD...E..KE SK..KWIMR. .....AAR.. GA.TGSRT..
Lp19 AQEIPD.EDA EEITTQHGRE KK..KWIMK. .....AAK.. ...VNSRS..
Gfuj ~~~~~~~M H EGCQGQHPQQ ....CCQ.. ....
Ater VEIIPS.... E.......KE DD..SWRLR. .....SPR.. EE..GSPR..
Spom SIALPSNLAN PPIDYFLDSS STVIQRILP. .....AIR.. EH.GISWS..
Hsap ~~~~~~~~~~ ~~~~~~~~~~ ~~MLSRLFRM HGLFVASHPW EVIVGTVTLT
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
251 300
Ppax ....ALKLWL TD....AWGS FVDLIKHAET I...DIVIMV LGYISMHLSF
Lp19 ....SITHWL SN....AWVG FIDLLKNAET L...DIVIMV LGYLSMHLTF
Gfuj ....WV SN....AWSE FLDLLKNAET L...DIVIML LGYIAMHLTF
Ater ....SLGHWL GS....SWLS FLHRVHHAET V...DLVIIG LSYLAMNMTV
Spom ....WLLQLI AR....TWMN TLKIASQASK T...ELLIVG TAYACMLISI
Hsap ICMMSMNMFT GNNKICGW.N Y.ECPKFEED VLSSDIIILT ITR.CIAILY
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~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Lp19 VSL.FLSMRR MGSNFWLGTS TLFSSVF.AF LFGLAVTTKL GVPISVILLS
Gfuj VSL.FLSMRK MGSKFWLGIC TLFSSVF.AF LFGLVVTTKL GVPISVILLS
Ater VSL.FRVMRH LGSRFWLAAS VLLSGAF.AF VLGLGITTTC DVPVDMLLLF
Spom VSL.YLKMRR LGSKFWLFFS VLLSTLF.SV QFAMTLVRAS GVRISLVSLI
Hsap IYFQFQNLRQ LGSKYILGIA GLFT.IFSSF VFSTVVIHFL DK..ELTGLN
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
351 400
Ppax EGLPFLVVTI GFEKPIMFTR A.VLNASVDN RRPHPGAAPR .PL.ASS...
Lp19 EGLPFLVVTI GFEKNIVLTR A.VLSHAVEH RRTQGGREVQ .PGN.KSG..
Gfuj EGLPFLVVTI GFEKNIVLTR A.VMSHAIEH RRIQ....AQ ...NSKSGKR
Ater EGIPYLVLTV GFEKPIQLTR A.VL..CVS. EELWGGGQRQ VPNGASS...
Spom ESLPFLINVV ALDKAAELTR Q.VITRC... ....SVSDSH
Hsap EALPFFLLLI ..D....LSR ASTL..... ....AK FALSSNSQ..
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
401 450
Ppax TP.SSIQD.S I..A..TAIK QQG..FEIIQ HYCIEIGLLT IGAASGVQGG
Lp19 .GDKS.QN.I ISYAIQAAIK DKG..YEILR DYAIEILILS LGAASGVQGG
Gfuj SPDGSTQN.M IQYAVQAAIK EKG..FEIIR DYAIEIVILV IGAASGVQGG
Ater ..DDSRQNQL IPNIIQLAVD REG..WYIVR SYLLEIGALA LGAVLRPKDS
Spom SP....MHED IAKACRNAAP P......ILR HFSFGIVVLA I..FSYCNFG
Hsap ..DEVREN.. IARGM..AI. .LGPTFTL.. DALVECLVIG VGTMSGVR.Q
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
451 500
Ppax LQ.QFCF..L AAWIL..FFD CVLLFTFYTT ILCIKLE..I TRIKR..HVA
Lp19 LQ.QFCF..L AAWIL..FFD CILLFTFYTA ILSIKLE..I NRIKR..HYE
Gfuj LQ.QFCF..L AAWTL..FFD FILLFTFYTA ILSIKLRSTV SSVMSICVWP
Ater LG.HFCF..L AAWTL..LID AVLLFTFYAT ILCVKLE..I TRI.......
Spom IK.QF.F..L FAAVM..IYD LLLLFSFFVA ILTLKLE..M RRYNAKD..D
Hsap LEIMCCFGCM S..VLANYF. .VFM.TFFPA CVSLVLE..L SRESREG...
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
501 550
Ppax LRKALEEDGI THSVAENVA. SNNDWPNIGS DGGEG.DTSI FGRKIKSSNV
Lp19 MRMALEADGV SRRVAEKVAK SNDDW.T.QS SGSESKNTTL FGR.MRSSSV
Gfuj LRM.M.A... SRRVAENVAK GDDEL.N.RV RG....DAPL FGR..KSSSI
Ater .R...SPGGL G.QV..N.AK H....P.... SG.......I FGHKVKSTNI
Spom VRKVLIEEGL SESTARHVAD GNDSSATT.S AGSRYFKVR. YGTKI.....
Hsap .RPIWQ...L SH.FAR.VLE EEENKPN... ....PV ......TQRV
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
551 600
Ppax RRFKIL VGG LILINVVNLS AIPF..RNTG N.GAL...IS RLSNVM.A.P
Lp19 PKFKVLMISG FVLINVINIC TIPF..RSAS SLSTLRSWAG GLGGVVSA.P
Gfuj PKFKVLMILG FIFVNIVNIC SIPF..RNPS SMSTIRTWAS SLGGVI.A.P
Ater TWWKLLTVGG FVLCHFLQLS ..PFFYR... ...VMGEYMA ..NGTLP..P
Spom ....ILFI.. FIAFNLFELC SIPFKHYAAT SAAAAR..LI PL..VRSQYP
Hsap ...KMIMSLG LVLVH..... ....AH S....R.WI. ....ADP
Atha ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~MEDLRRRFP

```
        601                                                        650
Ppax    APIDPFKVAE NGL.DTIYVT AKSQKQETMV TI.IPP.IKY KLE.YPSV..
Lp19    .PVDPVKVAG KGL.DAILTA AKASGKATLV TV.LTP.IKY ELE.YPSV..
Gfuj    LSVDPFKVAS NGL.DAILPT AKSNNRPTLV TV.LTP.IKY ELE.YPSI..
Ater    TAVSPFKEAA NGL.NEIYLT ARVEGFETRV TV.LPP.LQY VLE...S...
Spom    DFKSQ.RLLD DGVFDDVL.S AISS....MS NIESPS.VR. ...LLPAV..
Hsap    ...SP....Q NSTAD....T SK.......V SLGLDENVSK RIE..PSVSL
Atha    ...TK.K... NG..EE.... .ISN....VA .VD.PP.LRK ASDALPLP..

        651                                                        700
Ppax    ..HY...AAG DA...PASFE IEYTD....Q F.LD..AVGG KVLESL.LKS
Lp19    ..HY...ALS SSLRDGAA.G .AVSSAV..Q F..DNYGVGG RMVGSL.LKS
Gfuj    ..HY...ALG SA....AS.N PAYNDAFHHH F..QGYGVGG RMVGGI.LKS
Ater    .......... ......AGFN ISATK..RST F..D..GV.. .......LDG
Spom    ..FY.....G AELSS.TSF. LS....TIHS F.INNW.... ....S...HY
Hsap    WQFYLSKMIS MDIEQVITLS LALLLAVKYI FFEQT..... .ETEST.L.S
Atha    ..LY...... ..LTN.TFF. LSLFFATVY. FLLSRW..RE KIRNSTPLHV

        701                                                        750
Ppax    VEDPIISKWI IA.ALTLS.I ILNGYLFNAA RW.SIKEP.. ........E.
Lp19    LEDPVLSKWI VI.ALALS.V GLNGYLFNVA RW.SIKDPNV PDHGIDRKEL
Gfuj    LEDPVLSKWI VI.ALALS.V ALNGYLFNVA RW.GIKDPNV PEHNIDRNEL
Ater    LESP.LGRLC LMGALVVS.L VLNNHLIHAA RW........ ..HA.....W
Spom    ISASFLSKWI V.CALSLS.I AVNVFLLNAA RLNSIKEE.. PEKKV.....
Hsap    LKNPITSP.V V....TQKKV P.D....NCC R....REPML VRNNQKCDSV
Atha    VD...LSE.. I.CAL.IGFV ASFIYLLGFC ...GI..... ..........

        751                                                        800
Ppax    ..A....... ..A...P... ...APP.... KEPA..PKV. ..........
Lp19    ARAQRFN.ET ESATL.PLGE Y..VPPTPSC TEPAT.PAL. TDD..EGDGL
Gfuj    ARAREFN.DT GSATL.PLGE Y..VPPTPMR TQPST.PAI. TDD..EAEGL
Ater    PQARE..... .SA.V.PDGS YLSVP....C S..ATAPEVC T.........
Spom    ..VEKVV.E. ...VV....K Y..IP....S SNSSSI.... .DD.IQKDEI
Hsap    EEETGINRER KVEVIKPLVA ETDTP..... .NRATF.VVG NSSLLDTSSV
Atha    ........D. ...LI..... F......... .RSSS..... .DD....D.V

        801                                                        850
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Lp19    SMSKLRSSQS RSQFE.H.RS IEEL.EKL.I VEK....... .RTHELNDEE
Gfuj    HMTKARP... .ANLP.N.RS NEEL.EKL.L SEN....... .ALREMTDEE
Ater    .....RP... ....P..... .EET.EAL.L KSN....... .QAESLTDDE
Spom    .A.......Q ESVV....RS LEEC...ITL Y.NN...... GQISTLNDEE
Hsap    LVTQ.EP... EIELPREPRP NEECLQILG. ..NAEKGAKF .....LSDAE
Atha    WV.......N DGMIPCN.QS L.DCREVLPI KPNSVDPPRE SELDSVEDEE

        851                                                        900
Ppax    LIDLSLKGK. LPGYALEKTM E.....DENL MSRVDAFTRA VKIRRAVIAR
Lp19    VVTMSMRGK. VPGYALEKAL K.....D... ......FTRA VKIRRTIISR
Gfuj    VISLSMRGK. IPGYALEKTL G.....D... ......FTRA VKIRRSIIAR
Ater    LVELCLRGK. IAGYSLEKTL ERIAAGSSRS VTRLEAFTRA VRIRRAAVSK
Spom    VVQLTLAKK. IPLYALERVL K.....DV.. .......TRA VVIRRTVVSR
Hsap    IIQL.VNAKH IPAYKLE.TL .......... ...METHERG VSIRRQLLSK
Atha    IVKLVIDGT. IPSYSLETKL G.....DC.. .......KRA AAIRREAVQR
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      901                                                        950
Ppax  TAATAETTGS LEASKLPYKH YNYGLVHGAC CENVIGYLPL PLGVAGPIKI
Lp19  TKATSEITNG LDRSKLPFEN YNWERVFGAC CENVIGYLPL PVGVAGPLVI
Gfuj  NKAAADITHS LDRSKLPYEN YNWERFFGAC CENVIGYMPL PVGVAGPLVI
Ater  TPSTQNLCSG LAESLLPYRD YNYELVHGAC CENVVGYLPL PLGVAGPMVI
Spom  SSRT.K...T LESSNCPVYH YDYSRVLNAC CENVIGYMPL PLGVAGPLII
Hsap  ..KLSE.PSS LQY..LPYRD YNYSLVMGAC CENVIGYMPI PVGVAGPLCL
Atha  I..TGK...S L..TGLPLEG FDYNSILGQC CEMPVGYVQI PVGIAGPLLL

      951                                                       1000
Ppax  DGQSYFIPMA TTEGVLVAST SRGSKAINAG GGAVT.VLTG DGMTRGPCVT
Lp19  DGQSYFIPMA TTEGVLVAST SRGCKAINSG GGAIT.VLTS DGMTRGPCVS
Gfuj  DGQSYFIPMA TTEGVLVASA SRGCKAINSG GGAIT.VLTA DGMTRGPCVA
Ater  DGQALFIPMA TTEGVLVASA SRGCKAINAG GGATT.MLKG DGMTRGPCLR
Spom  DGKPFYIPMA TTEGALVAST MRGCKAINAG GGAVT.VLTR DQMSRGPCVA
Hsap  DEKEFQVPMA TTEGCLVAST NRGCRAIGLG GGASSRVL.A DGMTRGPVVR
Atha  DGVEYSVPMA TTEGCLVAST NRGFKAIHLS GGAFS.VLVK DAMTRAPVVR

      1001                                                      1050
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Lp19  FETLERA...  GAAKLWLDSE AGQNTMKKAF NSTSRFARLQ HMKT.ALAGT
Gfuj  FETLERA...  GAAKLWLDSE AGQDMMKKAF NSTSRFARLQ SMKT.ALAGT
Ater  FPSAQRA...  AEAQRWVESP LGHEVLAAAF NATSRFARLQ TLTV.AQAGI
Spom  FPNLTRA...  GRAKIWLDSP EGQEVMKKAF NSTSRFARLQ HIKT.ALAGT
Hsap  LP...RACDS AEVKAWLETS EGFAVIKEAF DSTSRFARLQ KLHT.SIAGR
Atha  FPSARRA...  ALVMFYLQDP SNFERLSLIF NKSSRFARLQ SI.TCTIAGR

      1051                                                      1100
Ppax  YLYIRFK.TT TGDAMGMNMI SKGCEKALD. VMSKECGFDD MSIISLSGNF
Lp19  NLYIRFK.TT TGDAMGMNMI SKGVEHALN. VMATDGGFDD MNIITVSGNF
Gfuj  NLYIRFK.TT TGDAMGMNMI SKGVEHALS. VMANDGGFDD MQIISVSGNY
Ater  YLYIRFR.TT TGDAMGMNMI SKGVEKALE. AMAAEGGFPD MHTVTLSGNF
Spom  RLFIRF.CTS TGDAMGMNMI SKGVEHAL.V VMSNDAGFDD MQVISVSGNY
Hsap  NLYIRFQSRS .GDAMGMNMI SKGTEKALSK L..HEY.FPE MQILAVSGNY
Atha  NLYPRFAC.S TGDAMGMNMV SKGVQNVLDF VKS.E..FPD MDVIGISGNY

      1101                                                      1150
Ppax  CTDKKSAAIN WTDGRGKSVV AEAIIPGDVV KSVLKSDVNA LVELNVSKNL
Lp19  CIDKKPAAMN WIDGRGKGIV AEAIIPADVV KSVLKSDVDA LVELNIAKNL
Gfuj  CTDKKAAALN WIDGRGKGVV AEAIIPGEVV RSVLKSDVDS LVELNVAKNL
Ater  CSDKKSAAIN WIGGRGKSVI AEATIPAETV RQVLKTDVDA LVELNTAKNL
Spom  CTDKKPAAIN WIDGRGKSVI AEAIIPGDAV KSVLKTTVED LVKLNVDKNL
Hsap  CTDKKPAAIN WIEGRGKSVV CEAVIPAKVV REVLKTTTEA MIEVNINKNL
Atha  CSDKKASAVN WIEGRGKHVV CEAFIKAEIV EKVLKTSVEA LVELNTLKNL

      1151                                                      1200
Ppax  IGSAMAGSLG GFNAHASNIV SAIFLATGQD PAQNVESSSC ITTMKNN.NG
Lp19  IGSAMAGSIG GFNAHAANIV AAIFLATGQD PAQVVESCNC ITTMKNL.HG
Gfuj  IGSAMAGSVG GFNAHAANIV AAIFLATGQD PAQVVESANC ITIMKNL.NG
Ater  VGSAMAGSLG GFNAHASNLV QAVFLATGQD PAQNVESSSC ITTMKNI.DG
Spom  IGSAMAGSVG GFNAHAANIV TAVYLATGQD PAQNVESSNC ITLMDNV.DG
Hsap  VGSAMAGSIG GYNAHAANIV TAIYIACGQD AAQNVGSSNC ITLME..ASG
Atha  VGSAMAGSLG GFNAHSSNIV SAVFIATGQD PAQNVESSHC MTMI..LPDG
```

```
      1201                                                      1250
Ppax  ....NLQIAV SMPSIEVGTI GGGT.ILEAQ SAMLDLLGVR GAHPTTPGEN
Lp19  ....SLQIAV SMPSLEVGTL GGGT.ILEPQ SAMLDMLGVR GSHPTNPGDN
Gfuj  ....ALQISV SMPSLEVGTL GGGT.ILEPQ GAMLDILGVR GSHPTNPGDN
Ater  ....NLHIAV SMPSMEVGTI GGGT.ILEAQ GAMLDLLGVR GAHSTEPGAN
Spom  ....NLQLSV SMPSIEVGTI GGGT.VLEPQ GAMLDLLGVR GAHMTSPGDN
Hsap  PTNEDLYISC TMPSIEIGTV GGGTNLL.PQ QACLQMLGVQ GACKDNPGEN
Atha  ...DDLHISV SMPCIEVGTV GGGT.QLASQ AACLNLLGVK GSNNEKPGSN

      1251                                                      1300
Ppax  ARQLSRIIAA SVLAGELSLC SALAAGHLVK AHMAHNRSA. ..APTR.SST
Lp19  ARRLARIIGA SVLAGELSLC SALQAGHLVK AHMQHNRSA. ..APSR.STT
Gfuj  ARRLARIIGA AVLAGELSLC SALAAGHLVR AHMQHNRSA. ..APSR.STT
Ater  ARRLARIVAA AVLAGELSTC AALAAGHLVN AHMQHNRTS. ..KDAI.SGT
Spom  SRQLARVVAA AVMAGELSLC SALASGHLVK SHIGLNRSAL N.TPAMDSSA
Hsap  ARQLARIVCG TVMAGELSLM AALAAGHLVK SHMIHNRSKI NLQDLQGACT
Atha  AQQLARIVAG SVLAGELSLM SAIAAGQLVK SHMKYNRS.. ....SRDIG.

      1301                                                      1350
Ppax  ..P.......  VS..A..AVG  ...AARGLSM  TSK~~~~~~~  ~~~~~~~~~~
Lp19  ..PAPPPMTP  VSL.A.MTIA  QDKSSKSAA.  ..AQQRSKR~  ~~~~~~~~~~
Gfuj  ..PG....S.  .SHDARLT.G  HDQCPRALSV  NNVDER.RRY  SEVKAIDE~~
Ater  ..EYGAIRTP  V.YVVILEHA  GD..IHFVQI  EYKNTYLRRK  VPTLSCNLGR
Spom  KKPATDALKS  VNSRVPGR~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~
Hsap  KKTA~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~
Atha  ..PSS....Q  VN.R~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~
```

(residues 684 to 1139) is highly conserved and catalytically active residues were predicted by comparison to other *hmg* genes. The complete nucleotide and polypeptide sequence, including the position of the introns, transcription and translation start and stop sites, is shown in figure 3.7. Codon bias in the *P. paxilli hmg* gene is shown in Table 3.3. A summary of gene features in *P. paxilli* is shown in Table 3.7.

3.1.8 Confirmation of introns

Total RNA was isolated from samples of mycelia, removed at a number of time points, from a *P. paxilli* liquid culture (section 2.1.2 and section 2.17). The RNA was reverse transcribed into cDNA with random hexamer primers (section 2.18). RT-PCR was performed on cDNA to characterise the structure of the *hmg* mRNA. The removal of introns can be confirmed when products amplified from cDNA are compared to products amplified from gDNA with the same primers. Intron 3 was identified when cDNA, synthesised from RNA present in mycelia at 48 h, was amplified with primers ET22 and ET9 (Table 2.2). A routine PCR (section 2.11.2) was performed and the gDNA and cDNA products separated on a 2% (w/v) agarose gel (section 2.10.1 and 2.10.3) (Figure 3.9). The cDNA product was purified (section 2.5.4) and sequenced (section 2.12.3) to confirm the exact location of 5' donor and 3' acceptor sites. Intron 2 was identified and utilised as a control when the transcription start site was estimated (section 3.1.9). Primer ET5 is downstream of intron 2 and when used in combination with primers ET2, ET6 and ET8, results in a 71 bp size difference between the genomic PCR product and the cDNA product (Figure 3.10, lanes 10-12). The product amplified with ET5 and ET8 was purified from agarose gel (section 2.5.4), and sequenced (section 2.12.3) to confirm the exact intron/exon boundary of intron 2. The size difference between cDNA and gDNA products increases further when ET5 is paired with ET12 (Figure 3.10, lanes 6-8), indicating that a second intron has been removed. The position of this intron was confirmed when cDNA, synthesised from RNA present in mycelia at 60 h, was amplified with primers ET12 and ET15 (Figure 3.11). The product was cloned into a pGEM®-T vector (section 2.15) and sequenced (2.12.3) to show the exact location of the intron one 5' donor and 3' acceptor sites. On closer examination, intron consensus sequences were present in the Lp19 *hmg* 5'UTR sequence as well. RNA isolated from Lp19 mycelia grown in liquid culture for 14 days (McGill, 2000 In Prep)

Figure 3.9 Confirmation of hmg intron 3

RT-PCR (section 2.20) was performed on a 1/100 dilution of cDNA from 48 h cultures, using primers ET22 and ET9 (Table 2.2). Samples were separated on a 2% agarose gel (section 2.10.1 and 2.10.3) as follows. Lane 1, 100bp ladder (BioLabs). Lane 2, gDNA. Lane 3, 48 h cDNA. Lane 4, water control. Sizes shown are in bp.

Figure 3.10 Estimation of the transcription start site of hmg

RT-PCR (section 2.20) was performed on 1/100 dilutions of cDNA from 36 h and 60 h cultures. Standard PCR reactions (section 2.11.2) were performed with the 3' anchored primer ET5 (Table 2.2) and a set of 5' nested primers (ET4Bam, ET12, ET2, ET6 and ET8), on templates; gDNA, 36 h cDNA, 60 h cDNA and a water control. Products were separated on a 2% agarose gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 22, 100bp ladder (BRL). Lanes 2-5, ET4Bam and ET5. Lanes 6-9, ET12 and ET5. Lanes 10-13. ET2 and ET5. Lanes 14-17, ET6 and ET5. Lanes 18-21, ET8 and ET5. Sizes shown are in bp.

Figure 3.9

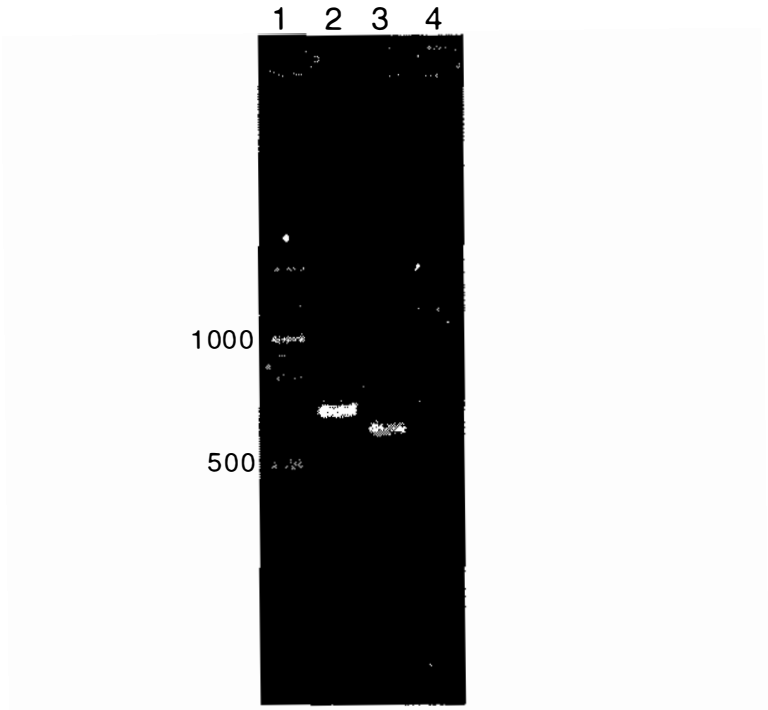


Figure 3.10

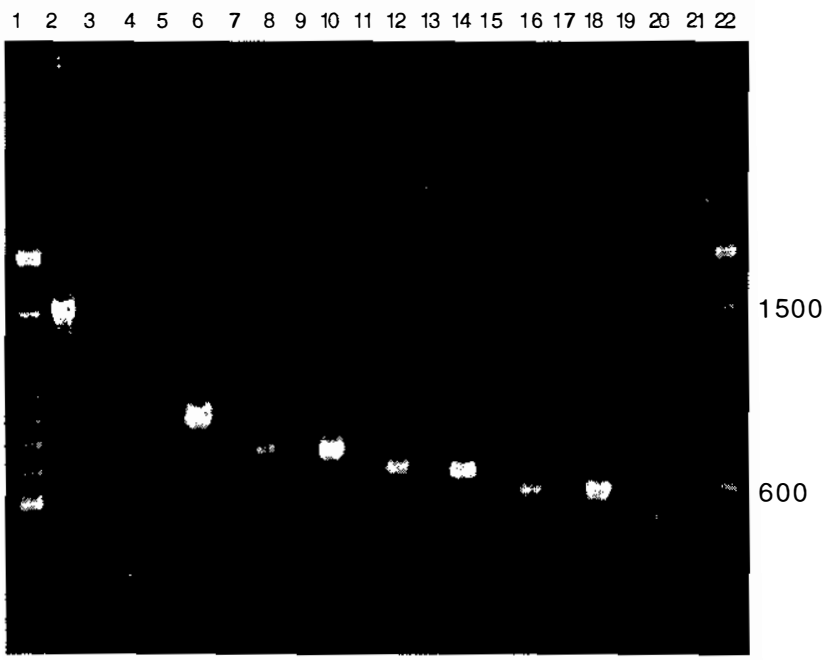


Figure 3.11 *Confirmation of 5' UTR intron 1 in P. paxilli hmg*

RT-PCR (section 2.20) was performed on a 1/10 dilution of cDNA. from a 60 h culture, with primers ET12 and ET15 in a standard PCR reaction (section 2.11.2). Samples were separated on a 2% agarose gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 5, 100bp ladder (BioLabs). Lane 2, gDNA. Lane 3, 60 h cDNA. Lane 4, water control. Sizes shown are in bp.

Figure 3.12 *Confirmation of 5' UTR intron 1 in N. lolii Lp19 hmg*

RT-PCR (section 2.20) was performed on a 1/10 dilution of cDNA from a 14 day *N. lolii* Lp19 culture. The standard PCR reactions (section 2.11.2) were set up using combinations of primers hmgeco, hmg2eco and hmgnco (Table 2.2). Samples were separated on a 2% agarose gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 13, 1kb+ ladder (BRL). Lane 2-6, (hmgeco and hmgnco) 14 day *N. lolii* Lp19 cDNA, 14 day *N. lolii* Lp19 RNA (-RT), Reverse transcriptase water control, *N. lolii* Lp19 gDNA, and a water control. Lanes 7-9, (hmg2eco and hmgnco) 14 day *N. lolii* Lp19 cDNA, *N. lolii* Lp19 gDNA, and a water control. Lanes 10-12, (hmg29 and hmg30) 14 day *N. lolii* Lp19 cDNA, *N. lolii* Lp19 gDNA, and a water control. Sizes shown are in bp.

Figure 3.11

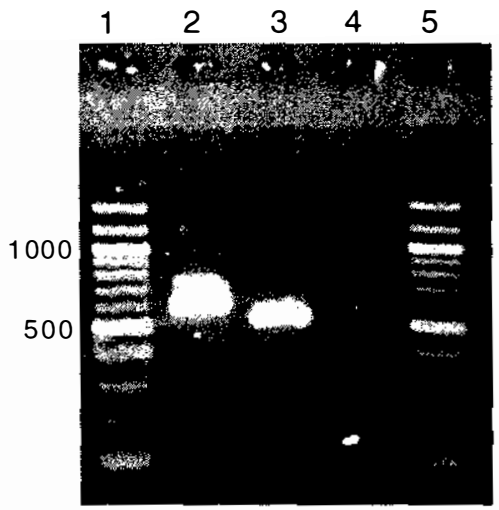


Figure 3.12

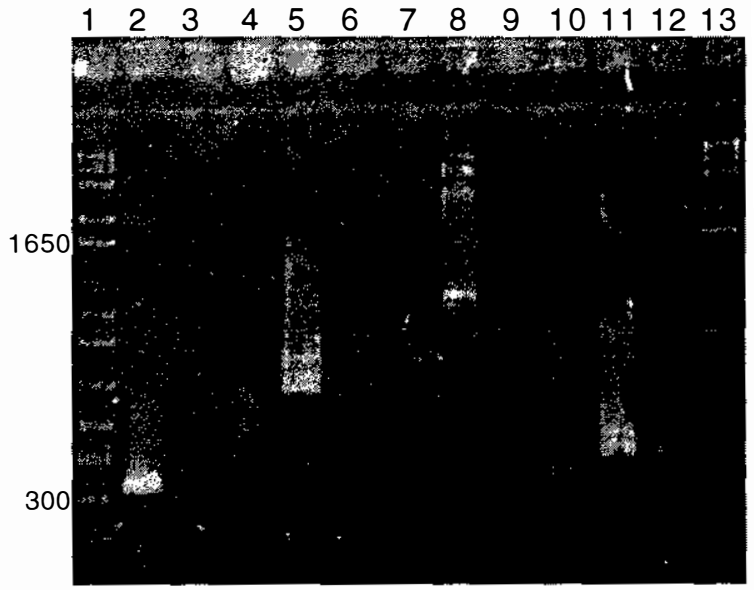
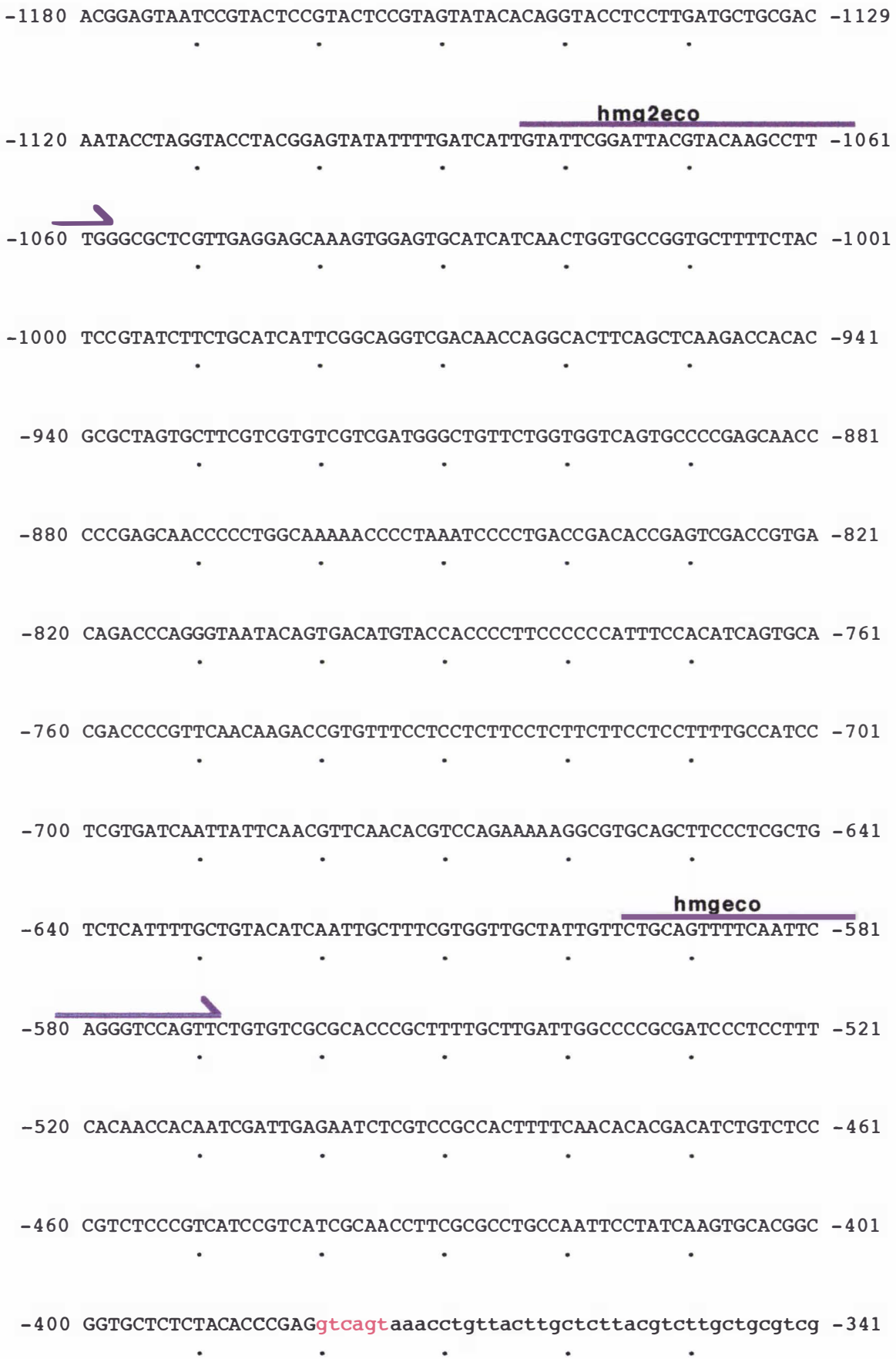


Figure 3.13 *Partial DNA sequence and deduced amino acid sequence of N. lolii Lp19 hmg*

The 5' UTR Sequence of *N. lolii* Lp19 hmg was determined to ¯287 bp by Dobson (1997), and further extended to ¯1180 bp by Lisa McMillan. The 5' UTR intron of 282 bp is shown in lower case, with the 5' and 3' splice junctions highlighted in red. The putative 3' and 5' splices for alternative splicing are shown in orange. The single lariat consensus sequence is shown in green. Primers used to determine the size of the 5' UTR are shown as purple lines. The translation start codon is shown in blue.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hmg2eco
-1120 AATACCTAGGTACCTACGGAGTATATTTTGATCATTGTATTCGGATTACGTACAAGCCTT -1061
-1060 TGGGCGCTCGTTGAGGAGCAAAGTGGAGTGCATCATCAACTGGTGCCGGTGCTTTTCTAC -1001
-1000 TCCGTATCTTCTGCATCATTCGGCAGGTCGACAACCAGGCACTTCAGCTCAAGACCACAC -941
-940 GCGCTAGTGCTTCGTCGTGTCGTCGATGGGCTGTTCTGGTGGTCAGTGCCCCGAGCAACC -881
-880 CCCGAGCAACCCCCTGGCAAAAACCCCTAAATCCCCTGACCGACACCGAGTCGACCGTGA -821
-820 CAGACCCAGGGTAATACAGTGACATGTACCACCCCTTCCCCCCATTTCCACATCAGTGCA -761
-760 CGACCCCGTTCAACAAGACCGTGTTTCCTCCTCTTCCTCTTCTTCCTCCTTTTGCCATCC -701
-700 TCGTGATCAATTATTCAACGTTCAACACGTCCAGAAAAAGGCGTGCAGCTTCCCTCGCTG -641
hmgeco
-640 TCTCATTTTGCTGTACATCAATTGCTTTCGTGGTTGCTATTGTTCTGCAGTTTTCAATTC -581
-580 AGGGTCCAGTTCTGTGTCGCGCACCCGCTTTTGCTTGATTGGCCCCGCGATCCCTCCTTT -521
-520 CACAACCACAATCGATTGAGAATCTCGTCCGCCACTTTTCAACACACGACATCTGTCTCC -461
-460 CGTCTCCCGTCATCCGTCATCGCAACCTTCGCGCCTGCCAATTCCTATCAAGTGCACGGC -401
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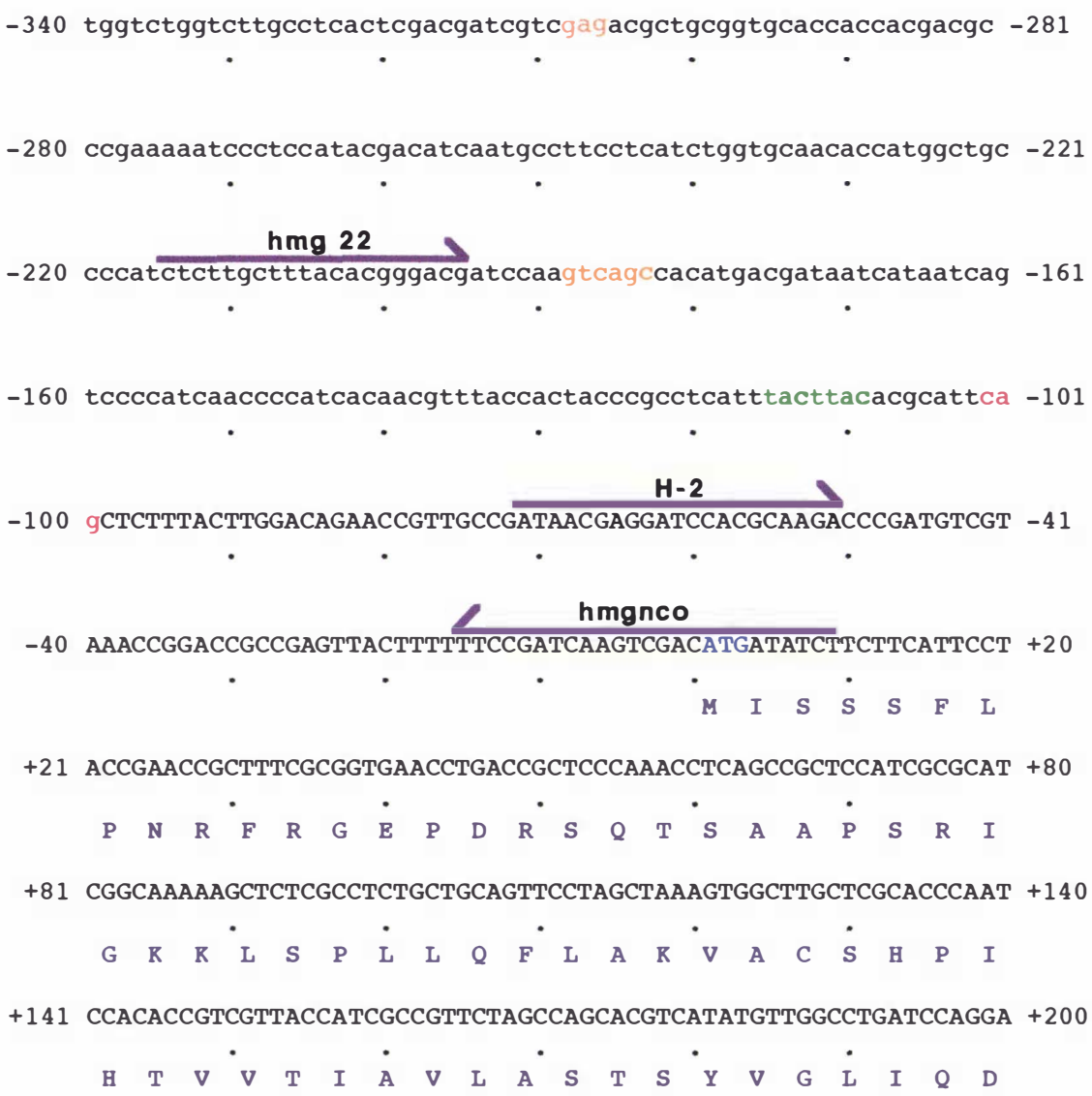
-340 tggtctggtcttgcctcactcgacgatcgtcgagacgctgcggtgcaccaccacgacgc -281
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hmg 22
-220 cccatctcttgctttacacgggacgatccaagtcagccacatgacgataatcataatcag -161
-160 tccccatcaaccccatcacaacgtttaccactacccgcctcatttacttacacgcattca -101
H-2
-100 gCTCTTTACTTGGACAGAACCGTTGCCGATAACGAGGATCCACGCAAGACCCGATGTCGT -41
hmgnco
-40 AAACCGGACCGCCGAGTTACTTTTTTCCGATCAAGTCGACATGATATCTTCTTCATTCCT +20
M I S S S F L
+21 ACCGAACCGCTTTCGCGGTGAACCTGACCGCTCCCAAACCTCAGCCGCTCCATCGCGCAT +80
P N R F R G E P D R S Q T S A A P S R I
+81 CGGCAAAAAGCTCTCGCCTCTGCTGCAGTTCCTAGCTAAAGTGGCTTGCTCGCACCCAAT +140
G K K L S P L L Q F L A K V A C S H P I
+141 CCACACCGTCGTTACCATCGCCGTTCTAGCCAGCACGTCATATGTTGGCCTGATCCAGGA +200
H T V V T I A V L A S T S Y V G L I Q D

was reverse transcribed into cDNA, using random hexamer primers. RT-PCR was performed on this cDNA with a set of Lp19 *hmg* specific primers (hmgeco, hmg2eco and hmgnco (Table 2.3)), and the products separated on a 2% (w/v) agarose gel (section 2.10.1 and 2.10.3). The size difference between gDNA and cDNA products confirms a large intron or several small introns had been removed between primers hmgeco and hmgnco (Figure 3.12, lanes 2-6). No cDNA products were amplified with primers hmg2eco and hmgnco (lanes 7-9), indicating that the transcription start site is between primers hmgeco and hmg2eco at this time point. Control reactions with primers hmg29 and hmg30 (lanes 10-12), amplified *hmg* cDNA in the coding region of the gene, confirming that *hmg* mRNA was present in mycelia at this time point. The cDNA product amplified with hmgnco and hmgeco was cloned into a pGEM®-T vector (section 2.15) and sequenced (section 2.12.3) to determine the number of introns removed and the exact location of intron/exon boundaries. Sequencing revealed a single intron of 282 bp had been removed (Figure 3.13). It is possible this may represent an alternatively spliced product encompassing the 5'donor site of one intron and the 3' acceptor site of a downstream intron. Putative 3' acceptor and 5' donor sites are present between the sites utilised in this instance.

3.1.9 Estimation of transcription start site with RT-PCR

Copy DNA synthesised from the RNA present in mycelia at 36 h and 60 h, was compared to determine if transcription of *hmg* mRNA is initiated at different sites during paxilline biosynthesis. During early growth experiments, paxilline was detected in mycelia after 60 h by HPLC (Figure 3.25 A). Therefore, if a different *hmg* transcript is present during paxilline biosynthesis, it should be detected at 60 h. Primer ET5 (Table 2.3) downstream of intron 2, was paired with a number of primers (ET4Bam, ET12, ET2, ET6 and ET8) upstream of the translation start site. A routine PCR reaction (section 2.11.2) was performed and products separated on a 2% (w/v) agarose gel (section 2.10.1 and 2.10.3) (Figure 3.10). Products were amplified only when the 5' primer sequence was present in the cDNA. The gDNA control included with each primer set confirms a successful PCR was achieved, and the size difference between gDNA and cDNA products confirms the origin of amplified products. Both templates produce a cDNA product with primer ET12, but not with ET4Bam, indicating that

transcription is initiated between these two primer sites at both time points. The level of *hmg* expression at 36 h is lower than that seen at 60 h, even though the concentration of the cDNA templates at both time points is equivalent. This was confirmed when *tub-2* specific primers were used to amplify products of equal intensity from the same two samples of cDNA (Figure 3.34).

3.1.10 Confirmation of transcription start site with 5' RACE

Although the approximate location of *hmg* transcription initiation had been estimated with RT-PCR (section 3.1.9), 5' RACE was used to pinpoint the exact location. As described above, RNA isolated from mycelia at two different time points was used to determine if transcription of *hmg* mRNA is initiated at a different site during paxilline biosynthesis. Analysis of expression levels from genes involved in paxilline biosynthesis, performed after the initial estimation of the transcription start site, showed transcription of *pax* genes as early as 36 h (section 3.4). Therefore, the time points selected were 24 h and 60 h. RACE was performed as described in section 2.19 (Figure 3.14 A), and after the terminal transferase reaction had been completed several rounds of nested PCR were performed. The final PCR products amplified with primers AUAP and ET3 (Table 2.3) were separated on a 2% (w/v) agarose gel (section 2.10.1 and 2.10.3) (Figure 3.15). Only the 60 h RNA produced any detectable RACE products. Gel stab purifications (section 2.11.4) were performed on the two products visible in Figure 3.15. However, after second round amplification, only the smaller 314 bp product was seen. The purified product was cleaned (section 2.11.5) and cloned into a pGEM®-T vector (section 2.15) for sequencing (section 2.12.3). The sequence data obtained confirmed the position of the transcription start site that had previously been estimated with RT-PCR (section 3.1.9). Due to time constraints further attempts to isolate the larger 60 h RNA RACE product, or a product from 24 h RNA were not possible. It is possible that other transcription start sites for *hmg* exist, but only one, shown in Figure 3.7, has been identified with 5' RACE at this time.

Figure 3.14 Strategy for RACE

A) 5' RACE

The transcription start site was determined using the primers indicated (Table 2.3)

B) 3' RACE

The transcription termination site was determined using the primers indicated (Table 2.3) (section 2.19).

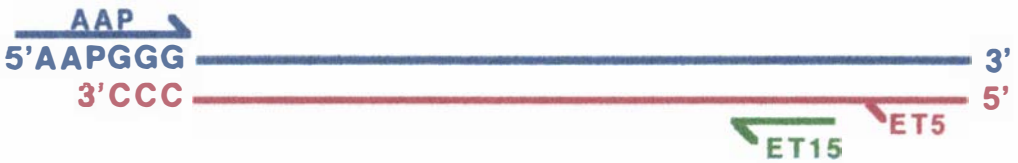
A

1. First strand synthesis

2. Degrade RNA strand with RNase H

3. Terminal Transferase

4. Second strand synthesis



5. Nested PCR

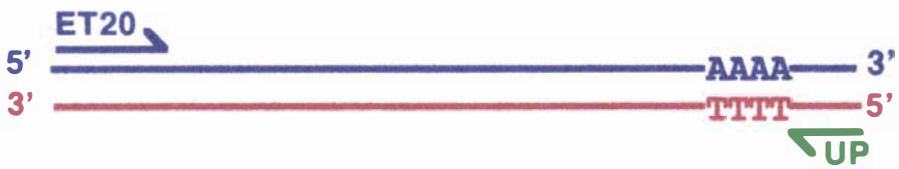
6. Clone PCR product for sequencing using Terminal A

B

1. First strand synthesis

2. Degrade RNA strand with RNase H

3. Second strand synthesis



4. Nested PCR

5. Clone PCR product for sequencing using Terminal A

Figure 3.15 P. paxilli hmg 5' RACE

5' RACE was performed (section 2.21) (Figure 3.14 A) on RNA from 24 h and 60 h cultures (section 2.19). After the terminal transferase reaction (section 2.19.3), the standard PCR reaction (section 2.11.2) was performed with primers AUAP and ET3 (Table 2.2). Products were separated on a 2% agarose gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 7, 100bp ladder (BioLabs). Lane 2, 24 h cDNA with terminal transferase added. Lane 3, 24 h cDNA without terminal transferase. Lane 4, 60 h cDNA with terminal transferase added. Lane 5, 60 h cDNA without terminal transferase. Lane 6, water control. Sizes shown are in bp.

Figure 3.16 P.paxilli hmg 3' RACE

3' RACE was performed on RNA from 60 h cultures (section 2.19) (Figure 3.14 B). The standard PCR reactions (section 2.11.2) were set up with combinations of primers ET22, ET9 and UP (Table 2.2) and samples separated on a 2% agarose gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 7, 1kb+ ladder (BRL). Lanes 2 and 3, (ET22 and UP) 3' RACE cDNA and water control. Lanes 4-6, (ET22 and ET9) 3' RACE cDNA, gDNA, and water control. Sizes shown are in bp.

Figure 3.15

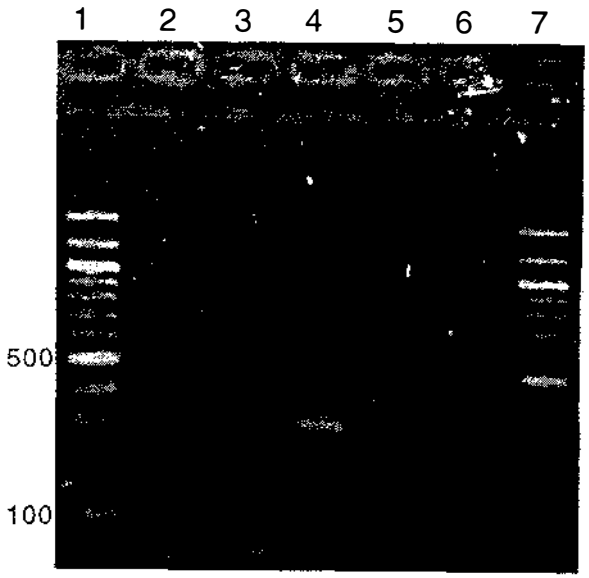
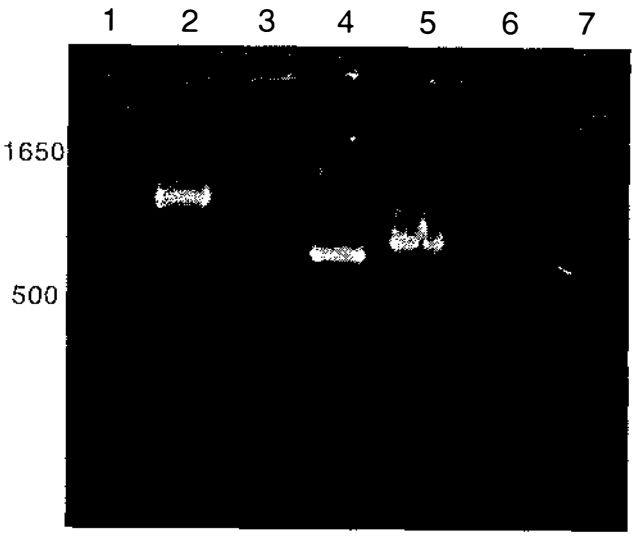


Figure 3.16



3.1.11 Confirmation of the transcription stop site with 3' RACE

Many filamentous fungal genes are transcribed past the signal for polyadenylation (Gurr *et al.*, 1987). The signal for polyadenylation in filamentous fungal genes is frequently the consensus 5'-AAUAAA-3'. The closest match to this sequence in the *P. paxilli hmg* 3' UTR, is the motif 5'-ATAAAA-3', found 483 bp downstream of the translation termination codon (Figure 3.7). The RACE technique was used to determine the 3' end of transcription using RNA isolated from mycelia grown in liquid culture for 60 h. The first round of 3' RACE amplification is non specific, and uses an oligo dT primer (Table 2.3) to reverse transcribe a pool of mRNA (Figure 3.14 B). As 3' RACE was being used to determine the transcription stop site of a number of *P. paxilli* genes, a single first round reaction was performed by Lisa McMillan and stored at -20°C. Second round amplification was then performed twice with a set of nested gene specific 5' primers and the UP primer. Amplification with gene specific internal primers ET22 and ET9, and separation of the products on a 2% (w/v) agarose gel (section 2.10.1 and 2.10.3) confirmed the specificity of the product (Figure 3.16). The single product shown (lane 2), was purified (section 2.11.5) and cloned (section 2.15) into a pGEM®-T EASY vector for sequencing (section 2.12.3). The 3'UTR region was 563 bp long (Figure 3.7).

3.1.12 Chromosomal location of *P. paxilli hmg*

The chromosomal location of *hmg* was identified by probing a nylon membrane (section 2.13), containing a contour-clamped homogenous electric field (CHEF)-gel electrophoresis separation (Figure 3.17 A) previously performed by Carolyn Young, with a PCR product of *hmg* amplified with ET5 and ET6. Chromosomes of *P. paxilli* were separated by CHEF electrophoresis, and transferred to nylon membrane by Southern blotting. The conditions for CHEF separation are described in the legend of Figure 6, in the paper by Young *et al.* (1998). This gel included several Pax$^-$ mutants of *P. paxilli* as well as wild type, but in all cases, the *hmg* probe hybridised to chromosome Vb (Figure 3.17 B).

Figure 3.17 CHEF analysis of P. paxilli chromosomes

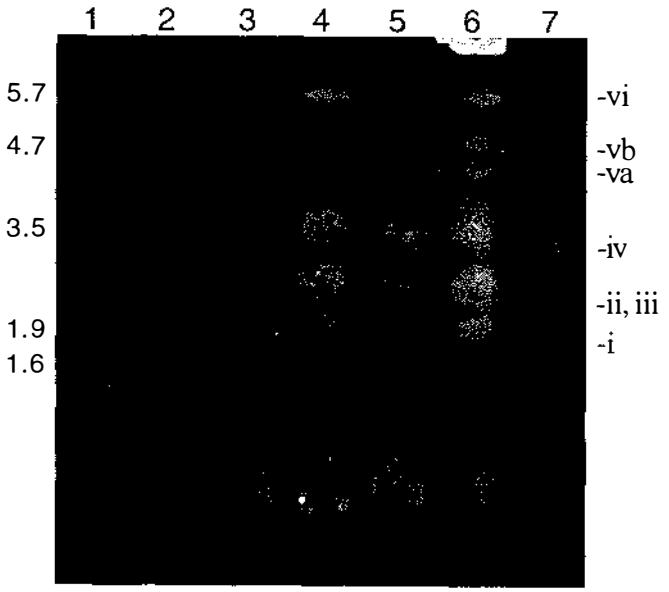
A) Ethidium bromide stained CHEF gel

This is the same gel as that shown in Figure 6 B in Young *et al.* (1998). Running conditions for CHEF electrophoresis are described in the legend of this figure. Lane 1, *S. cerevisiae*. Lane 2, *S. pombe*. Lanes 3-7, *P. paxilli* wild-type, YI-20, CY-2, CY-102, and CY-35. Sizes shown are in Mb.

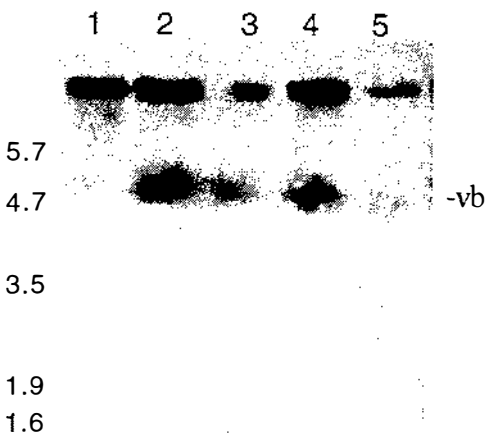
B) Chromosomal location of hmg

Autoradiograph of gel shown in Figure 3.15 A probed with a [^{32}P]-labelled PCR product of *hmg*, amplified with primers ET5 and ET6. Southern blotting and probe hybridisation is described in section 2.13. Sizes shown are in Mb.

A



B



3.2 Isolation and Characterisation of *P. paxilli tub-2*

3.2.1 Preparation of *tub-2* genomic probe from pBT6

A bacterial culture containing plasmid pBT6 (Table 2.1 Appendix A 1.2) was plated for single colonies (section 2.1.2) and DNA isolated using an alkaline lysis based mini-prep kit (section 2.5.5). After quantification by fluorometry (section 2.6.2), the plasmid was cut with *Eco*RI (section 2.7) and separated on a 0.7% (w/v) agarose gel (section 2.10.1 and 2.10.3). The 1.8 kb fragment, containing part of the *Neurospora crassa* β-tubulin gene, was isolated from the gel (section 2.5.4), purified with phenol/chloroform (section 2.8) and precipitated with ethanol (section 2.9).

3.2.2 Confirmation a single *tub-2* exists in the *P. paxilli* genome

The *P. paxilli* genomic blot (Figure 3.18 A) originally probed with the 359 bp *hmg* product was stripped (section 2.13.4), and re-probed with the [α-^{32}P]-labelled 1.8 kb *Eco*RI fragment of pBT6 as described in section 2.13 (Figure 3.18 B). The *tub-2* gene is extremely conserved, therefore, the conditions for heterologous probing with the *N. crassa tub-2* were identical to the homologous probing of this blot with the unique *hmg* discussed in section 3.1.4

Table 3.4 Size of *P. paxilli* gDNA bands hybridising with 1.8 kb *tub-2* probe

Size of Bands (kb)						
Restriction enzyme						
*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII	*Pst*I	*Sal*I	*Sst*I
6.2 5.6	20.5	14.1	3.2 2.5	7.7	13.9	0.7

Figure 3.18 *Southern analysis of tub-2 in the P. paxilli genome*

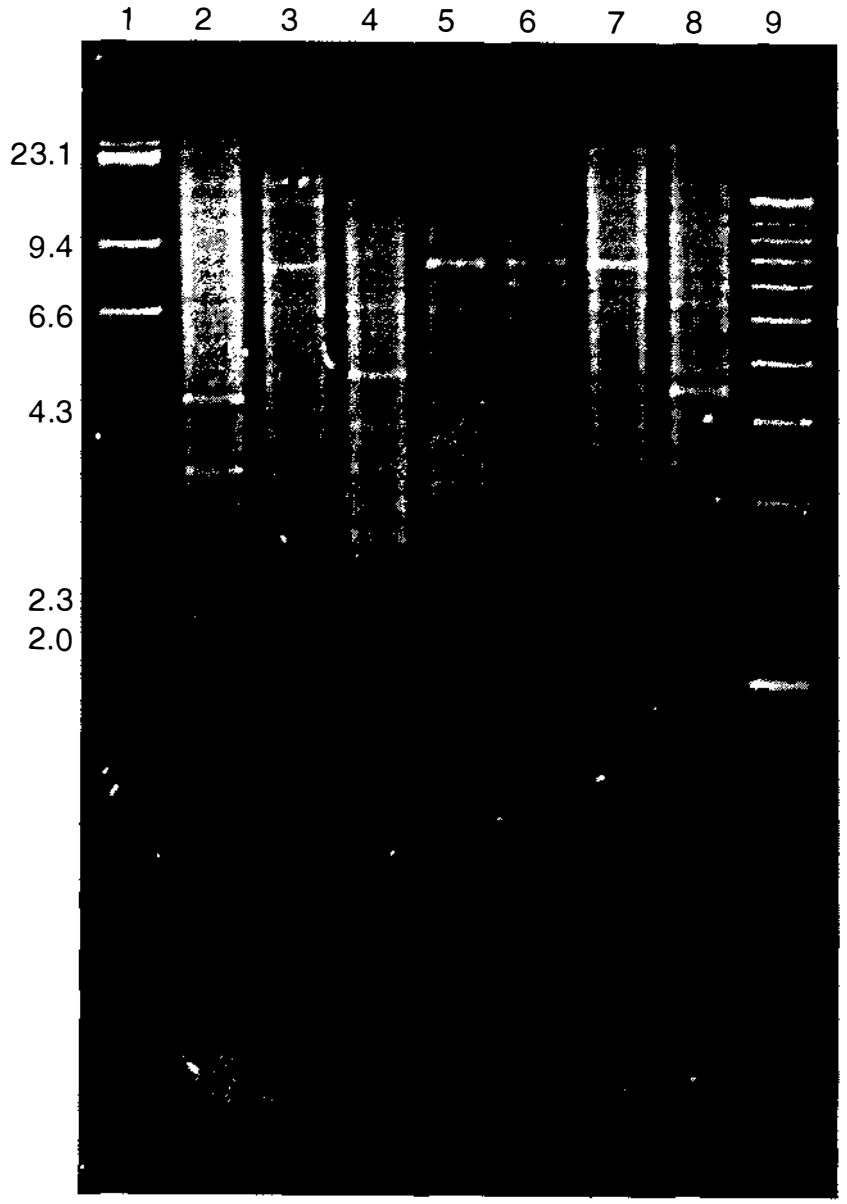
A) Restriction endonuclease digestion of P. paxilli gDNA

This is the same gel seen in Figure 3.3 A. *P. paxilli* genomic DNA (2 μg) (section 2.5.1) was digested with restriction endonucleases (section 2.7). Each reaction was separated on a 0.7% agarose gel (section 2.10.2 and 2.10.3) as follows. Lane 1, λ/*Hin*dIII. Lanes 2-8, *P. paxilli* gDNA cut with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Sst*I. Lane 9, 1kb ladder (BRL). Sizes shown are in kb.

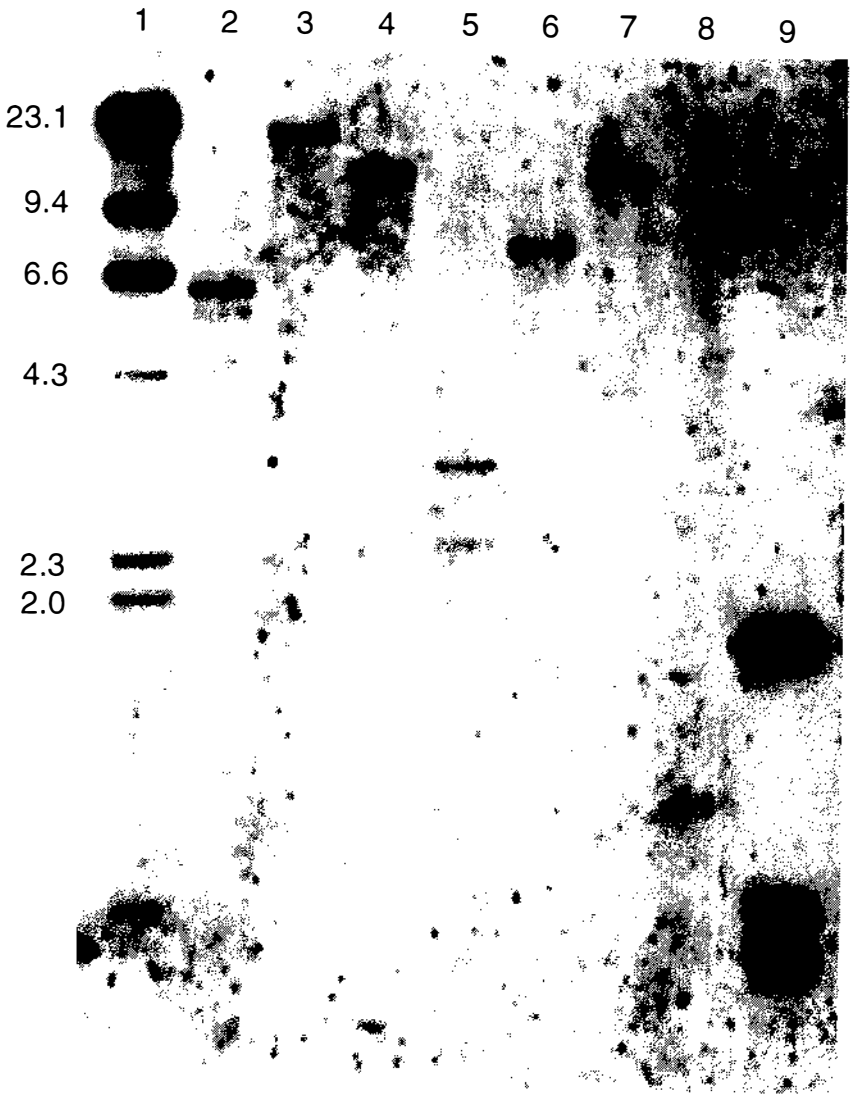
B) Confirmation that sequence heterologous to the 1.8kb EcoRI fragment of pBT6 is part of the P. paxilli genome

Autoradiograph, showing hybridisation of *N. crassa tub-2* to the *P. paxilli* genome. Ethidium bromide stained gel seen in Figure 3.3 A was Southern blotted and probed with the [^{32}P]-labelled 1.8 kb *Eco*RI fragment of pBT6 (section 2.13). Sizes shown are in kb.

A



B



The single bands hybridising in most lanes confirm the presence of a single copy of *tub-2* in the *P. paxilli* genome. The multiple bands present in the *Bam*HI and *Hin*dIII digests (Figure 3.16 B, lanes 2 and 5) result from the presence of these restriction enzyme sites within the *tub-2* sequence.

3.2.3 Library screening

The first round library filters used to isolate *hmg* were stripped (section 2.13.4) and re-probed with the 1.8 kb *Eco*RI fragment of pBT6 (section 2.14.5). Ten positive clones (λET20, λET21, λET22, λET23, λET24, λET25, λET26, λET27, λET28, and λET29) were isolated (section 2.14.6) and plated for second round screening (section 2.14.8). Only six of these clones (λET22, λET23, λET24, λET25, λET27, and λET29) were positive after second round screening. Duplicates clones were isolated (section 2.14.6) and one of these was plated for further screening. All six clones were positive after third round screening, and duplicate samples of each clone were isolated and stored at 4°C.

3.2.4 Physical mapping of *tub-2* λ clones

DNA was isolated (section 2.5.2) from each clone, digested with *Sst*I (section 2.7) and separated by gel electrophoresis (section 2.10.2 and 2.10.3) (Figure 3.19 A). A Southern blot of this gel was probed with the 1.8 kb *Eco*RI fragment of pBT6 (section 2.13) (Figure 3.19 B). Band sizes and patterns of hybridisation are represented in Table 3.5. A *Sst*I restriction map based on these sizes was produced (Figure 3.20), and used to determine the best clone to continue sequencing of *tub-2*.

3.2.5 Sub-cloning λET22 into a sequencing vector

Clone λET22 was chosen for sub-cloning and sequencing, as it was the most central of the clones with respect to probe hybridisation. DNA from this clone was digested with *Sst*I, purified with phenol/chloroform (section 2.8) and precipitated with ethanol

Figure 3.19 Southern analysis of tub-2 lambda clones

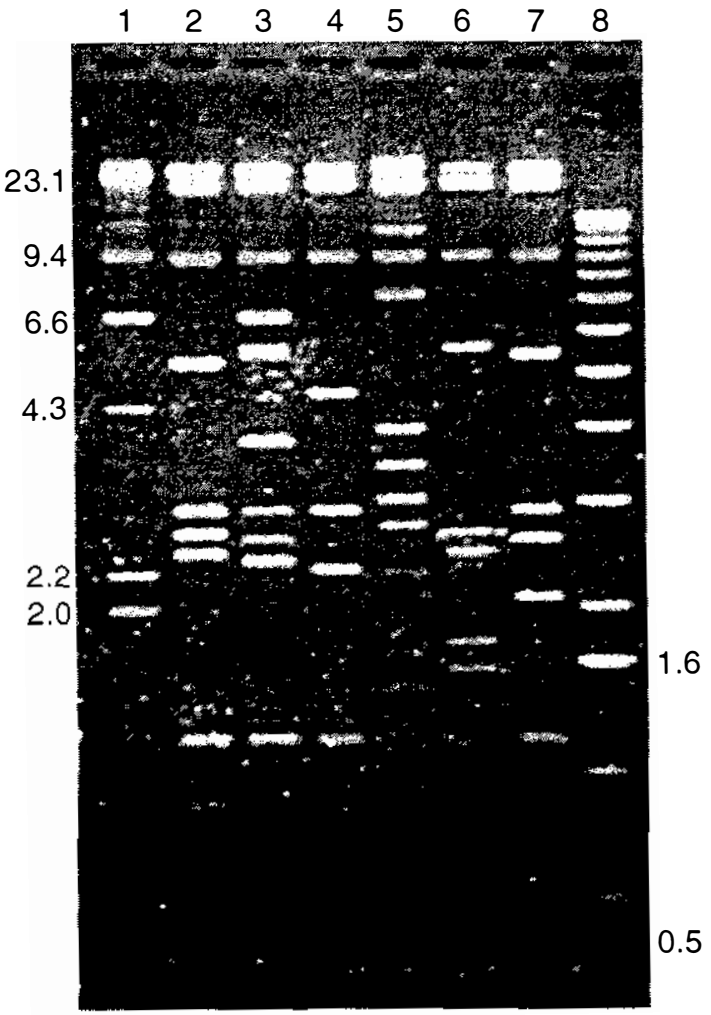
A) Restriction endonuclease digestion of tub-2 lambda clones with SstI

DNA (~500 ng) from medium scale λ preps (section 2.5.2) was digested with *Sst*I (section 2.7) and separated on a 0.7% agarose gel (section 2.10.2 and 2.10.3) as follows. Lane 1, λ/*Hin*dIII. Lanes 2-7, *Sst*I digestion of, λET22, λET23, λET24, λET25, λET27 and λE29. Lane 8, 1 kb ladder (BRL). Sizes shown are in kb.

B) Mapping the position of tub2 on lambda clones

Autoradiograph showing hybridisation of *N. crassa tub-2* to the digested λ clones. Ethidium bromide stained gel seen if Figure 3.4 A was Southern blotted and probed with [^{32}P]-labelled 1.8kb *Eco*RI fragment of pBT6 (section 2.13). Sizes shown are in kb

A



B

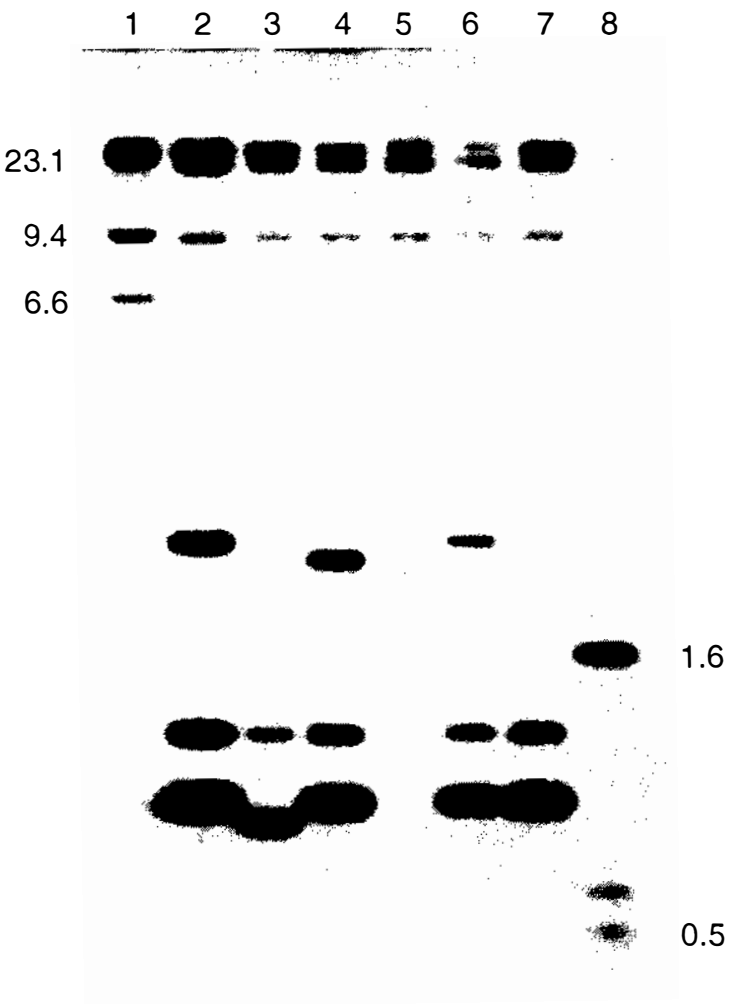


Table 3.5 *Size and hybridisation pattern of SstI digests of tub2 λ Clones probed with 1.8kb EcoRI fragment of pBT6*

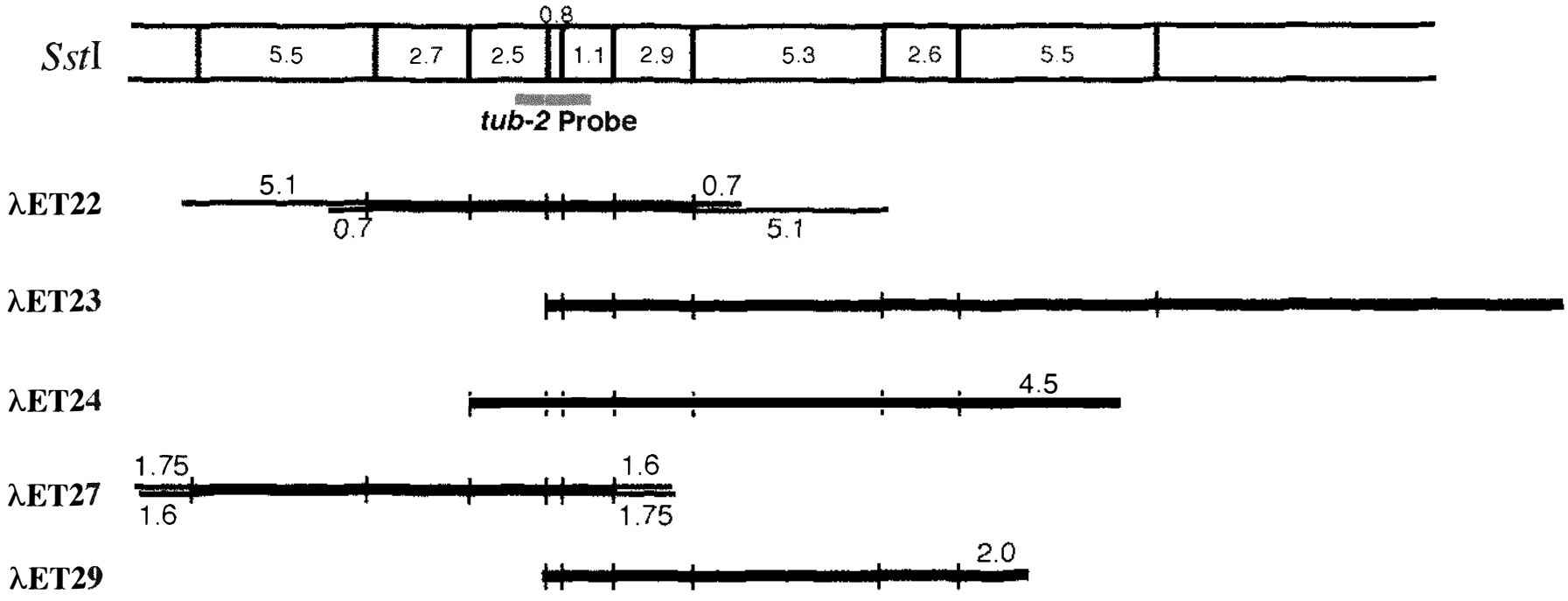
	λ Clones					
Size (kb)	λET22	λET23	λET24	λET25	λET27	λET29
23.1	—	—	—	—	—	—
17.2	—	—	—	—	—	—
10.7				—		
9.4	—	—	—	—	—	—
7.0				—		
6.4		—				
5.5		—			—	
5.3		—				—
5.1	—					
4.5			—			
3.84				—		
3.76		—				
3.4				—		
3.0				—		
2.9	—	—	—			—
2.72				—		
2.67	—				—	
2.6		—				—
2.5	—				—	
2.4		—				
2.3			—	—		
2.0						—
1.75					—	
1.67				—		
1.6					—	
1.45				—		
1.2				—		
1.14	—	—	—		—	—
0.84	—		—		—	—
0.81		—		—		
0.70	—				—	

● Band Stained with ethidium Bromide and Hybridises to ^{32}P probe

* Band Hybridises to ^{32}P probe

Figure 3.20 ***SstI Restriction map of tub2 λ Clones tub2***

All sizes shown are in kb.



(section 2.9). The purified DNA was ligated into pUC118 and transformed into *E. coli* XL-1 (section 2.15). Due to the size of the *tub-2* gene, there was no need to reduce sub-clones further with restriction enzymes as was the case with sequencing of *hmg* (section 3.1.7). Only a few novel primers (Table 2.3) were required to complete double stranded sequencing. The sub-clones used for sequencing are listed in Table 2.1. The restriction map of *P. paxilli tub-2* locus and relative position of the sub-clones used for sequencing is shown in Figure 3.21.

3.2.6 Sequencing λET22 sub-clones

P. paxilli tub-2 was sequenced and analysed as described in section 3.1.7. Junctions between individual fragments were sequenced directly from λET22 and λET27 with the appropriate primers. Templates used to assemble the *tub-2* contig, are displayed in Appendix A.2.4 with the Sequencher™ software. Sequence analysis and polypeptide sequence of *tub-2* translation revealed a 447 amino acid protein comprised of 7 ORF. Open reading frame 1 extends from +1 to +12 bp, ORF 2 extends from +120 to +140 bp, ORF 3 extends from +261 to + 286 bp, ORF 4 extends from +355 to +386 bp, ORF 5 extends from +463 to +517 bp, ORF 6 extends from +588 to +1044 bp, and ORF 7 extend from +1107 to a termination codon at + 1896 bp. This overall gene structure is depicted in Figure 3.21. The complete nucleotide and polypeptide sequence, including the position of introns and translation start and stop sites, is shown in Figure 3.22. The translation start and stop sites were determined by homology to other *tub-2* genes and sequence surrounding the translation start codon (Ballance, 1986; Kozak, 1984). Codon bias in *P. paxilli tub-2* is shown in Table 3.6. A summary of gene features in *P. paxilli* is shown in Table 3.7.

3.2.7 Confirmations of introns

As described previously (section 3.1.8), cDNA was synthesised from RNA isolated from mycelia at different time points. The presence of introns was determined by comparing products amplified with the same primers from cDNA and gDNA. The standard PCR reaction (section 2.11.2) was set up with primers Etub5 and Etub6 (Table

Figure 3.21 Physical map of P. paxilli tub-2 locus

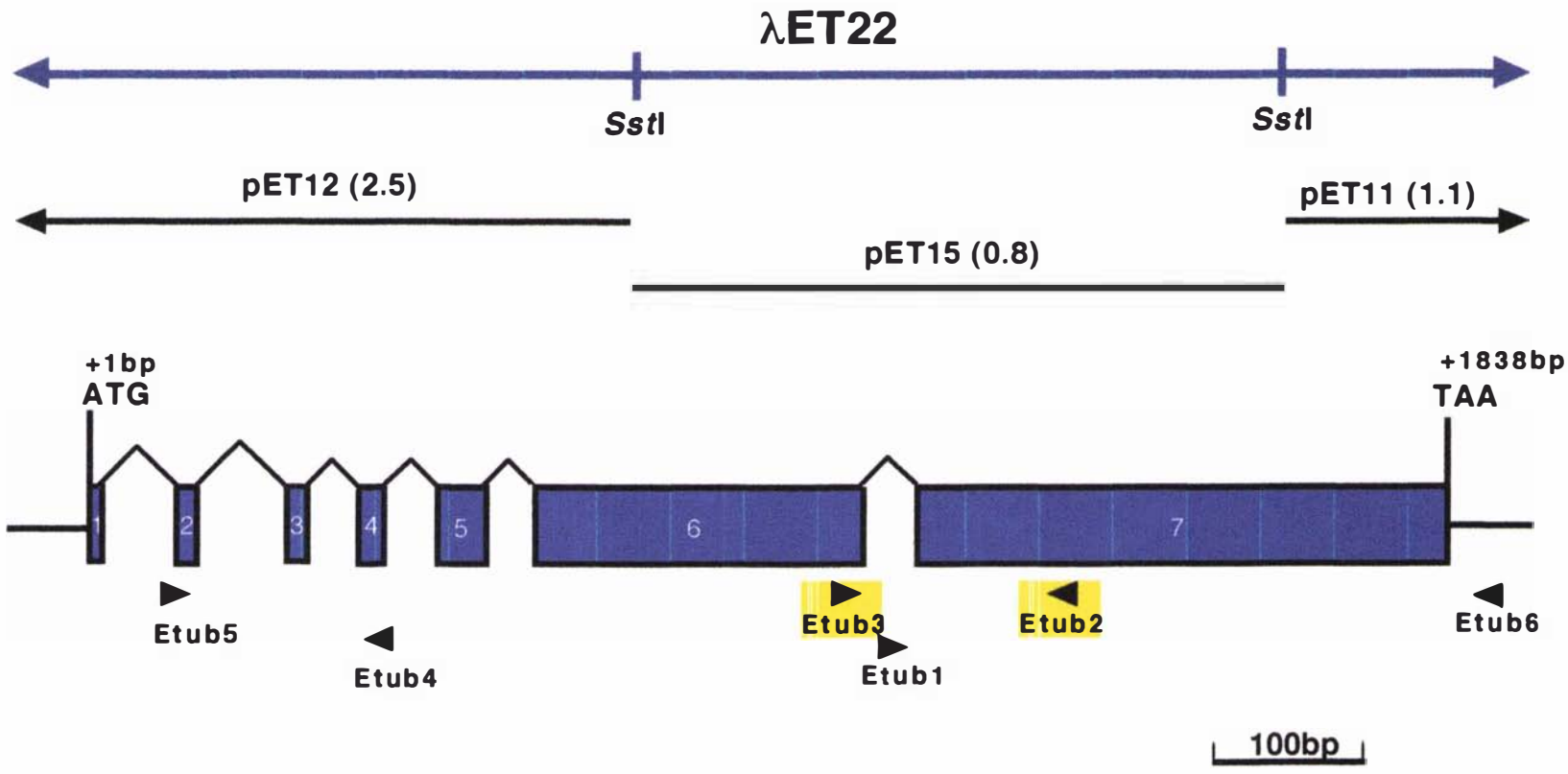
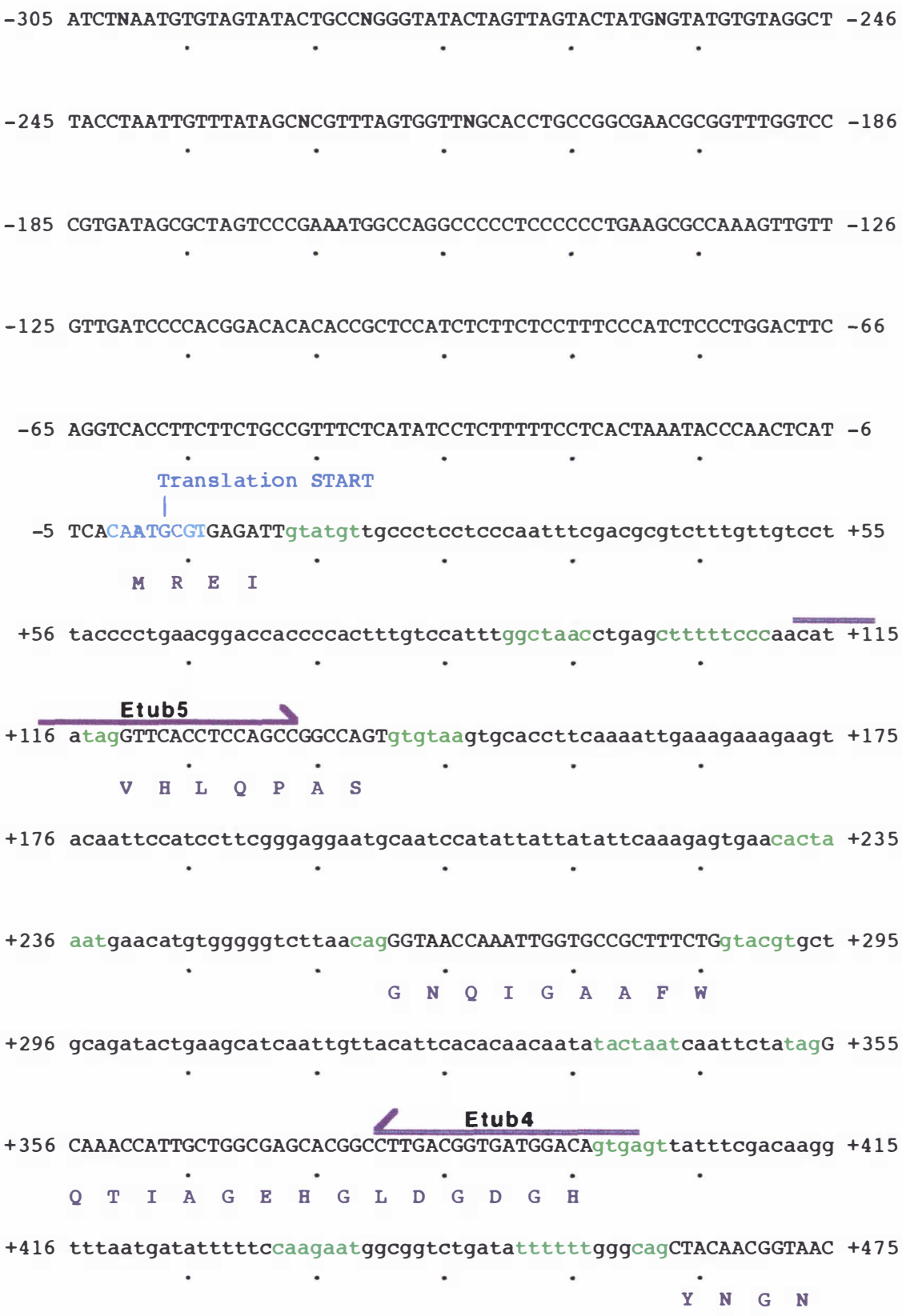
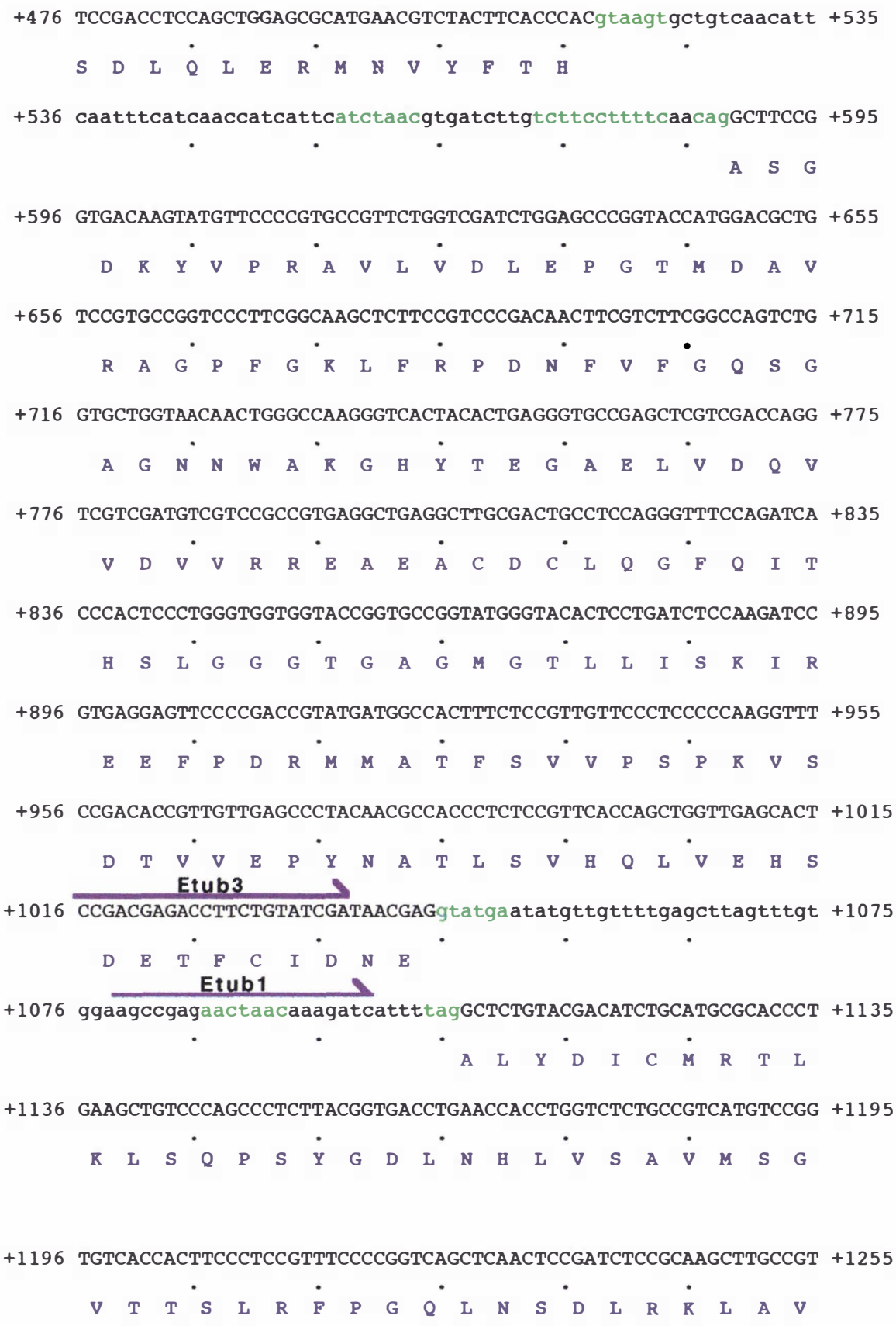


Figure 3.22 *DNA sequence and deduced amino acid sequence of P. paxilli tub-2*

Sequence of *P. paxilli tub-2* was determined as described in section 3.1.7. The 2220 bp of sequence isolated is shown here, including 305 bp of 5' UTR (-305 to -1), 1839 bp of coding sequence (+1 to +1838), and 76 bp of 3' UTR (+1839 to 1915). The predicted 447 amino acids are shown beneath their respective codons. Introns are shown in lower case, with 5' and 3' splice junctions, and the lariat consensus sequence highlighted in green. Where present, intronic polypyrimidine tracts are highlighted in green as well. The translation start and stop codons are highlighted in blue, with the Kozak consensus sequence surrounding the translation start codon highlighted in light blue. Primers used for sequencing and gene analysis are shown as purple lines.


-305 ATCTNAATGTGTAGTATACTGCCNGGGTATACTAGTTAGTACTATGNGTATGTGTAGGCT -246
-245 TACCTAATTGTTTATAGCNCGTTTAGTGGTTNGCACCTGCCGGCGAACGCGGTTTGGTCC -186
-185 CGTGATAGCGCTAGTCCCGAAATGGCCAGGCCCCCTCCCCCCTGAAGCGCCAAAGTTGTT -126
-125 GTTGATCCCCACGGACACACACCGCTCCATCTCTTCTCCTTTCCCATCTCCCTGGACTTC -66
-65 AGGTCACCTTCTTCTGCCGTTTCTCATATCCTCTTTTTCCTCACTAAATACCCAACTCAT -6
Translation START
-5 TCACAATGCGTGAGATTgtatgttgccctcctcccaatttcgacgcgtctttgttgtcct +55
M R E I
+56 tacccctgaacggaccaccccactttgtccatttggctaacctgagctttttcccaacat +115
Etub5
+116 atagGTTCACCTCCAGCCGGCCAGTgtgtaagtgcaccttcaaaattgaaagaaagaagt +175
V H L Q P A S
+176 acaattccatccttcgggaggaatgcaatccatattattatattcaaagagtgaacacta +235
+236 aatgaacatgtgggggtcttaacagGGTAACCAAATTGGTGCCGCTTTCTGgtacgtgct +295
G N Q I G A A F W
+296 gcagatactgaagcatcaattgttacattcacacaacaatatactaatcaattctatagG +355
Etub4
+356 CAAACCATTGCTGGCGAGCACGGCCTTGACGGTGATGGACAgtgagttatttcgacaagg +415
Q T I A G E H G L D G D G H
+416 tttaatgatatttttccaagaatggcggtctgatatttttgggcagCTACAACGGTAAC +475
Y N G N


+476 TCCGACCTCCAGCTGGAGCGCATGAACGTCTACTTCACCCACgtaagtgctgtcaacatt +535
S D L Q L E R M N V Y F T H
+536 caatttcatcaaccatcattcatctaacgtgatcttgtcttccttttcaacagGCTTCCG +595
A S G
+596 GTGACAAGTATGTTCCCCGTGCCGTTCTGGTCGATCTGGAGCCCGGTACCATGGACGCTG +655
D K Y V P R A V L V D L E P G T M D A V
+656 TCCGTGCCGGTCCCTTCGGCAAGCTCTTCCGTCCCGACAACTTCGTCTTCGGCCAGTCTG +715
R A G P F G K L F R P D N F V F G Q S G
+716 GTGCTGGTAACAACTGGGCCAAGGGTCACTACACTGAGGGTGCCGAGCTCGTCGACCAGG +775
A G N N W A K G H Y T E G A E L V D Q V
+776 TCGTCGATGTCGTCCGCCGTGAGGCTGAGGCTTGCGACTGCCTCCAGGGTTTCCAGATCA +835
V D V V R R E A E A C D C L Q G F Q I T
+836 CCCACTCCCTGGGTGGTGGTACCGGTGCCGGTATGGGTACACTCCTGATCTCCAAGATCC +895
H S L G G G T G A G M G T L L I S K I R
+896 GTGAGGAGTTCCCCGACCGTATGATGGCCACTTTCTCCGTTGTTCCCTCCCCCAAGGTTT +955
E E F P D R M M A T F S V V P S P K V S
+956 CCGACACCGTTGTTGAGCCCTACAACGCCACCCTCTCCGTTCACCAGCTGGTTGAGCACT +1015
D T V V E P Y N A T L S V H Q L V E H S
Etub3
+1016 CCGACGAGACCTTCTGTATCGATAACGAGgtatgaatatgttgttttgagcttagtttgt +1075
D E T F C I D N E
Etub1
+1076 ggaagccgagaactaacaaagatcatttagGCTCTGTACGACATCTGCATGCGCACCCT +1135
A L Y D I C M R T L
+1136 GAAGCTGTCCCAGCCCTCTTACGGTGACCTGAACCACCTGGTCTCTGCCGTCATGTCCGG +1195
K L S Q P S Y G D L N H L V S A V M S G
+1196 TGTCACCACTTCCCTCCGTTTCCCCGGTCAGCTCAACTCCGATCTCCGCAAGCTTGCCGT +1255
V T T S L R F P G Q L N S D L R K L A V

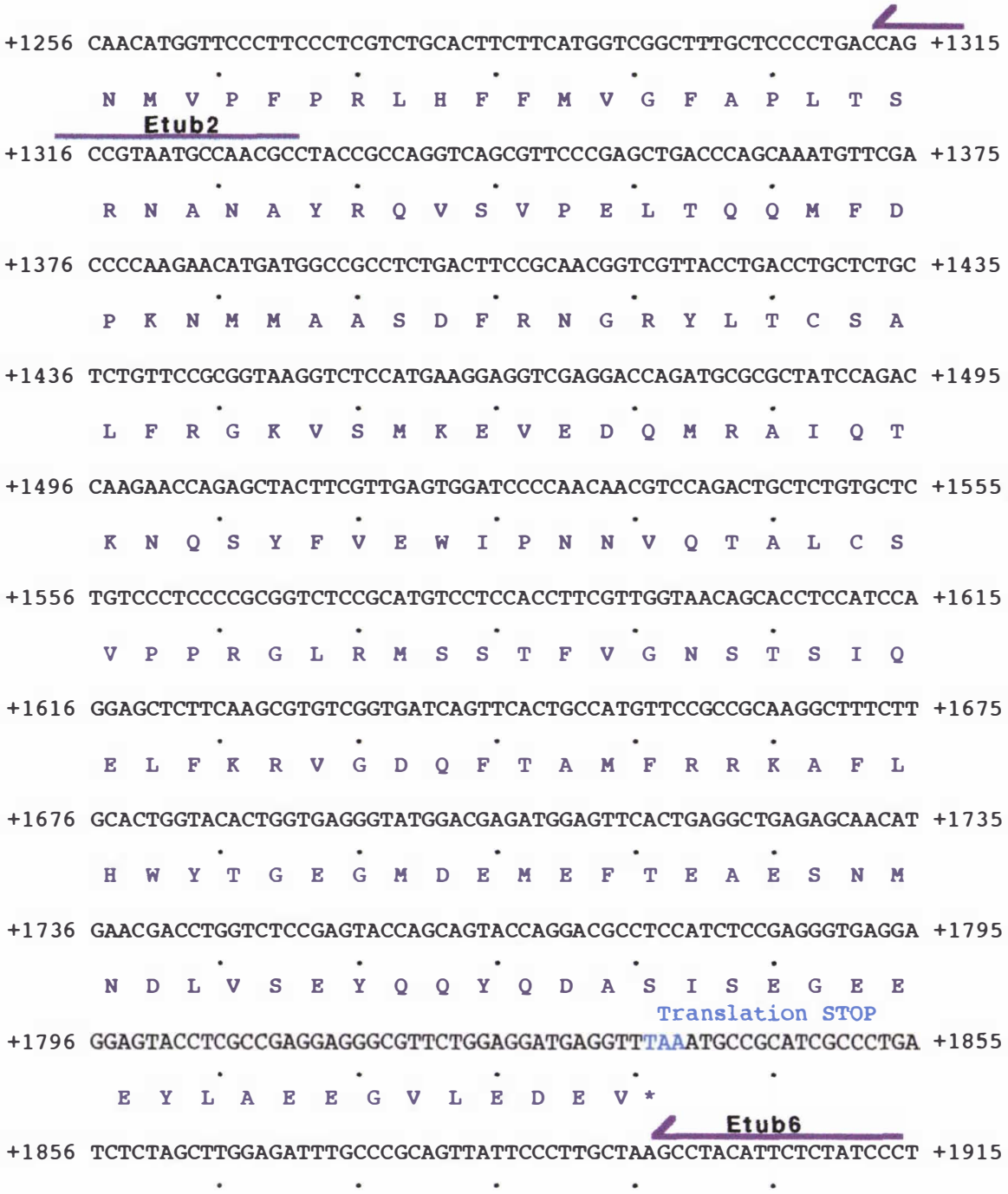

+1256 CAACATGGTTCCCTTCCCTCGTCTGCACTTCTTCATGGTCGGCTTTGCTCCCCTGACCAG +1315
N M V P F P R L H F F M V G F A P L T S
Etub2
+1316 CCGTAATGCCAACGCCTACCGCCAGGTCAGCGTTCCCGAGCTGACCCAGCAAATGTTCGA +1375
R N A N A Y R Q V S V P E L T Q Q M F D
+1376 CCCCAAGAACATGATGGCCGCCTCTGACTTCCGCAACGGTCGTTACCTGACCTGCTCTGC +1435
P K N M M A A S D F R N G R Y L T C S A
+1436 TCTGTTCCGCGGTAAGGTCTCCATGAAGGAGGTCGAGGACCAGATGCGCGCTATCCAGAC +1495
L F R G K V S M K E V E D Q M R A I Q T
+1496 CAAGAACCAGAGCTACTTCGTTGAGTGGATCCCCAACAACGTCCAGACTGCTCTGTGCTC +1555
K N Q S Y F V E W I P N N V Q T A L C S
+1556 TGTCCCTCCCCGCGGTCTCCGCATGTCCTCCACCTTCGTTGGTAACAGCACCTCCATCCA +1615
V P P R G L R M S S T F V G N S T S I Q
+1616 GGAGCTCTTCAAGCGTGTCGGTGATCAGTTCACTGCCATGTTCCGCCGCAAGGCTTTCTT +1675
E L F K R V G D Q F T A M F R R K A F L
+1676 GCACTGGTACACTGGTGAGGGTATGGACGAGATGGAGTTCACTGAGGCTGAGAGCAACAT +1735
H W Y T G E G M D E M E F T E A E S N M
+1736 GAACGACCTGGTCTCCGAGTACCAGCAGTACCAGGACGCCTCCATCTCCGAGGGTGAGGA +1795
N D L V S E Y Q Q Y Q D A S I S E G E E
Translation STOP
+1796 GGAGTACCTCGCCGAGGAGGGCGTTCTGGAGGATGAGGTTTAAATGCCGCATCGCCCTGA +1855
E Y L A E E G V L E D E V *
Etub6
+1856 TCTCTAGCTTGGAGATTTGCCCGCAGTTATTCCCTTGCTAAGCCTACATTCTCTATCCCT +1915

Table 3.6 Codon bias in *P. paxilli tub-2*

Amino Acid	Codon	Number	Frequency	Amino Acid	Codon	Number	Frequency
Gly	GGG	0	0.00	Trp	TGG	4	1.00
	GGA	1	0.03	STOP	TGA	0	0.00
	GGT	29	0.81	Cys	TGT	1	0.17
	GGC	6	0.17		TGC	5	0.83
Glu	GAG	33	1.00	STOP	TAG	0	0.00
	GAA	0	0.00	STOP	TAA	1	1.00
Asp	GAT	7	0.28	Tyr	TAT	1	0.07
	GAC	18	0.72		TAC	13	0.93
Val	GTG	0	0.00	Leu	TTG	1	0.03
	GTA	0	0.00		TTA	0	0.00
	GTT	16	0.43	Phe	TTT	1	0.04
	GTC	21	0.57		TTC	24	0.96
Ala	GCG	0	0.00	Ser	TCG	0	0.00
	GCA	0	0.00		TCA	0	0.00
	GCT	14	0.44		TCT	6	0.19
	GCC	18	0.56		TCC	20	0.62
Arg	AGG	0	0.00	Arg	CGG	0	0.00
	AGA	0	0.00		CGA	0	0.00
Ser	AGT	1	0.03		CGT	12	0.50
	AGC	5	0.16		CGC	12	0.50
Lys	AAG	13	1.00	Gln	CAG	20	0.87
	AAA	0	0.00		CAA	3	0.13
Asn	AAT	1	0.05	His	CAT	0	0.00
	AAC	21	0.95		CAC	11	1.00
Met	ATG	20	1.00	Leu	CTG	19	0.54
Ile	ATA	0	0.00		CTA	0	0.00
	ATT	3	0.25		CTT	2	0.06
	ATC	9	0.75		CTC	13	0.37
Thr	ACG	0	0.00	Pro	CCG	1	0.05
	ACA	1	0.04		CCA	0	0.00
	ACT	7	0.29		CCT	2	0.11
	ACC	16	0.67		CCC	16	0.84

Third position nucleotide G (111) 25%, A (6) 1%, T (103) 23%, C (228) 51%.

Table 3.7 Gene structure in *P. paxilli*

Feature			Sequence			
Translation start site			**Kozak Sequence**			
Eukaryote consensus			CC(A/G)CC**ATG**GC[1]			
Fungal consensus			TCA(C/A)(A/C)**ATG**(G/T)C[2]			
hmg			TCACC**ATG**GC			
tub-2			TCACA**ATG**CG			
Introns		**Size**	**5' Splice site**	**Putative Lariat**	**Polypyr**	**3' Splice site**
Yeast consensus				TACTAAC[3]		
Fungal consensus			GTANGT[4]	PyGCTAAC[5]		PyAG[6]
hmg	intron 1	77 nt	GTATAT	GGCTAAC	19 nt	AAG
	intron 2	71 nt	GTGAGC	TACTAAT	13 nt	AAG
	intron 3	68 nt	GTGAGT	TACTAAC	4 nt	CAG
tub-2	intron 1	107 nt	GTATGT	GGCTAAC	9 nt	TAG
	intron 2	120 nt	GTGTAA	CACTAAT	4 nt	CAG
	intron 3	68 nt	GTACGT	TACTAAT	4 nt	TAG
	intron 4	66 nt	GTGAGT	CAAGAAT	6 nt	CAG
	intron 5	71 nt	GTAAGT	ATCTAAC	10 nt	CAG
	intron 6	62 nt	GTATGA	AACTAAC	3 nt	TAG
P. paxilli proposed consensus			GT(A/G)NGT	NACTAAPy		PyAG

1 Kozak, 1984
2 Ballance, 1986
3 Voet & Voet, 1990
4 Gurr *et al.* 1987
5 Gurr *et al.* 1987
6 Gurr *et al.* 1987

2.2) and a 10-fold dilution of cDNA from 60 h RNA as a template. The products were separated on a 1% (w/v) agarose gel (section 2.10.1 and 2.10.3). In Figure 3.23, the cDNA product is the predicted 490 bp smaller than the gDNA product. The cDNA PCR reaction was then purified (section 2.11.5), cloned into a pGEM®-T vector (section 2.15) and sequenced (section 2.12.3) to confirm the exact location of intron/exon boundaries. No sequence data was available for the intron/exon boundary for intron 1 and 2, as the initial ORF is upstream of the sequencing primer, and the second intron is too close to the sequencing primer. However, this is an extremely conserved gene, and these ORF have been predicted from the *tub-2* sequence of other species.

3.2.8 Chromosomal location of *P. paxilli tub-2*

The chromosomal location of *tub-2* was determined by probing a nylon membrane containing a CHEF separation of *P. paxilli* chromosomes (Figure 3.24 A), previously discussed in section 3.1.12, with a PCR product of *tub-2* amplified with Etub2 and Etub3. All strains, including the Pax$^-$ mutants and wild type *P. paxilli*, show the *tub-2* probe hybridising to chromosome IV (Figure 3.24 B).

Figure 3.23 Confirmation of P. paxilli tub-2 introns

RT-PCR (section 2.20) was performed on 1/100 dilution of cDNA from a 60 h culture, using primers Etub5 and Etub6 (Table 2,2). Products were separated on a 1% agarose gel (section 2.10.1 and 2.10.3) as follows. Lanes 1 and 4, 1kb ladder (BRL). Lane 2, gDNA. Lane 3, 60 h cDNA. Lane 3, water control. Sizes shown are in kb.

Figure 3.23

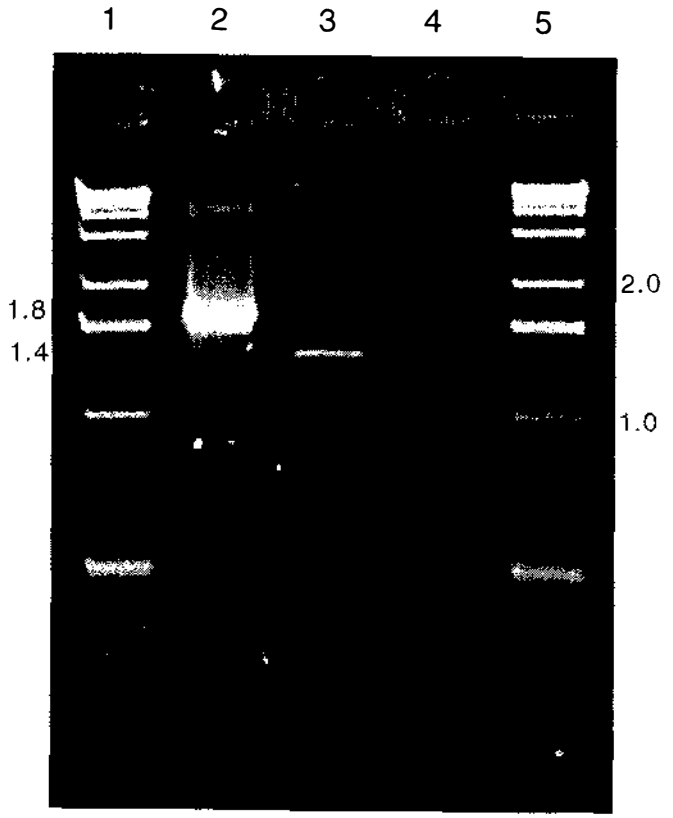


Figure 3.24 *CHEF analysis of P. paxilli chromosomes*

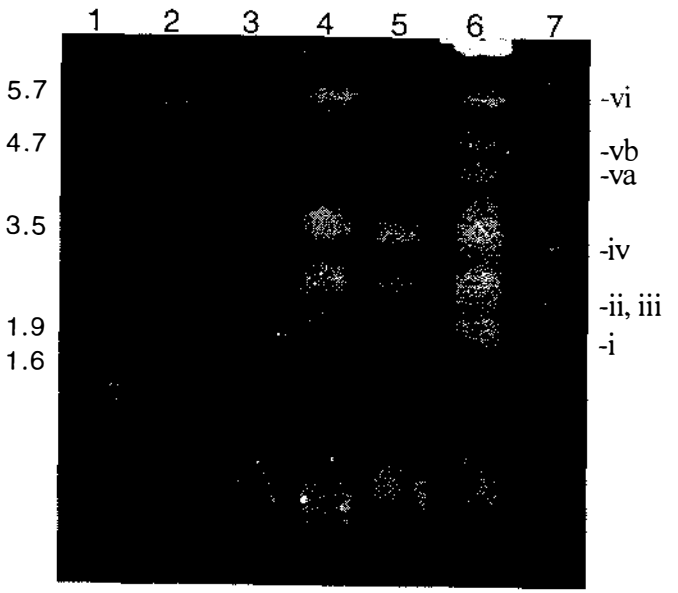
A) Ethidium bromide stained CHEF gel

This is the same gel as that shown in Figure 6 B in Young *et al.* (1998). Running conditions for CHEF electrophoresis are described in the legend of this figure. Lane 1, *S. cerevisiae*. Lane 2, *S. pombe*. Lanes 3-7, *P. paxilli* wild-type, YI-20, CY-2, CY-102, and CY-35. Sizes shown are in Mb.

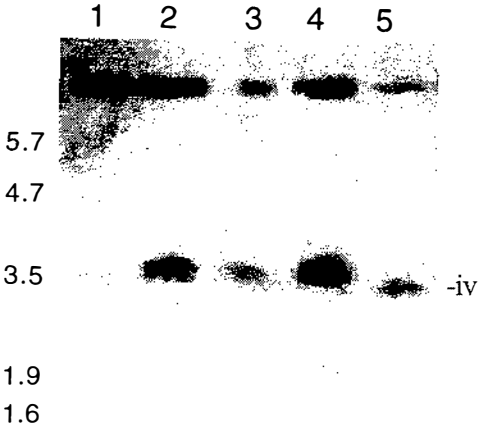
B) Chromosomal location of tub-2

Autoradiograph of gel shown in Figure 3.22 A probed with a [^{32}P]-labelled PCR product of *tub-2,* amplified with primers Etub2 and Etub3. Southern blotting and probe hybridisation as described in section 2.13. Sizes shown are in Mb.

A



B



3.3 P. paxilli physiology and conditions for induction of paxilline

The growth of filamentous fungi in submerged liquid culture is an important process for both researchers and commercial enterprises. However, the optimal conditions for production of fungal secondary metabolites in liquid culture can vary with different species and even different strains. To study the regulation of paxilline biosynthesis in liquid culture, three fundamental requirements had to be met. Firstly, paxilline must be produced at some time in the course of the experiment. Secondly, experiments must be reproducible, with a limited level of inherent variability. Thirdly, the gross morphology of mycelia in liquid culture should be such that a small sample volume is still representative of the entire culture, and that all mycelia in the culture have equal access to nutrients and oxygen.

3.3.1 Spore viability

Liquid cultures of *P. paxilli* were inoculated from a spore suspension. Once a fresh spore suspension had been prepared (section 2.1.5), the concentration of spores was determined by haemocytometer counting and the spore suspension stored at 4°C. Although spores are hardy structures, the percentage of viable spores can drop over time. To ensure that spore viability did not overly affect the growth rate of liquid cultures, the viability of spores over time was determined. Serial dilutions of each spore suspension were plated onto PD plates (section 2.1.2) on a regular basis, until a significant drop in viability was recorded. In all spore suspensions tested, the initial percentage of viable spores fell by 50% in approximately 60 days. For future experiments, spore suspensions were stored at 4°C for no more than 6 weeks, before a new suspension was prepared.

3.3.2 Cultures inoculated from spore suspensions

In the initial growth experiments, cultures were inoculated directly from a spore suspension (section 2.1.7). Typical results for growth rate (dry weight) and paxilline content are shown in Figure 3.25 A. Using these conditions there was considerable

Figure 3.25 Comparision of inoculation protocols

All cultures were grown in a 2 L Erlenmeyer flask in 300 mL of CDYE media (section 2.1.2). Samples of mycelia (10 mL) were removed every 12 h (section 2.16.1), freeze-dried and the dry weight determined. Paxilline content was determined by HPLC (section 2.16). An example of a typical HPLC trace is shown in Figure 3.26.

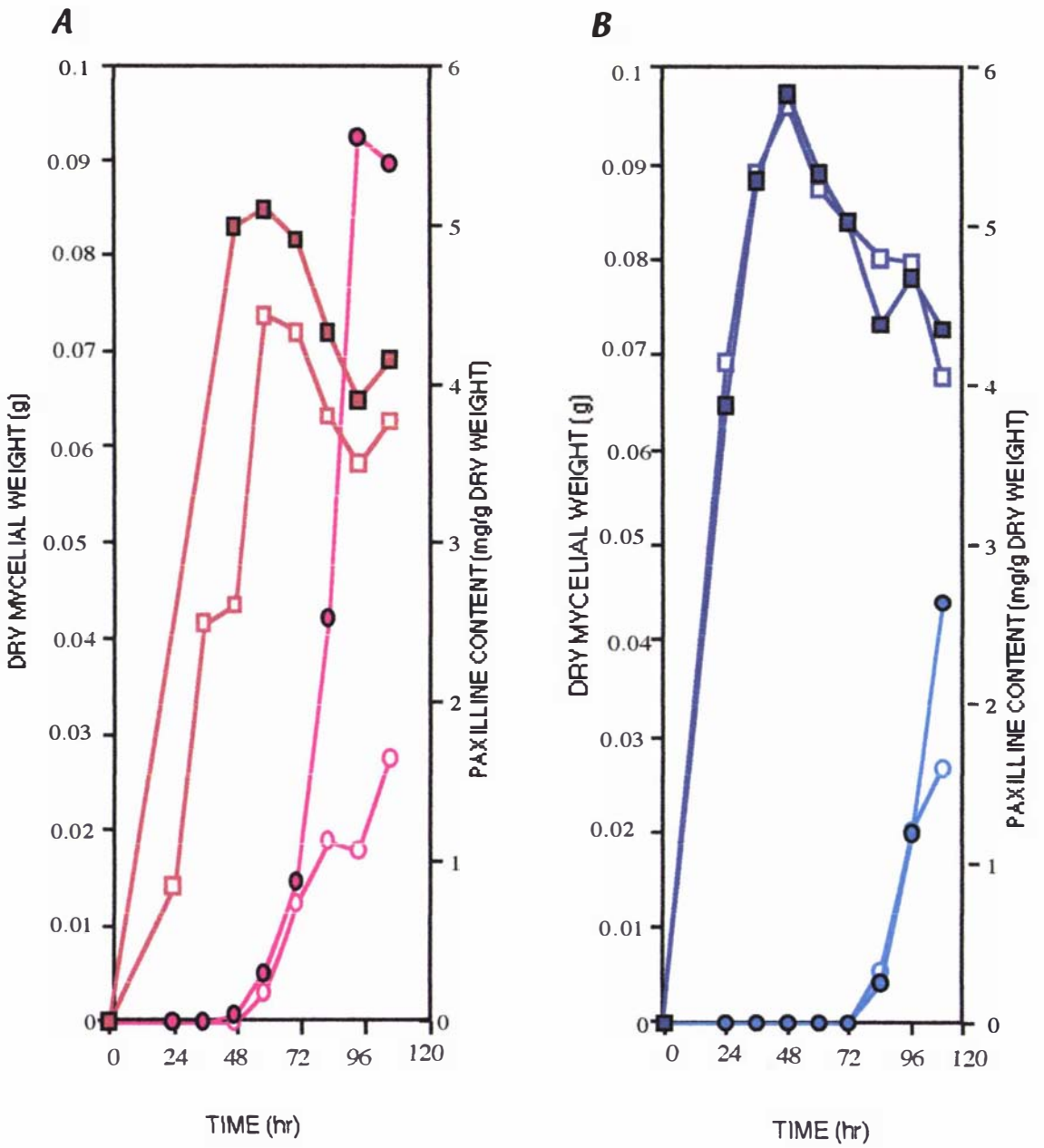
A) Inoculation by spore suspension

The replica cultures were inoculated from a spore suspension (section 2.1.7). Flask A, dry weight (—■—). Flask B, dry weight (··□··). Flask A, paxilline content (··●···). Flask B, paxilline content (—○ -).

B) Inoculation by seed culture

The replica cultures were inoculated from a seed culture (section 2.1.8). Flask A, dry weight (—■—). Flask B, dry weight (··□··). Flask A, paxilline content (··●···). Flask B, paxilline content (—○ -).

Figure 3.25 Comparision of Two Inoculation Protocols.



variation between both the time of paxilline induction and the yield of paxilline. This variability was considered unsatisfactory for identifying real differences between cultures due to variations in growth conditions or strain genealogies. The source of variation was thought to be the spore suspension itself as the concentration of spores was very high. Therefore, small variations in inoculum volume could result in a dramatic increase in the final concentration of viable spores.

The morphology of mycelia in liquid culture was also variable. A number of factors were identified as sources of variation, including the culture volume, the shake speed of the incubator and the concentration of the inoculum. Small 25 mL cultures grown at 230 rpm have large mycelial pellets, which settle rapidly when removed from the shaker. In the large 300 mL cultures grown at 350 rpm, only loose mycelial fragments were present. Decreasing the culture volume, the shaker speed, or the inoculum concentration resulted in the formation of pellets. The larger pellets are hollow, which results in a mass transfer gradient across the pellet, of nutrients and oxygen in, and of waste and secreted metabolites out of the pellet (Prosser & Tough, 1991). A number of protocols were trialed to improve the reproducibility of replica cultures with respect to morphology (loose mycelia fragments) and gross physiological markers (dry weight and paxilline induction).

3.3.3 Cultures inoculated from seed culture

A number of seed culture protocols were trialed to eliminate the variation seen in cultures inoculated directly from a spore suspension. Seed cultures of 100 mL were grown for 24 h, and used to inoculate 300 mL CDYE (section 2.2) cultures (section 2.1.8), but this resulted in the large pellet morphology. Pellets had formed in the smaller culture volume, even at 350 rpm. Oat flour has been used in filamentous fungal seed cultures to inhibit the formation of pellets (Tkacz *et al.*, 1993). However, seed cultures containing 1% (w/v) oat flour still formed pellets. It appears that once the pellets have formed, sub-culturing in to a larger volume does not change the morphology. The number of pellets is also fixed. Therefore if 24 mL of seed culture is sub-cultured into a 300 mL volume, the number of pellets does not increase. The growth rate and subsequent accumulation of biomass is severely hampered by

inoculation with mycelial pellets. Finally the incubation period of the seed culture was reduced from 24 h to 12 h. At this time spores have begun to germinate, but no gross culture morphology has been established (Figure 3.29, photograph A and B). Replica cultures, inoculated from the same seed culture showed a significant decrease in variability with respect to both growth rate and paxilline induction (Figure 3.25 B), when compared to cultures inoculated by spore suspensions (Figure 3.25 A). This particular protocol (section 2.1.8) was subsequently used for all other growth experiments.

3.3.4 Growth of *P. paxilli* in paxilline-inducing media

3.3.4.1 Experiment 1

After establishing a reliable and reproducible inoculation protocol (section 3.3.3), a large scale physiological investigation was conducted in two separate experiments. CDYE cultures of 300 mL were inoculated from a 100 mL seed culture (section 2.1.8) and grown as described in section 2.1.2. Analysis of culture physiology in Experiment 1 is shown in Figure 3.27. Biomass accumulation, peaked after approximately 42 h (A), at which time the average dry weight in a 5 mL sample of culture was 0.1 g or 20 g/L. The pH of the broth declined after 48 h (B) in both cultures, 24 h before paxilline was first detected. However, a slight increase in pH is detected in both flasks after 96 h. Paxilline was first detected by HPLC at 72 h (C), and reaches an average content of 3.25 mg/g of mycelia after 96 h. Production of paxilline by *P. paxilli* grown in CDYE is equivalent to 65 mg/L.

3.3.4.2 Experiment 2

The growth experiment was repeated (Experiment 2) and the results are shown in Figure 3.28. Although the seed culture inoculation protocol was used, there is still evidence of variation between cultures. This was most apparent when dry weight was analysed. When both cultures are averaged, the biomass accumulation peaks after 45 h (A), is comparable to the 42 h peak seen in Experiment 1. At this time the average maximum

Figure 3.26 Analysis of paxilline by HPLC

Paxilline samples from Experiment #2 were prepared as described in section 2.18 and analysed by HPLC (section 2.18.3). The vertical axis in all three traces is absorbency at 230 nm. Calculations for determining paxilline content of mycelia samples are given in Appendix A3.0

A) Paxilline standard

Trace for a 10μL sample of a 25μg/mL paxilline stock.

B) Paxilline extracted from P. paxilli mycelia after 60 h

Trace for a 10μL sample extracted from 60 h mycelia as described in section 2.16.

C) Paxilline extracted from P. paxilli mycelia after 120 h

Trace for a 10μL sample extracted from 120 h mycelia as described in section 2.16.

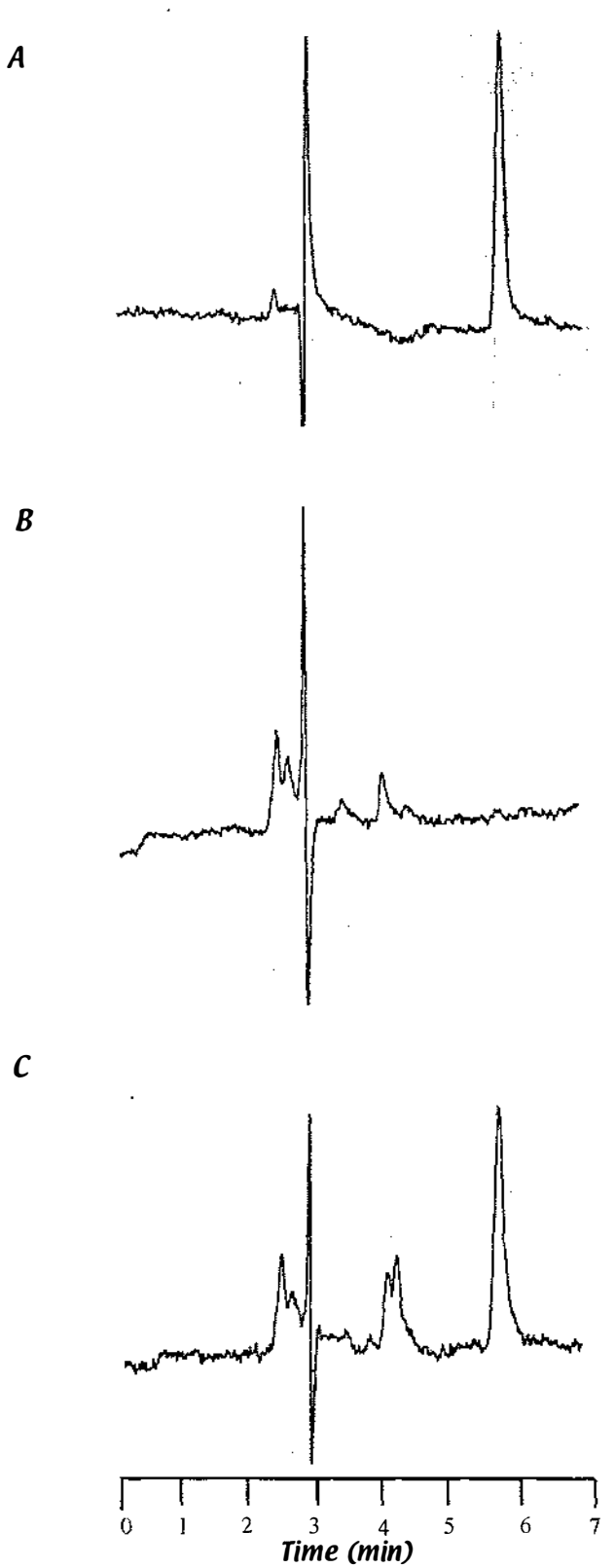

A
B
C
0
1
2
3
4
5
6
7
Time (min)

Figure 3.27 *Growth analysis and paxilline production of P. paxilli in Experiment 1*

Liquid cultures of *P. paxilli* were grown (section 2.1.2) in a 2 L Erlenmeyer flask containing 300 mL of CDYE media, inoculated from a seed culture (section 2.1.8). Samples of 10 mL were removed for analysis of dry weight and paxilline content.

A) Analysis of mycelial dry weight

The dry weight of 10 mL of freeze-dried mycelia was determined. Flask A (—■—), Flask B (···●···).

B) Analysis of broth pH

The pH was determined on the mycelia-free supernatant of the samples removed for paxilline analysis and RNA isolation, with an Orion research digital ionalyzer/501. Flask A (—■—), Flask B (···●···).

C) Analysis of mycelial paxilline content

The paxilline content of freeze-dried 10 mL mycelia samples was determined by HPLC (section 2.16). Flask A (—■—), Flask B (···●···).

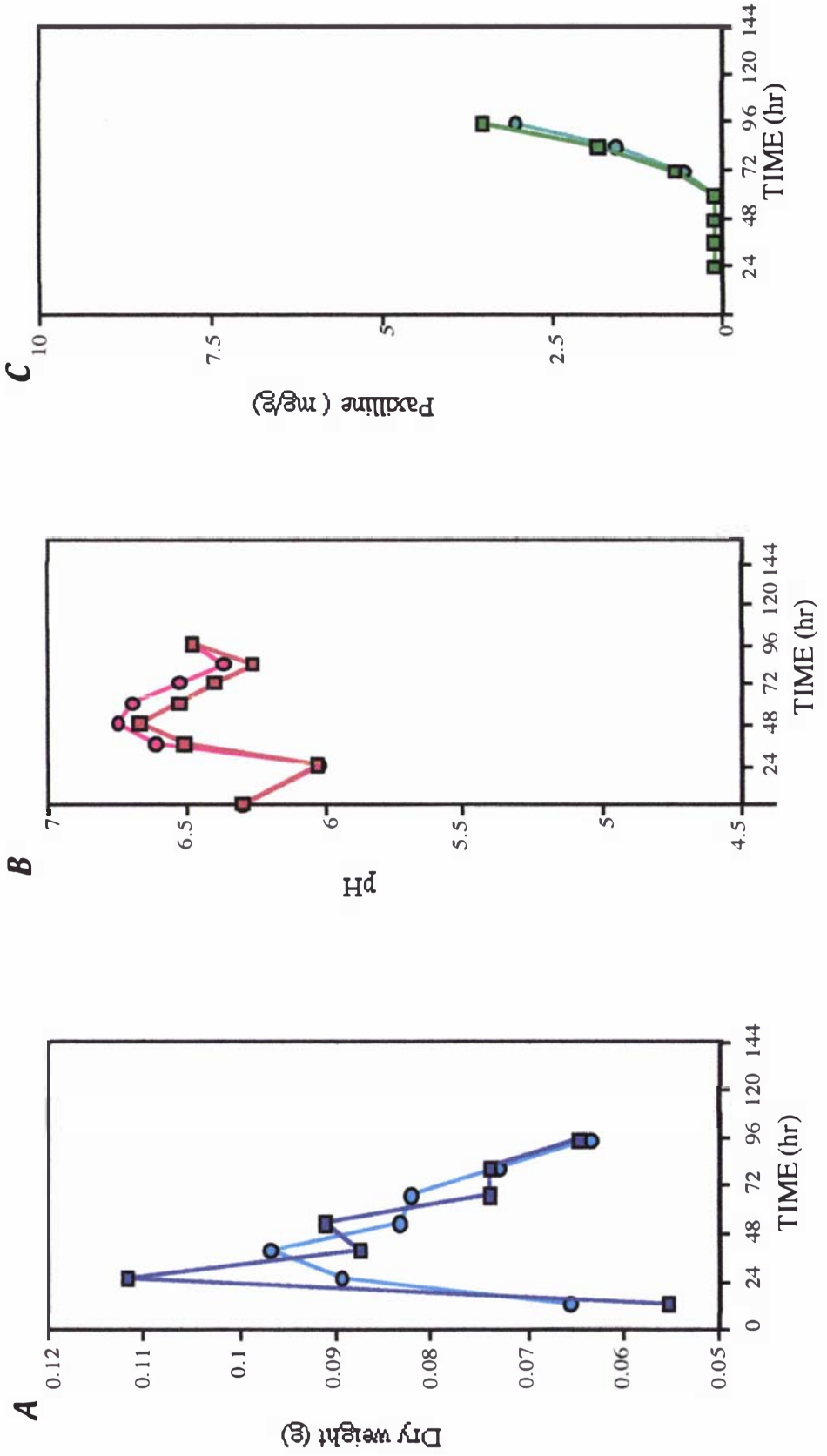

A
Dry weight (g)
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0
24
48
72
96
120
144
TIME (hr)
B
pH
7
6.5
6
5.5
5
4.5
TIME (hr)
C
Paxilline (mg/g)
10
7.5
5
2.5
0
TIME (hr)

Figure 3.28 *Growth analysis and paxilline production of P. paxilli in Experiment 2*

Liquid cultures of *P. paxilli* were grown (section 2.1.2) in a 2 L Erlenmeyer flask containing 300 mL of CDYE media, inoculated from a seed culture (section 2.1.8). Samples of 10 mL were removed for analysis of dry weight and determination of paxilline content.

A) Analysis of mycelial dry weight

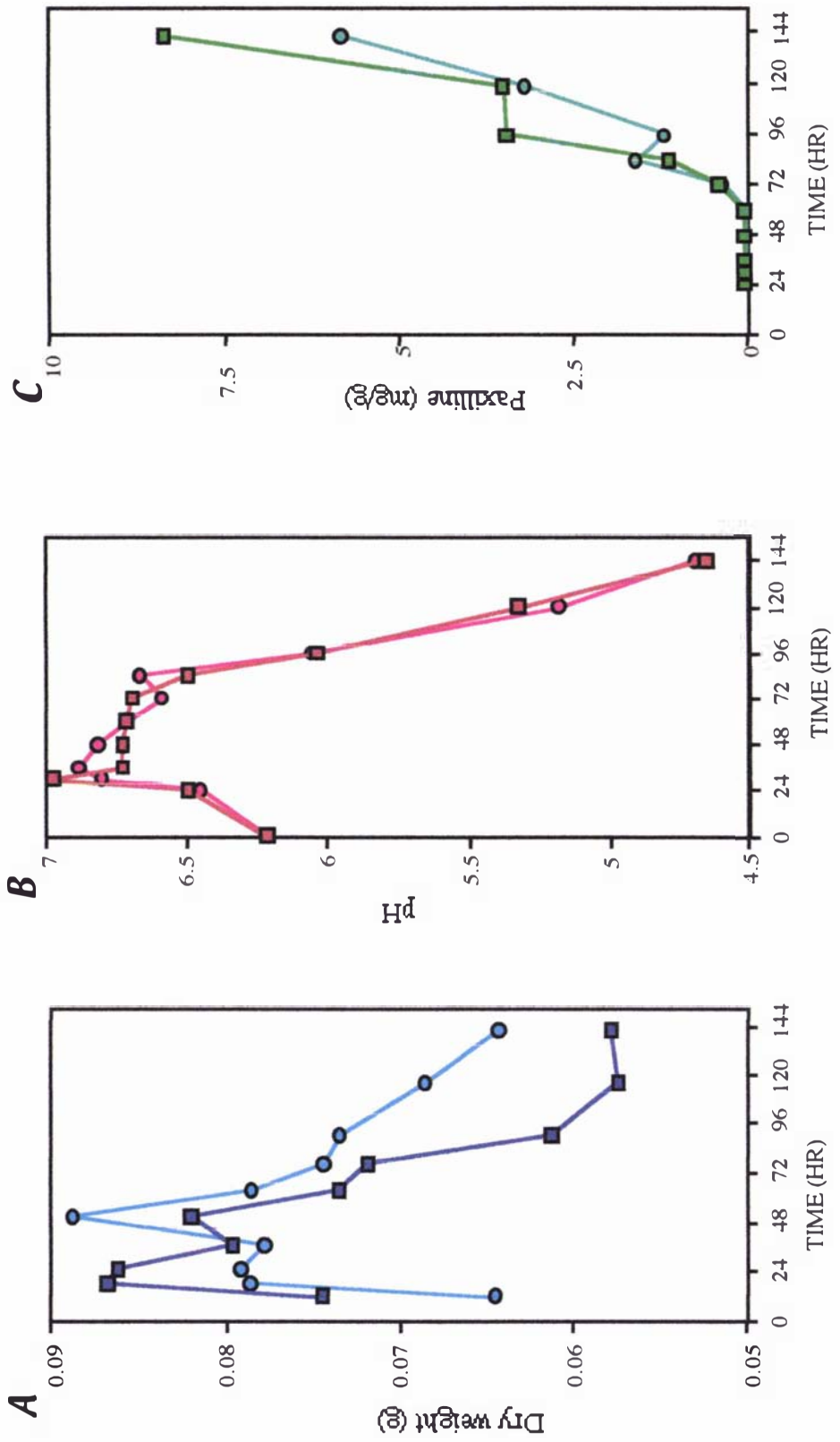
The dry weight of 10 mL of freeze-dried mycelia was determined. Flask A (), Flask B ().

B) Analysis of broth pH

The pH was determined on the mycelia-free supernatant of the samples removed for paxilline analysis and RNA isolation, with an Orion research digital ionalyzer/501.
Flask A (), Flask B ().

C) Analysis of mycelial paxilline content

The paxilline content of freeze-dried 10 mL mycelia samples was determined by HPLC (section 2.16). Flask A (), Flask B ( ).


A
Dry weight (g)
0.09
0.08
0.07
0.06
0.05
0
24
48
72
96
120
144
TIME (HR)
B
pH
7
6.5
6
5.5
5
4.5
0
24
48
72
96
120
144
TIME (HR)
C
Paxilline (mg/g)
10
7.5
5
2.5
0
0
24
48
72
96
120
144
TIME (HR)

dry weight in a 5 mL sample was 0.09 g, or 18 g/L. The pH of the broth declined after 33 h (B), with a more dramatic decrease apparent after 84 h. The increase in pH, recorded at 96h in Experiment 1, was not seen in Experiment 2 and remains unexplained at this time. Paxilline was first detected at 72 h (C). For comparison with Experiment 1, where the final paxilline sample was taken at 96 h, the average paxilline content at this time in Experiment 2 is 2.35 mg/g of mycelia. However, the paxilline content of flask B, registered a decrease in paxilline after 96 h. In all paxilline analyses conducted by HPLC, this is the only example where a drop in paxilline was recorded. If this result is discarded, the paxilline production in flask A after 96 h is 3.49 mg/g of mycelia. The overall paxilline production is then equivalent to 61 mg/L, comparable with that seen in Experiment 1. A typical set of results for paxilline analysis by HPLC (section 2.16) is shown in Figure 3.26. Authentic paxilline was eluted from the HPLC column after 5.8 min (Figure 3.26 A). When *P. paxilli* mycelial extracts were analysed, a second peak, with an elution time of 4.25 min was seen. This peak has been identified as ergosterol (Sarah Munday-Finch pers. comm.). By comparing the 60 h mycelia sample (B) with the 120 h mycelia sample (C) a dramatic increase in paxilline is seen, but the peak of ergosterol increased only slightly. The method for calculating the concentration of paxilline from the peak area is described in appendix A.3.0.

3.3.4.3 Analysis of paxilline in broth

An analysis of paxilline in broth was performed during Experiment 2. Broth removed from mycelia samples was collected, freeze-dried and the paxilline content determined by HPLC. Paxilline was not detected until 144 h. There was no steady increase in paxilline over time, although a small peak was present in all samples from 84 h to 120 h. The elution time of this peak was the same as the elution time for paxilline, but it was too small to register as a true peak with the Shimadzu C-R3A Chromatopac detector. If paxilline was being actively secreted into the media, a steady increase in broth paxilline should have been detected.

3.3.4.4 Microscopic analysis of culture morphology

The culture morphology was also monitored in Experiment 2. Mycelia were removed every 24 h, examined microscopically with a Nikon Diaphot inverted microscope and photographed with a Nikon F-601 camera using Kodak TMY 400 film. Examples of the morphology observed are shown in Figure 3.29. Photographs A and B show the morphology of mycelia, 4 h after inoculation by seed culture. The germinating spores, both singly or in aggregates, can be seen and are also an example of the typical morphology seen in seed cultures after 12 h. The clumps of mycelia are presumed to be the initial structure that eventually forms a mycelial pellet. After 28 h, the cultures appear very viscous with fine micropellets (0.5-1 mm diameter) suspended in loose mycelial fragments. Pellets in photograph C appear quite dense, especially when compared to the fragmented pellets seen in the culture after 100 h (photograph J). Previous reports had stated that *P. paxilli* does not sporulate when grown in CDYE media (Ibba *et al.*, 1987), however, a conidiophore structure, with a single conidia still attached is seen in photograph D. Conidia are seen in the broth after 28 h. These must be a result of conidiation that has occurred after inoculation, as all the conidia seen in photographs A and B are germinating, whereas ungerminated conidia are present in photograph C. After 56 h, ungerminated conidia are still visible in the broth (photograph E). A 'penicillius', the conidiophore structure typical of *Penicillium* spp (Bold *et al.*, 1987), is shown in photograph F. After 76 h (photograph G), discrete hyphal fragments can be seen suspended in the broth, some of which undergo cryptic growth or regeneration, (photograph H). Vacuolation is also occurring at this time, and can be identified by the banding pattern seen on hyphae in photograph I. By 100 h, the culture has become less viscous, and enters what is known as the death phase (Prosser & Tough, 1991). Pellets are less dense, and have begun to fragment (Photograph J). Vacuolation becomes more evident (photographs K and L) and there is evidence of autolysis, as hyphae appear to shrink and break (photographs M and N).

Figure 3.29 Microscopic analysis of P. paxilli liquid culture

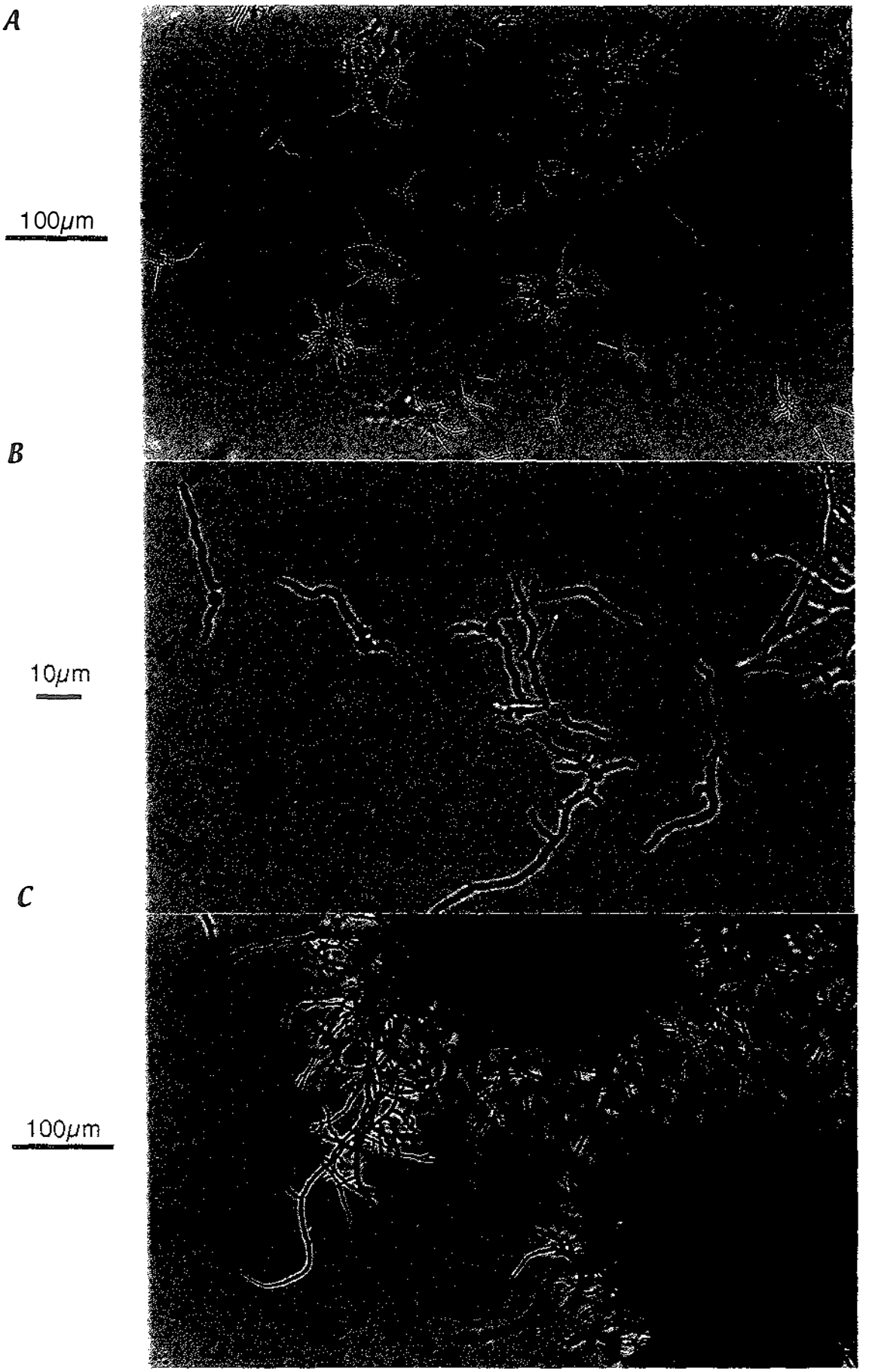
Photographs were taken from a Nikon Diaphot inverted microscope with a Nikon F-601 camera using Kodak TMY 400 film.

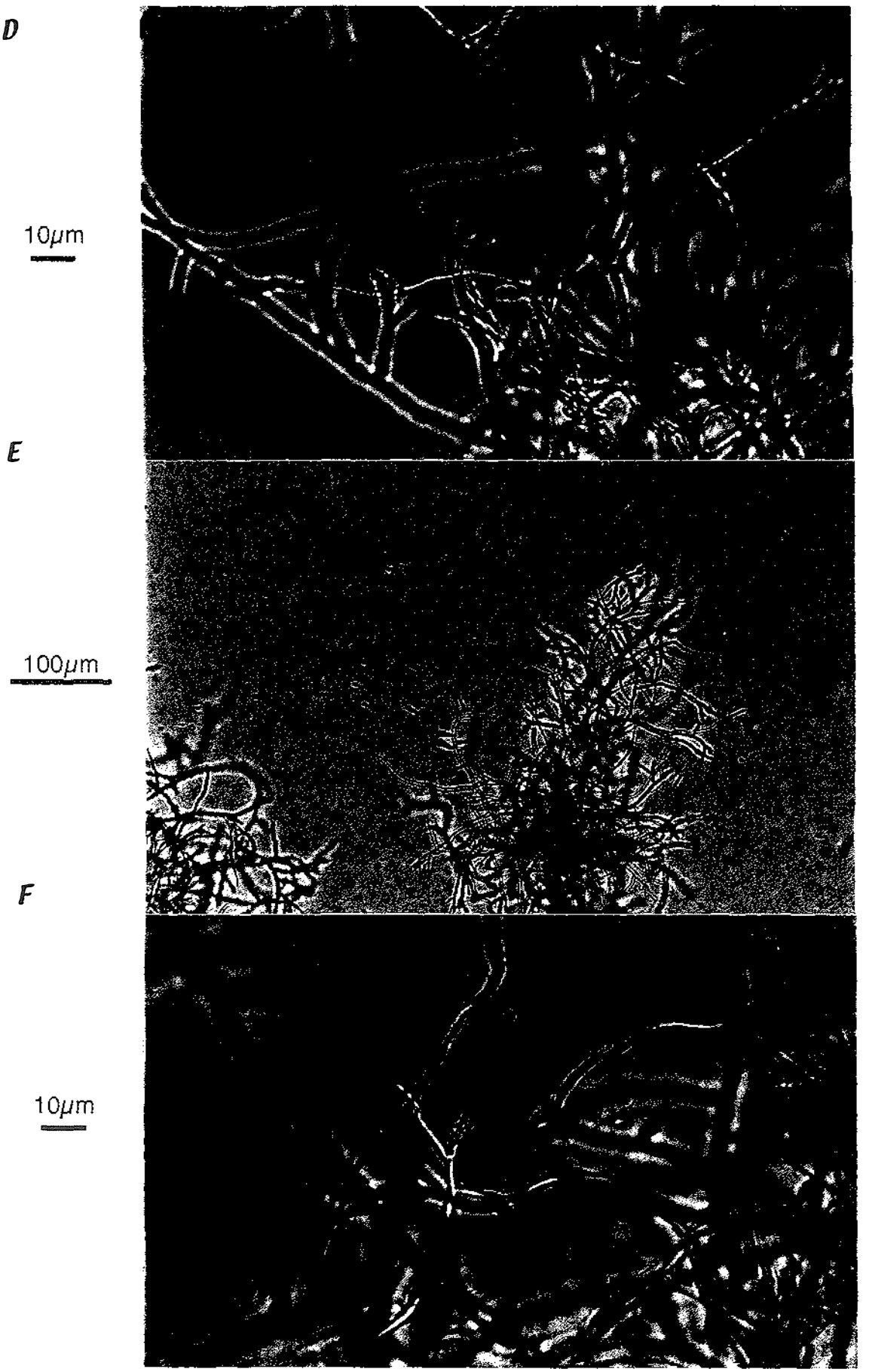
Key to symbols

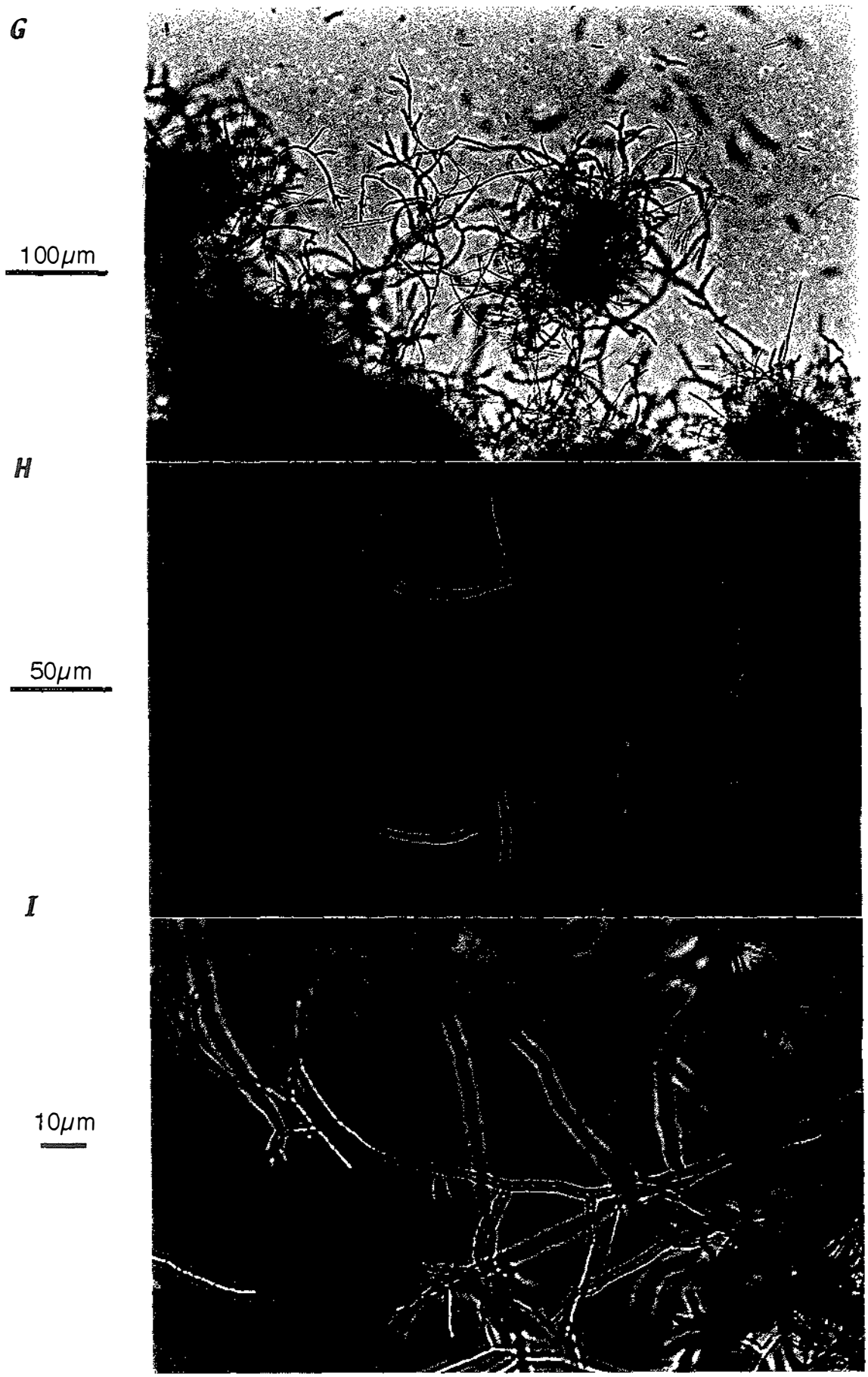
C Conidiophore structure
U Ungerminated spore (conidia)
G Germinating spore (conidia)
R Regenerating hyphal fragment
V Vacuolation
A Autolysis

Photographs

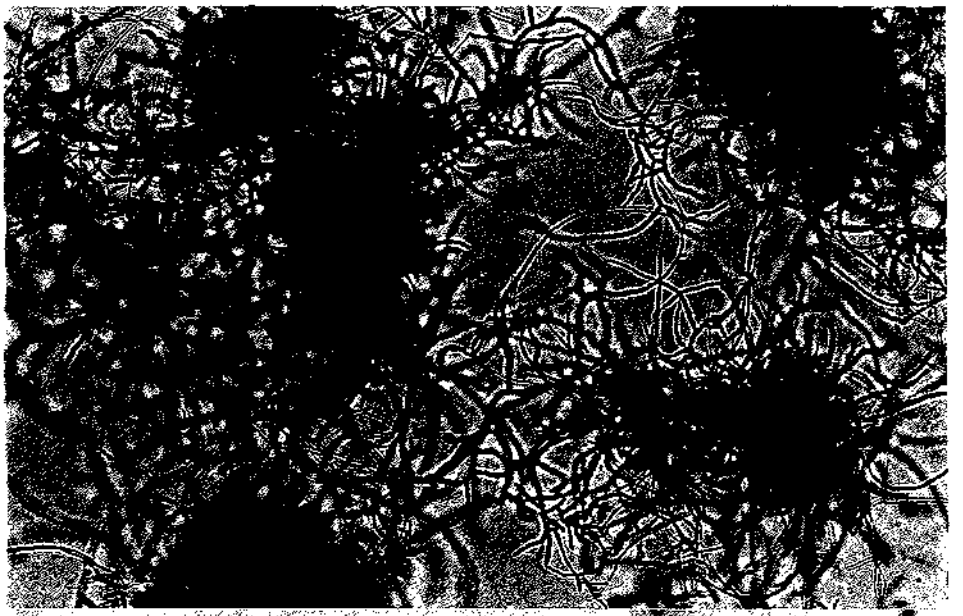
A) 4h culture at 90 x magnification
B) 4h culture at 360 x magnification
C) 28h culture at 90 x magnification
D) 28h culture at 360 x magnification
E) 52h culture at 90 x magnification
F) 52h culture at 360 x magnification
G) 76h culture at 90 x magnification
H) 76h culture at 180 x magnification
I) 76h culture at 360 x magnification
J) 100h culture at 90 x magnification
K) 100h culture at 360 x magnification
L) 100h culture at 360 x magnification
M) 100h culture at 360 x magnification
N) 100h culture at 360 x magnification


A
100μm
B
10μm
C
100μm


D
10μm
E
100μm
F
10μm

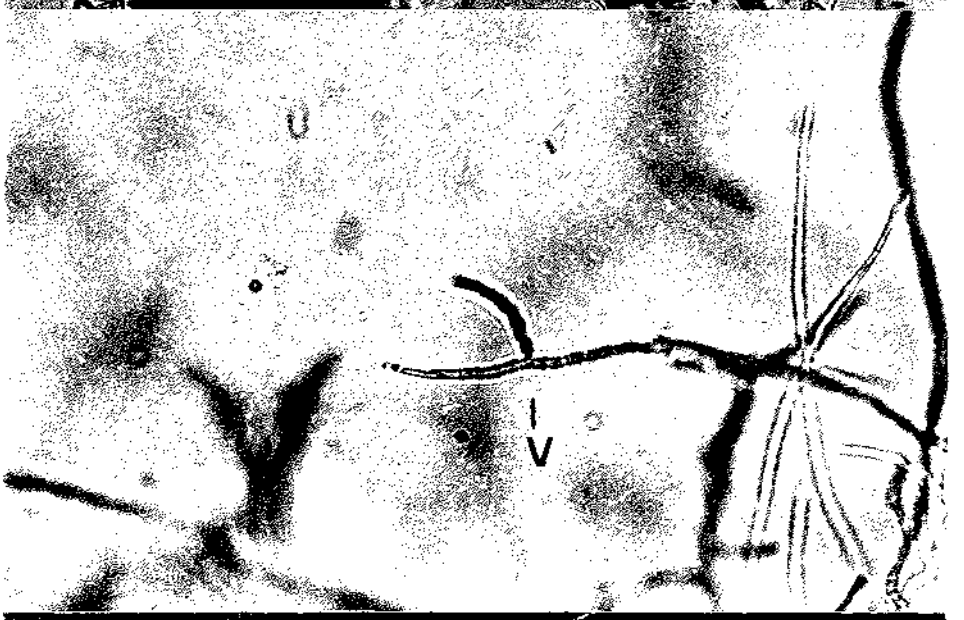

G
100μm
H
50μm
I
10μm

J



100μm

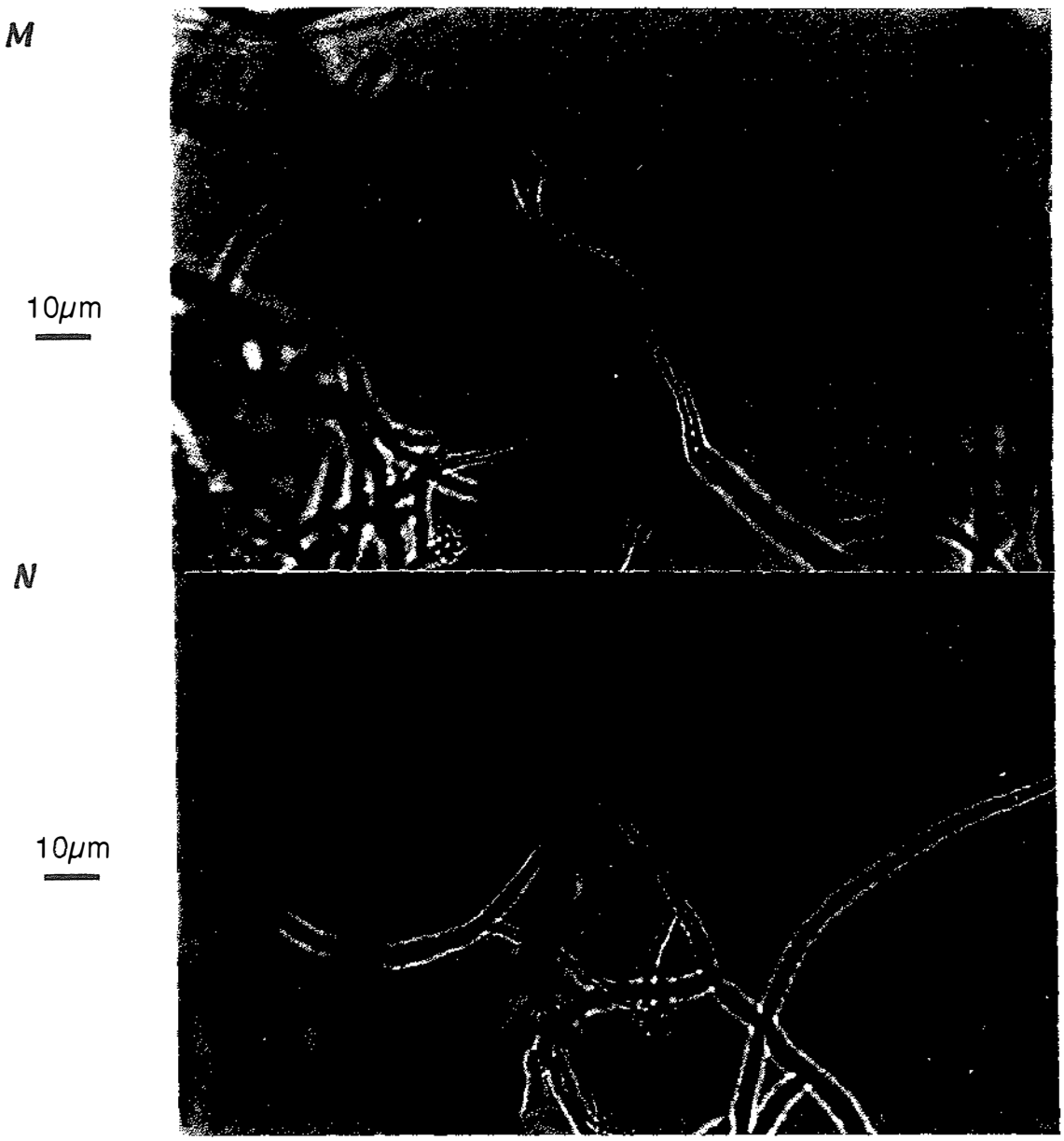
K



10μm

L

10μm


M
10μm
N
10μm

3.3.5 Growth of *P. paxilli* in supplemented media

A number of experiments were conducted to determine the effect of various media supplements on the growth and paxilline production of wild type *P. paxilli* in liquid culture. The scale of the experiments was reduced to maximise the number of variables that could be tested. The culture volume was reduced to 100 mL and all experiments were inoculated with 8 mL of a 100 mL seed culture (section 2.1.8). Samples (10 mL) were taken at two time points (60 h and 120 h) except in cultures grown in CD and CD + 0.25% tryptophan, where biomass accumulation was significantly reduced. A single sample, of 100 mL was removed from these two cultures after 120 h., and the excess broth removed to a final volume of 10 mL. Paxilline was extracted from 10 mL samples (section 2.16.2) and separated by TLC (section 2.16.4). The gross morphology of mycelia, grown in a 100 mL volume at 350 rpm, was fine pellets of approximately 1-2 mm diameter.

3.3.5.1 Calcium

Calcium-induced sporulation was reported to inhibit the synthesis of paxilline (Ibba *et al.*, 1987). However, microscopic analysis of *P. paxilli* in the paxilline-inducing media CDYE, confirmed that sporulation was occurring (Figure 3.29 D and F). To test this theory, *P. paxilli* was grown in the sporulation medium PD (section 2.2) and in a CDYE media (section 2.2) containing 2% (w/v) $CaCl_2$. The concentration of spores in the broth after 120 h was determined by haemocytometer counting. In the control CDYE media, the spore concentration was 1.4×10^5 spores/mL. At this concentration, the culture is cream coloured. When *P. paxilli* was grown in PD or CDYE + 2% (w/v) $CaCl_2$, the culture is coloured dark green. The spore concentration in PD media and CDYE + 2% (w/v) $CaCl_2$ after 120 h was 2.5×10^7 spores/mL and 9.3×10^7 spores/mL respectively. The paxilline content of the cultures was determined by TLC (section 2.16.4). Results are qualitative only, and are shown in Figure 3.30. The control culture grown in CDYE is seen in lanes 2 and 3, with paxilline staining dark green when exposed to alcoholic Erhlich's reagent. Paxilline was detected at 60 h and increases at

Figure 3.30 *TLC analysis of paxilline production with media supplements*

P. paxilli cultures were grown as described in section 2.1.2, in 500mL Erlenmeyer flasks containing 100mL of test media. The test media used the Czapek Dox medium from Oxoid as a base. The constituents of CD medium from Oxoid are slightly different from those in the CD salts used for other experiments and therefore should not be compared. Paxilline was extracted from 10 mL of culture into 12 mL of chloroform:methanol (2:1) (section 2.16.1). An aliquot of 1.5 mL was removed and evaporated overnight. The dried sample was then resuspended in 50 μL of chloroform:methanol (2:1) and run on a TLC plate (section 2.16.4). Lanes 1 and 12, 40μg of Paxilline standard. Lanes 2-3, CDYE culture at 60 h and 120 h. Lane 4, CD culture at 120 h. Lane 5, CD + 0.25% Tryptophan culture at 120 h. Lanes 6-7, PD culture at 60 h and 120 h. Lanes 8-9, CDYE + 2% $CaCl_2$ culture at 60 h and 120 h. Lanes 10-11, CDYE + 50mM MOPS culture at 60 h and 120 h.

Figure 3.30 TLC analysis of paxilline production by *P. paxilli* with media supplements

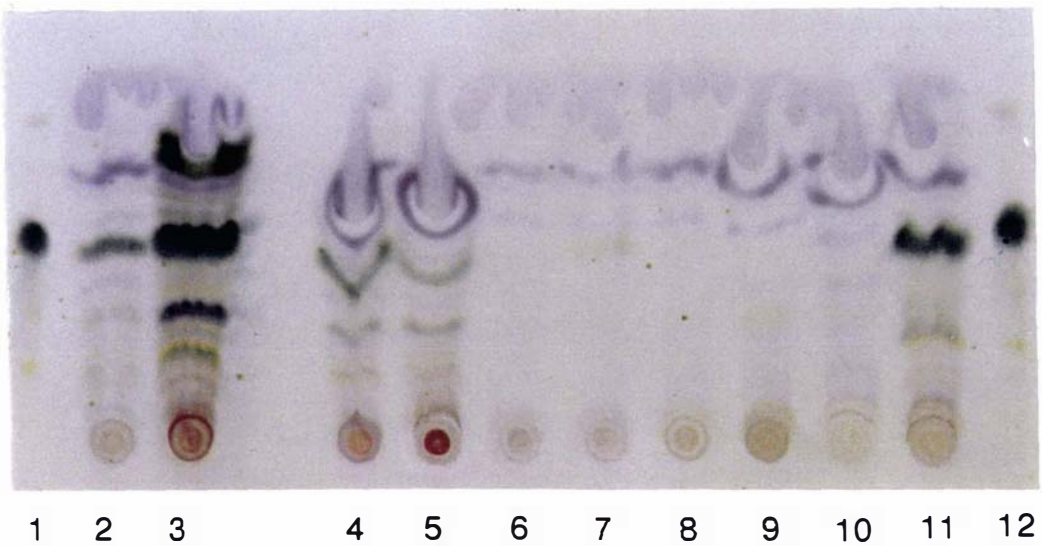


Table 3.8 Summary of growth in supplemented media

Lane	Media	Age (hours)	Sample Volume	Paxilline*	Biomass (g/L)	pH
	CDYE	0				6.48
2	CDYE	60	10 mL	+++	16.2	6.85
3	CDYE	120	10 mL	++++++	16.2	5.62
	CD	0				6.76
4	CD	120	100 mL	+++	1.9	7.29
	CD + 0.25% Trp	0				6.71
5	CD + 0.25% Trp	120	100 mL	++	3.2	7.01
	PD	0				5.72
6	PD	60	10 mL	-	6.4	5.29
7	PD	120	10 mL	+	6.4	5.25
	CDYE + 2% CaCl2	0				6.00
8	CDYE + 2% CaCl2	60	10 mL	-	11.9	6.22
9	CDYE + 2% CaCl2	120	10 mL	-	16.2	5.30
	CDYE + 50 mM MOPS	0				5.66
10	CDYE + 50 mM MOPS	60	10 mL	-	19.1	6.47
11	CDYE + 50 mM MOPS	120	10 mL	++++	17.1	6.33

* semi quantitative interpretation of TLC results seen in Figure 3.30.

120 h. In lanes 6 and 7 the samples taken at 60 h and 120 h, from the culture grown in PD, are shown. Paxilline was not detected at 60 h, although a very faint band is discernible at 120 h. Cultures grown in CDYE + 2% (w/v) $CaCl_2$ showed no trace of paxilline, even after 120 h of growth (lanes 8 and 9). Interestingly, the biomass accumulation in PD is quite low (6.4 g/L), compared to both the control CDYE culture (16.2 g/L) and the CDYE + 2% (w/v) $CaCl_2$ culture (16.2 g/L) (Table 3.8). However, even with the lowered biomass, a faint trace of paxilline was still detected. The cause of this decrease in biomass accumulation could be the absence of yeast extract in the PD media.

3.3.5.2 Yeast Extract

The putative source of the indole moiety in paxilline is tryptophan (Laws & Mantle, 1989). The yeast extract supplement added to CD media, although undefined, is a source of many amino acids. To examine the effect of yeast extract on paxilline production, a *P. paxilli* culture was grown in CD only media. A second culture was supplemented with 0.25% (w/v) tryptophan, to determine if yeast extract was the source of tryptophan during paxilline biosynthesis. Biomass accumulation in both experimental media, was severely reduced, therefore the entire culture volume was collected after 120 h and analysed for paxilline content. Both cultures were pink in colour, although the colour was more intense in the CD + 0.25% (w/v) tryptophan culture. Occasionally, cultures grown in CDYE are coloured pink, but this is usually in cultures of less than 100 mL. Paxilline was detected in both cultures after 120 h, however, the paxilline seen in Figure 3.30, lanes 4 and 5 represents a sample volume 10 times that of the other lanes.

3.3.5.3 Biological buffer

Changes in pH have been associated with the onset of secondary metabolite biosynthesis in a number of filamentous fungi. During the growth experiments discussed in section 3.3.4, the pH of the culture broth was seen to change over time

(Figure 3.27 B and 3.28 B). A drop in pH is seen approximately 30 h before paxilline was detected in the culture. The biological buffer MOPS, was supplemented to 50 mM in CDYE to examine the effect of pH on paxilline biosynthesis. Cultures grown in CDYE + 50 mM MOPS achieved the greatest biomass (19.1 g/L) of all the supplements tested, including the control culture grown in CDYE, which had a biomass of 16.2 g/L. The pH of the broth from both CDYE cultures and CDYE + 50 mM MOPS is shown in Table 3.8. The cultures grown in CDYE and CDYE + 50 mM MOPS both reach a pH of over 6.5 after 60 h. Between 60 h and 120 h, pH in the control culture decreased 18%, while the pH in the culture supplemented with MOPS only decreased 2% during the same time. The paxilline content of the culture grown in CDYE + 50 mM MOPS is shown in Figure 3.30, lanes 10 and 11. Even with a greater overall biomass, paxilline was not detected until 120 h, at which time it is approximately equivalent to the amount of paxilline seen in the control culture at 60 h (Figure 3.30, lane 2). The results from all these experiments are summarised in Table 3.8.

3.3.6 Growth of *P. paxilli* in variable carbon sources

Utilisation of a preferred carbon source can effect the growth rate and biosynthesis of secondary metabolites by fungal species in liquid culture. To examine the effect of carbon source on production of paxilline, *P. paxilli* was grown in CD salts (section 2.2) supplemented with 0.5% (w/v) YE and a range of carbon sources at 3% (w/v). The standard CDYE media from Oxoid contains 3% sucrose as the carbon source. The culture grown in 3% (w/v) sucrose accumulated the highest biomass after 60 h and was the only culture to record a drop in biomass after 120 h. At the end of 120 h, the culture grown in 3% (w/v) glucose, had accumulated the greatest biomass (11.9 g/L) (Table 3.9). The paxilline content was determined by TLC (section 2.16.4) and the results are shown in Figure 3.31 and Table 3.9. The most interesting result is the paxilline content of the culture grown in 3% (w/v) glucose (lane 6-7). The level of paxilline in this culture was less than the control culture, containing 3% (w/v) sucrose, after 60 h (lanes 2-3), but surpassed it after 120 h. The number of bands seen in lane 6 indicates that the biosynthesis of other indole-diterpenoid moieties has also increased. Four indole-diterpenoid products (stained green with alcoholic Erhlich's reagent), with a R_f greater than paxilline are less polar than paxilline, and may represent precursors of paxilline.

Figure 3.31 *TLC analysis of paxilline production in variable carbon sources*

P. paxilli cultures were grown as described in section 2.1.2, in 500mL Erlenmeyer flasks containing 100mL of test media. The basic media contained CD salts (section 2.2.) + 0.5% Yeast Extract (Oxoid), + 3% (w/v) carbon source. The constituents of the CD salts medium used, vary from those in the CD medium supplied by Oxoid, which was used for the experiments shown in figure 3.30 and should not be compared. Paxilline was extracted from 10 mL of culture into 12 mL of chloroform:methanol (2:1) (section 2.16.1). An aliquot of 1.5 mL was removed and evaporated overnight. The dried sample was then resuspended in 50 μL of chloroform:methanol (2:1) and run on a TLC plate (section 2.16.4). Lanes 1 and 10, 5μg of paxilline standard. Lanes 2-5, 60 h cultures with 3% sucrose, 3% glucose, 3% glycerol, and 3% mannitol. Lanes 6-9, 120 h cultures with 3% sucrose, 3% glucose, 3% glycerol, and 3% mannitol.

Figure 3.31 *TLC analysis of paxilline production by P. paxilli in different carbon sources*

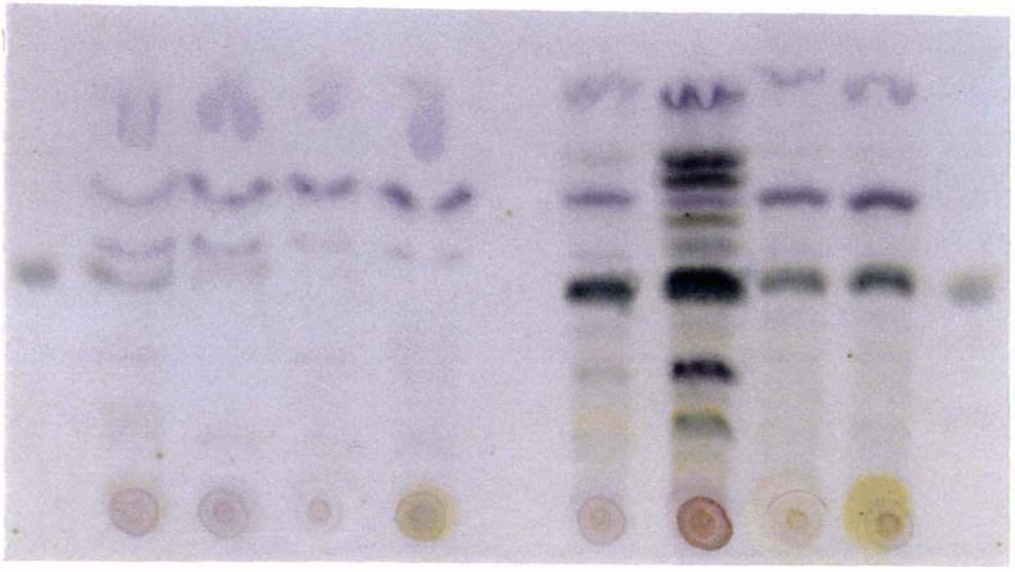


Table 3.9 *Paxilline production of P. paxilli cultures in variable carbon sources*

Lane	Carbon	Age (hours)	Paxilline* (g/L)	Biomass
2	Sucrose	60	++	12.0
3	Glucose	60	+	9.6
4	Glycerol	60	-	9.9
5	Mannitol	60	-	9.9
6	Sucrose	120	++++	8.1
7	Glucose	120	++++++	11.9
8	Glycerol	120	+++	10.4
9	Mannitol	120	+++	13.0

*semi quantitative interpretation of TLC results shown in Figure 3.31.

Two indole-diterpenoid products seen below paxilline, are structures more polar than paxilline that may represent products from additional oxidation steps after the synthesis of paxilline. Paxilline was not detected in cultures supplemented with 3% (v/v) glycerol or 3% (w/v) mannitol (lanes 7 and 8) until 120 h, although the rate of biomass accumulation was equivalent to that seen in the culture supplemented with 3% (w/v) glucose.

3.3.7 Growth of P. paxilli during carbon and nitrogen starvation

Biosynthesis of paxilline occurs during secondary metabolism. Regulation of many secondary metabolite pathways is under nitrogen or carbon repression (Drew & Demain, 1977; Marzluf, 1981). Experiments were performed to examine the effect of nitrogen and carbon repression on paxilline biosynthesis in *P. paxilli.* It was postulated that cultures prematurely released from repression by nitrogen or carbon catabolite regulatory proteins would initiate biosynthesis of paxilline earlier, resulting in an increased amount of paxilline at both 60 h and 120 h.

Test media, [CD salts + 0.5% (w/v) YE + 3% (w/v) sucrose] (100 mL), was inoculated from a seed culture (section 2.1.8) and allowed to grow for 24 h. The mycelia were then harvested and transferred to media lacking either nitrogen or carbon. The media was supplemented with 0.5% (w/v) yeast extract. The standard CDYE media contains 3% (w/v) sucrose and 0.2% (w/v) sodium nitrate. Samples were collected at 60 h and 120 h. After 60 h, the culture grown in the absence of nitrate had a typical biomass accumulation of 13.5 g/L (Table 3.10), compared to the control CD salts + YE + sucrose culture which had a biomass of 11.9 g/L after 60 h (Table 3.9). Unfortunately the culture lacking nitrogen became contaminated and a final sample was not prepared. Paxilline was detected after 60 h (Figure 3.32, lane 2) and is a similar quantity to that seen in controls CDYE (Figure 3.30, lane 2) and CD salts + YE + sucrose cultures (Figure 3.31, lane 2). Lack of nitrogen did not seem to effect the growth or production of paxilline significantly, although yeast extract is probably a compensatory source of nitrogen. Carbon starved cultures failed to gain biomass after being transferred into the experimental media (Table 3.10). Paxilline was not detected at either 60 h or 120 h

Figure 3.32 *TLC analysis of paxilline production under conditions of nitrogen and carbon starvation*

P. paxilli cultures were grown as described in section 2.1.2. After 24 h in CDYE media, the mycelia were transferred to media lacking either carbon or nitrogen. The constituents of the CD salts medium used in the second day of the experiment, vary slightly from those in the CD medium supplied by Oxoid. Paxilline was extracted from 10 mL of culture into 12 mL of chloroform:methanol (2:1) (section 2.16.1). An aliquot of 1.5 mL was removed and evaporated overnight. The dried sample was then resuspended in 50 μL of chloroform:methanol (2:1) and run on a TLC plate (section 2.16.4). Lanes 1 and 5, 5μg of paxilline standard. Lane 2, CD salts (-NaNO3) + 0.5% YE + 3% sucrose culture at 60 h. Lanes 3-4. CD salts + 0.5% YE at 60 h and 120 h.

Figure 3.32 TLC analysis of paxilline production by *P. paxilli* during carbon and nitrogen starvation

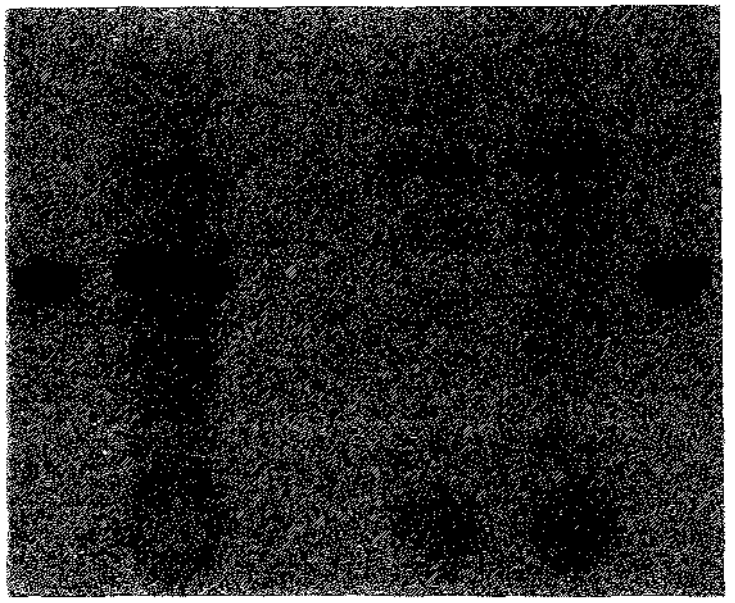


Table 3.10 *P. paxilli* cultures under carbon and nitrogen starvation

Lane	Media	Age (hours)	Paxilline*	Biomass (g/L)
2	- Nitrogen	60	+++	13.48
3	- Carbon	60	-	2.33
4	- Carbon	120	-	2.41

*semi quantitative interpretation of TLC results shown in Figure 3.32

(Figure 3.32, lanes 3 and 4). Yeast extract does not appear to compensate for a lack of carbon in the media.

3.4 Analysis of Paxilline Biosynthetic Gene Expression

3.4.1 RT-PCR gene expression assay

The level of gene expression in *P. paxilli*, grown in paxilline-inducing media, was determined with a semi-quantitative RT-PCR assay. Total RNA was isolated from mycelia, sampled at different time points from Experiment 1 and 2 (section 2.17). In both cases, flask B was used as the source of mycelia. The RNA was then reverse transcribed into cDNA (section 2.18) and stored at -20°C until required. Gene specific primers for each gene of interest were selected with two main requirements. Firstly, to distinguish cDNA products from gDNA products, primers were selected to amplify regions where introns were present. Several genes of interest lacked introns, however, stringent controls during reverse transcription (section 2.18.2) eliminated the likelihood of any gDNA contaminating these pools of cDNA. Secondly, the average size of the cDNA amplicon was approximately 400 bp, so that the amplification efficiency of each gene product would be roughly equivalent. The number of PCR amplification cycles was optimised for each gene and the individual specifications for each gene are summarised in Table 3.11. Products were separated on a 2.5% (w/v) agarose gel (section 2.10.1 and 2.10.3) and are shown in Figures 3.33 and 3.34. A frequent problem was the failure of an individual PCR reaction, which meant that the complete set (all time points at three dilutions) had to be repeated. In some cases (Figure 3.33 C), if the pattern of expression could be determined, a single set of reactions has been shown.

3.4.2 Standardisation of the assay with *tub-2*

The first gene analysed for the level of steady state RNA was *tub-2*. The design of this assay is semi-quantitative, as RNA levels are standardised after RNA isolation (120 µg removed for DNase I treatment) and after removal of DNA (1 µg is reverse transcribed).

Table 3.11 Summary of RT-PCR Gene expression

Gene	Putative Function	Primers	PCR Cycles	gDNA size (bp)	cDNA size (bp)	Exp# 1	Exp# 2
tub-2	β-Tubulin	Etub2 & Etub3	30	317	255	√	√
paxG	GGPP Synthase	ggpps4 & ggpps9	30	773	707	√	√
paxM1	Monooxygenase	mono3 & mono4	30	340	274	√	√
paxP1	P450 monooxygenase	cyp1 & cyp8	30	532	409	√	√
paxR1	Zinc finger transcription factor	Pax39 & Pax40	40	376	316	√	√
hmg	HMG CoA reductase	ET9 & ET22	30	671	604	√	√
ggs-1	GGPP Synthase	ggpps36 & ggpps40	40	480	400	√	√
paxH	Dehydrogenase	pax112 & pax113	40	404	404		√
paxU1	Cyclase /prenyl transferase	cyp6 & pax58	30	309	247		√
paxD	DMAT synthase	dmat15 & dmat4	30	568	479		√
paxA	pH responsive protein	pax81 & pax84	40	404	343		√
paxM2	Monooxygenase	pax133 & pax138	40	617	617		√
paxP2	P450 monooxygenase	pax60 & paxP2p1	30	509	443		√
amy	α-Amylase	pax7 & pax8	35	320	320		√
ara	Arabinase	dmat7 & dmat10	35	560	490		√
lip1	Lipase	pax3 & pax9	40	343	343		√
lip 2	Lipase	pax134 & pax135	40	283	283		√

Therefore the gene expression visualised by ethidium bromide staining, reflects the amount of a particular mRNA species in a fixed amount of total RNA. It is possible to think of the expression levels as a percentage of the total expression. For a gene such as *tub-2*, the percentage of expression does not change over time. This is highlighted in Figure 3.33 A and 3.34, where the *tub-2* product fluoresces with equal intensity in all lanes.

3.4.3 Identification of the Pax pattern of expression

The expression pattern of genes involved in paxilline biosynthesis is best exemplified in Experiment 2 by *paxG* (Figure 3.33 A) and reproduced using RNA isolated from Experiment 1 (Figure 3.34). Even at the highest cDNA concentration, there is no cDNA product visible until 36 h. At lower cDNA concentrations, the expression of paxG is seen to peak at 60 h, and then slowly decreases again. This pattern of expression is also seen when *paxP1* cDNA is amplified (Figure 3.33 A and 3.34), although the decrease after 60 h is not apparent. Gene replacement knockout mutants of both *paxG* and *paxP1* have a Pax$^-$ phenotype (Young *et al.*, 2000). Therefore this pattern of expression was established as the paxilline biosynthetic expression pattern (Pax pattern). An interesting example of this is *paxM1* (Figure 3.33 A and 3.34), although expression of *paxM1* is detected as early as 24 h, the lower cDNA dilutions clearly reveal an increase in expression at 36 h. Due to this increase, occurring at the same time as the onset of expression of *paxG* and *paxP1*, *paxM1* was considered to have the Pax pattern of expression. Additional genes that exhibit the Pax pattern of expression are *paxU1*, *lip2*, and *ara* in Figure 3.33 B, and *paxD*, *paxP2* and possibly *paxM2* in Figure 3.33 C. Some genes that were not predicted to be involved in paxilline biosynthesis have shown the Pax pattern of expression. For example, *lip2* and *ara* do not have predicted functions required for paxilline biosynthesis, although they clearly exhibit the Pax pattern of expression. Interestingly, *lip2* and *ara* are found close to the predicted boundaries of the cluster. Genes *lip1* and *amy* have predicted functions similar to *lip2* and *ara*, although they are located further away from the predicted boundaries of the cluster. The expression patterns of *amy* and *lip1* were included for comparison and neither exhibited the Pax pattern of expression (Figure 3.33 B and 3.33 C).

Not all genes predicted to function in the paxilline biosynthetic cluster exhibit the Pax pattern of expression. Genes *paxH,* a putative dehydrogenase and *paxA,* a putative pH-responsive protein, were proposed to function during paxilline biosynthesis. However, the expression of both these genes, seen in Figure 3.33 C, showed no change over time. The gene expression pattern of *paxR1*, the putative transcription regulator, is seen in Figure 3.33 A and 3.34. Expression of this gene does not conform to the Pax pattern, but does appear to change over time. Expression of *paxR1* was very faint at 24 h and increases to a peak level from 30 to 48 h and then decreases.

The expression pattern of *hmg* from Experiment 1 and 2 is shown in Figure 3.33 B and 3.34. In Figure 3.34, the expression of *hmg* appears to resemble the Pax pattern of expression, with an increase in expression after 36 h. However, when the pattern from Experiment 2 is examined (Figure 3.33 B) and the time points 24 h and 30 h are included, a new pattern emerges. Expression of hmg is high at 24 h, and then decreases at 30 h. After 36 h, the familiar pattern of increasing expression is seen, with expression decreasing again after 72 h. Therefore *hmg* was considered to have a modified form of the Pax pattern of expression.

The final gene to consider is *ggs-1*, the gene for primary geranylgeranyl pyrophosphate synthase. This was included for comparison with *paxG*, the secondary geranylgeranyl pyrophosphate synthase. Although these genes encode products of homologous function, the patterns of expression are quite different (Figure 3.33 A). Gene replacement knockout mutants of *paxG* do not synthesis paxilline, and the product of *ggs-1* does not appear to compensate the loss of the *paxG* product (Young *et al.*, 2000). Expression of *ggs-1* does not change significantly over time. A summary of the gene expression patterns of all the genes analysed is shown in Table 3.12.

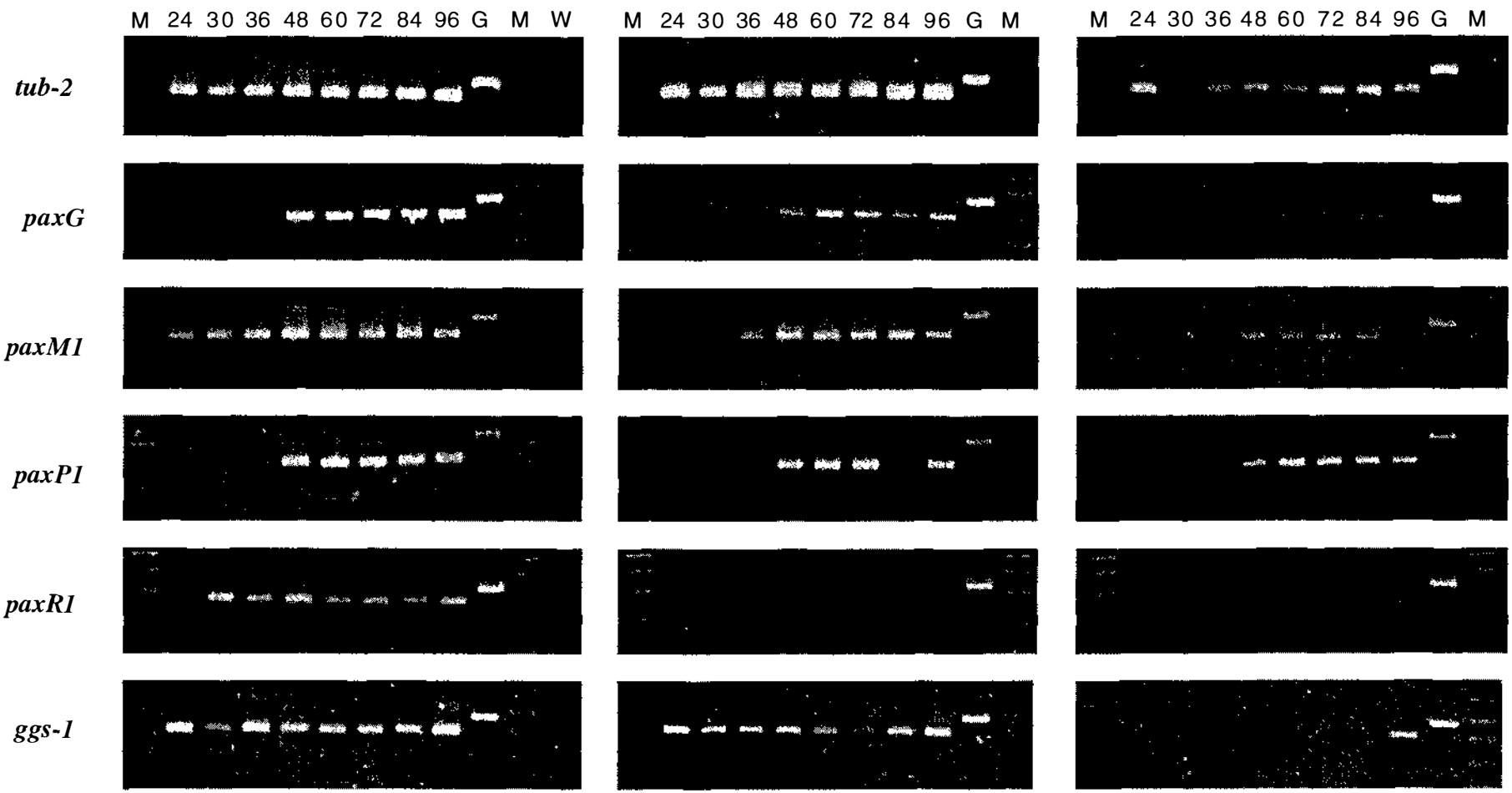
Figure 3.33 *Steady state mRNA levels detected by RT-PCR in RNA isolated from mycelia grown in Experiment 2*

Total RNA was isolated from mycelia harvested at different time points (section 2.17) and reverse transcribed with random hexamer primers (section 2.18). Gene specific primers were used to amplify cDNA from serial dilutions of template and PCR conditions were optimised for individual reactions (Table 3.11). Samples were run on a 2.5% (w/v) agarose gel (section 2.10.1 and 2.10.3). The time (h) at which the original mycelia sample was removed is shown above the corresponding lanes. The marker (M) used in all experiments was the 100 bp ladder (BioLabs). Lanes containing products amplified from a gDNA template are indicated with a 'G'. A 'W' indicates lanes that contain the water controls for each set of reactions.

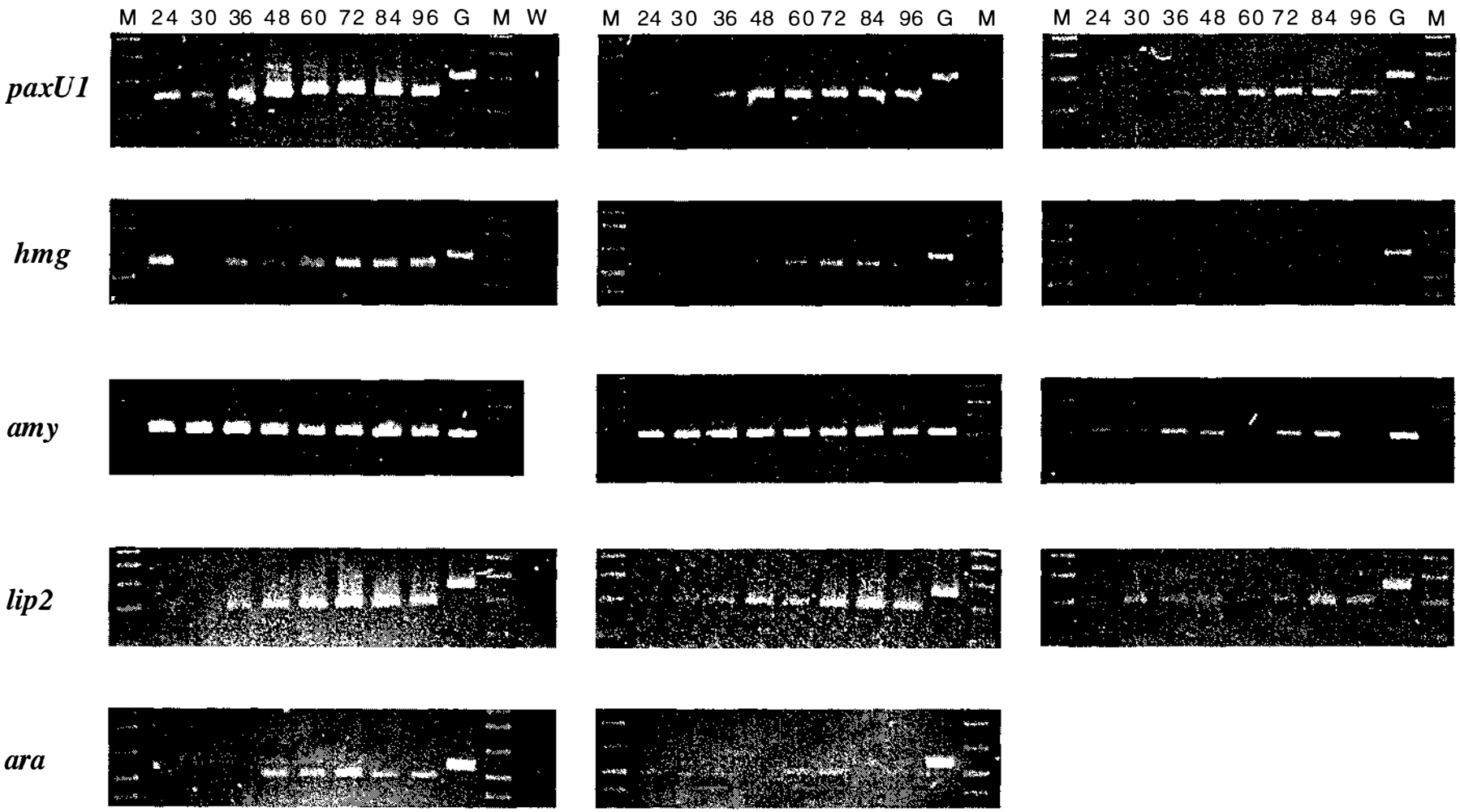
A) Steady state levels of mRNA from *tub-2, paxG, paxM1, paxP1, paxR1* and *ggs-1*.

B) Steady state levels of mRNA from *paxU1, hmg, amy, lip2* and *ara*.

C) Steady state levels of mRNA from *paxH, paxA, paxD, paxP2, paxM2* and *lip1*

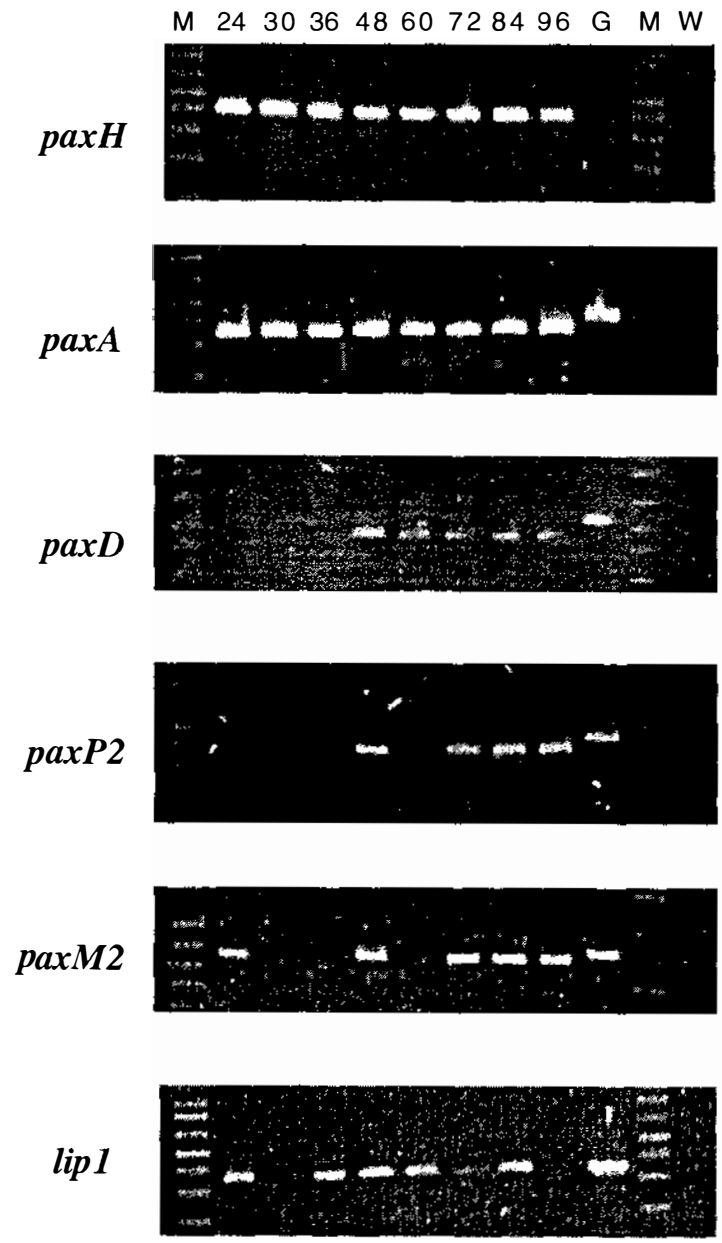

M 24 30 36 48 60 72 84 96 G M W
M 24 30 36 48 60 72 84 96 G M
M 24 30 36 48 60 72 84 96 G M
tub-2
paxG
paxM1
paxP1
paxR1
ggs-1
cDNA Dilution
1/10
1/100
1/1000

A


M 24 30 36 48 60 72 84 96 G M W
M 24 30 36 48 60 72 84 96 G M
M 24 30 36 48 60 72 84 96 G M
paxU1
hmg
amy
lip2
ara
cDNA Dilution
1/10
1/100
1/1000

B

C



cDNA Dilution **1/10**

Figure 3.34 *Steady state mRNA levels detected by RT-PCR in RNA isolated from mycelia grown in Experiment 1*

Total RNA was isolated from mycelia harvested at different time points (section 2.17) and reverse transcribed with random hexamer primers (section 2.18). Gene specific primers were used to amplify cDNA from serial dilutions of template and PCR conditions were optimised for individual reactions (Table 3.11). Samples were run on a 2.5% (w/v) agarose gel (section 2.10.1 and 2.10.3). The time (h) at which the original mycelia sample was removed is shown above the corresponding lanes. The marker (M) used in all experiments was the 100 bp ladder (BioLabs). Lanes containing products amplified from a gDNA template are indicated with a 'G'. A 'W' indicates lanes that contain the water controls for each set of reactions.

Steady state levels of mRNA from *tub-2, paxG, paxM1, paxP1, paxR1* and *hmg* are shown.

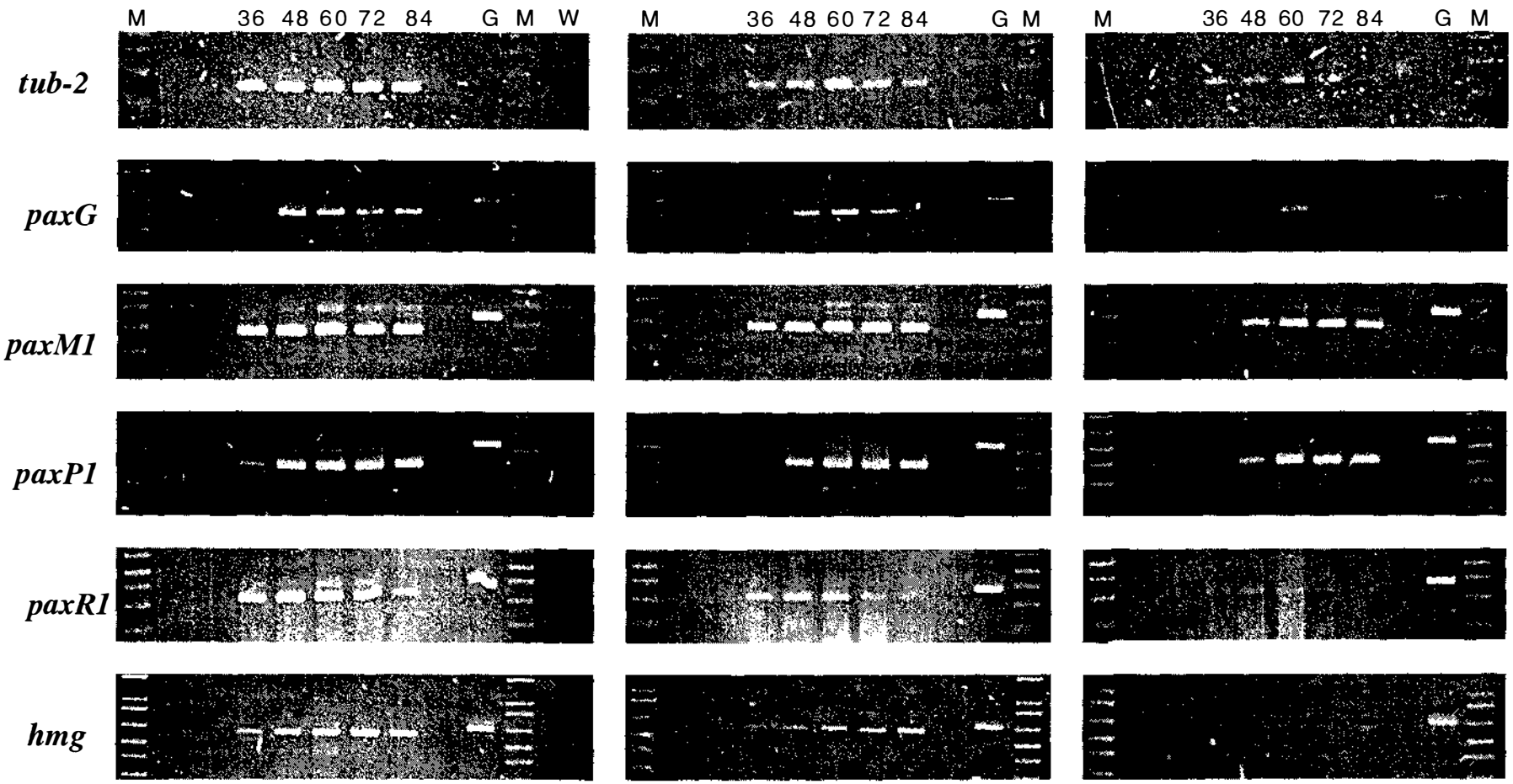

M 36 48 60 72 84 G M W
M 36 48 60 72 84 G M
M 36 48 60 72 84 G M
tub-2
paxG
paxM1
paxP1
paxR1
hmg
cDNA Dilution 1/10
1/100
1/1000

Figure 3.34

Table 3.12 Summary of gene expression

Gene	2° Pax Gene	Pax pattern	Figure
tub-2	No	X	3.33 A & 3.34
paxG	Yes	√	3.33 A & 3.34
paxM1	Yes	√	3.33 A & 3.34
paxM2	Yes	?	3.33 C
paxP1	Yes	√	3.33 A & 3.34
paxP2	Yes	√	3.3 C
paxR1	Yes	X	3.33 A & 3.34
paxU1	Yes	√	3.33 B
paxH	Yes	X	3.33 C
paxA	Yes	X	3.33 C
paxB	Yes	√	3.33 C
hmg	1° gene	√*	3.33 B & 3.34
ara	No	√	3.33 B
amy	No	X	3.33 B
lip1	No	X	3.33 C
lip2	No	√	3.33 B
ggs-1	No	X	3.33 A

*NB: Modified Pax pattern of expression.

4.1 Isolation and Characterisation of *P. paxilli hmg*

4.1.1 *P. paxilli hmg*

A unique *hmg* was successfully isolated from *P. paxilli* genomic DNA with degenerate primers designed to the conserved catalytic region of *hmg* genes (Figure 3.2). Isolation of genes with PCR is only possible when sufficient sequence data is available from other species to design PCR primers. When designing degenerate primers, a number of factors can enhance the probability of isolating a true product. These include primer length and the percentage of degenerate nucleotides (inosines). The presence of non-conserved introns can also affect the size of genuine products. Controls of a known size increase the likelihood of identifying the correct product. For example, six different products were amplified with HMG1 and HMG3 (Figure 3.1 C). It is interesting to note, the specific 359 bp *hmg* product isolated was not the most abundant product. The synthesis of additional products may be the result of inosine nucleotides in a primer and lowered annealing temperature.

Southern analysis of the *P. paxilli* genome indicates that a single copy of *hmg* is present (Figure 3.3), therefore, the mevalonate requirements for primary and secondary metabolism, are met by a single enzyme. Once secondary metabolism is initiated, enzyme activity may be increased to meet the new demands of paxilline biosynthesis, as well as the requirements for primary metabolism. This may occur via increased transcription, increased translation, decreased degradation or dephosphorylation of specific residues in the catalytic domain. The primary metabolic requirement for mevalonate may also be substantially lower after the onset of secondary metabolism, due to the cessation of exponential growth.

The chromosomal location of the *hmg* locus was identified by CHEF analysis of *P. paxilli* chromosomes. A gene specific probe for *hmg,* hybridised to chromosome Vb (Figure 3.18 A and B), indicating that *hmg* is not spatially linked to the paxilline biosynthetic gene cluster on chromosome Va (Young *et al.*, 1998a).

4.1.2 *P. paxilli hmg* nucleotide sequence

Double stranded sequence (4.6 kb) was determined for the *hmg* locus, including 1.1 kb of sequence upstream of the translation initiation codon. As discussed, *hmg* has a role in both primary and secondary metabolism, and this may necessitate separate promoter elements to regulate the dual requirements of this enzyme.

Sequencing of both genomic and copy DNA clones revealed three introns, one in the 5' UTR, and two within the coding region of the gene. These introns are labelled, intron 1, 2 and 3, and a summary of the sequence features is shown in Table 3.7. All introns are within the size range for filamentous fungal introns which are generally no greater than 100 bp in size (Gurr *et al.*, 1987). The GT/AG rule for intron splice sites is also conserved. At the 5' splice site the general consensus is GT, followed by ANGT (Ballance, 1986; Gurr *et al.*, 1987) although conservation of the last four nucleotides is less prominent in *hmg*.

At the 3' splice site, the consensus is a pyrimidine (Py), followed by AG. However, variations from this sequence invariably have an adenosine nucleotide in place of a pyrimidine (Ballance, 1986; Gurr *et al.*, 1987). In *hmg*, introns 1 and 2 have the sequence AAG at the 3' splice site, and intron 3 has the more conservative CAG sequence.

The internal consensus sequence, provides the branch site for lariat formation (Voet & Voet, 1990) and in yeast, the sequence 5'-TACTAAC-3' fulfils this function. The adenosine nucleotide in position 6 is absolutely required for intron splicing to occur. The branch site is located closer to the 3' splice site than the 5' splice site. Ballance (1986) reported that this exact sequence was rarely seen in filamentous fungi (only 3 out of 54 introns). However, the exact sequence is found in intron 3 of the *P. paxilli hmg* and a motif with a single mismatch was found in intron 2. There are no experimental data to show that these are functional branch sites, they have been identified only by homology to published consensus sequences. In filamentous fungi the consensus proposed by Gurr *et al.* (1987) is 5'-PyGCTAAC-3' with the most conserved nucleotides being the adenosines in position 5 and 6.

Another prominent feature of higher eukaryotic introns is the presence of a polypyrimidine tract located between the internal lariat sequence and the 3' branch site. In higher eukaryotes, the polypyrimidine tract is important for correct lariat formation and spliceosome assembly (Coolidge *et al.*, 1997). Ballance (1986) reported that most filamentous fungal introns lack a polypyrimidine tract. However, polypyrimidine tracts are present in intron 1 and intron 2. A smaller tract of 4 pyrimidines is seen in intron 3.

Filamentous fungi contain fewer introns than higher eukaryotes, for example, the *hmg* gene from humans and Chinese hamster, both contain 19 introns, including 1 intron in the 5' UTR (Luskey & Stevens, 1985; Reynolds *et al.*, 1984). In, *P. paxilli*, intron 3 is positionally conserved with introns in the *hmg* of *N. lolii* Lp19 and *G. fujikuroi* (Dobson, 1997; Woitek *et al.*, 1997), but is not conserved with any of the introns present in humans or Chinese hamster *hmg*. The *N. lolii* Lp19 intron is 73 bp in length, and has a small tract of 5 pyrimidines. In *G. fujikuroi,* the intron is 47 bp in length and lacks a distinctive polypyrimidine tract.

Codon preference in *P. paxilli hmg*, follows trends seen in the established filamentous fungal model organism, *Neurospora crassa* (Ballance, 1986; Gurr *et al.*, 1987). There is a preference for pyrimidines in the third position, particularly C nucleotides, which appear in 38% of all codons. The purine nucleotides, particularly adenosine, are rarely seen in the third position and occurred in only 8% of codons in *hmg* (Table 3.3). The coding sequence of *P. paxilli hmg,* from translation initiation to termination, including introns, has a GC content of 54%.

4.1.3 *P. paxilli hmg 5' sequence analysis*

4.1.3.1 *Initiation of transcription*

The transcription start site in the *P. paxilli hmg* was determined by 5' RACE, after first establishing the approximate position by RT-PCR. Positional techniques like 5' RACE and primer extension rely on the presence of a full-length 5' UTR and efficient reverse transcription. Multiple transcription initiation sites are often found in filamentous fungal genes (Gurr *et al.*, 1987) and given the dual role *hmg* has in both primary and

secondary metabolism, it is possible that this gene also has multiple transcription start sites. The RT-PCR estimation of the transcription start site was performed with RNA isolated from cultures grown for 36 h and 60 h. Nested RT-PCR gave a product with primers ET5 and ET12, but not with primers ET5 and ET4Bam, indicating that transcription is initiated between ET12 and ET4Bam (Figure 3.10). No apparent difference in 5' UTR length was seen between these two time points with the primers available. However, as will be discussed in section 4.4, the expression of genes involved in paxilline biosynthesis is seen at 36 h, and an earlier time point may have been required to show differences in the transcription of *hmg* for primary and secondary metabolism. In addition, the distance between primers ET4Bam and ET12 is large enough that multiple transcripts could initiate between them and go undetected.

In this study, 5' RACE was performed on RNA isolated from cultures grown for 24 h and 60 h. Unfortunately, 5' RACE products were only obtained from the 60 h RNA, and time constraints meant a second attempt to obtain a 24 h 5' RACE product was not possible. Two 5' RACE products were detected at 60 h (Figure 3.15), an abundant product of 285 bp and a less abundant product of 345 bp. Due to the weak amplification of the larger product, it was unable to be purified and sequenced in the time available. However, the presence of the two products would indicate either the presence of a second transcription initiation site or an alternatively spliced mRNA from the same transcription initiation site. The smaller product was sequenced and confirmed to start 436 bp upstream of the ATG. A putative TATA motif is located 22 bp upstream from this site. This 5' UTR sequence has a GC content of 51.3 %.

4.1.3.2 5' UTR introns

The cloning and sequencing of the 5' RACE product confirmed the presence of intron 1 in the 5' UTR. A second 63 bp 5' UTR intron is predicted in the *P. paxilli hmg*, due to the presence of consensus splice sites (Figure 3.7). At position -97 bp there is a 5' splice motif (5'-GTAATT-3'), at position -64 bp a lariat consensus sequence (5'-TCATAAC-3') is present, and at position -37 bp there is a 3' splice site (5'-CAG-3'). A 9 bp polypyrimidine tract is also present at position -58 bp. Many potential 5' and 3' splice sites are present in the 5' UTR of *P. paxilli hmg* (Figure 4.1), however, the

presence of all four consensus motifs in the correct spatial organisation, suggests that an unspliced intron is present in this position. Splicing of this intron was not detected and may be extremely rare or developmentally regulated. Alternatively spliced products may be present at earlier time points.

After the discovery of an intron in the 5' UTR of *hmg* from *P. paxilli*, the 5' UTR of *hmg* from *N. lolii* Lp19 was examined in closer detail. The site of transcription initiation had been estimated using RT-PCR, and was predicted to fall between primers hmg22 and H-2. Nested RT-PCR gave a cDNA product with H-2, but not with hmg22 (Dobson, 1997). A number of intron consensus sequences are present in this area, including a predicted intron that encompasses the site where hmg22 anneals (Figure 3.13). The 5' UTR sequence of Lp19 *hmg* has been extended out to -1180 bp beyond the translation initiation codon by Lisa McMillan, and a number of new primers were designed to this region. Using these new primers, a single large intron of 280 bp was detected by RT-PCR and confirmed by sequencing of a *N. lolii* Lp19 cDNA clone (Figure 3.12 and 3.13). Filamentous fungal introns are usually under 100 bp in length (Gurr *et al.*, 1987), although Ballance (1986) reports a range of 48-240 bp for filamentous fungi. As was seen in *P. paxilli*, there are multiple 5' and 3' intron splice sites in the 5' UTR of the *N. lolii* Lp19 *hmg* gene (Figure 4.1), including sites within the 280 bp intron (Figure 3.13). It is possible that the splice product isolated encompasses two introns and an intervening exon. A 3' splice site was identified 73 bp downstream from the confirmed 5' splice site, however there is no lariat branch site upstream of this site. This site may have been lost over time, and spliceosome machinery has moved to the next available lariat consensus and 3' splice junction at position -116 bp and -103 bp respectively. A second 5' splice site was also identified 87 bp upstream of the confirmed 3' splice site, provided further evidence that two separate introns may be present. While 5'UTR introns have been observed in *hmg* genes from higher eukaryotic systems, this is the first report of a 5' UTR intron in *hmg* genes from fungi.

4.1.3.3 5' UTR heterogeneity

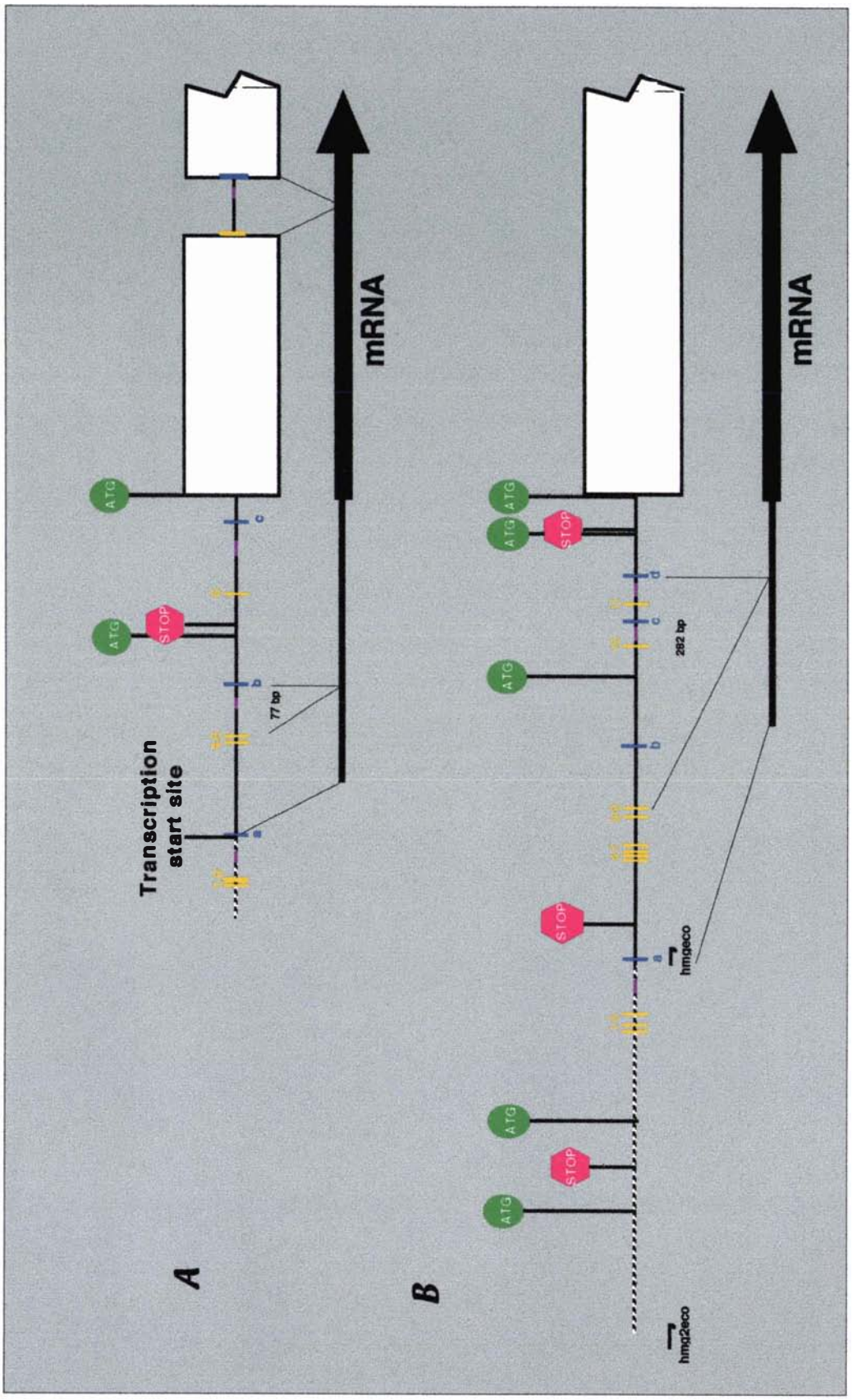
When present, 5' UTR introns are frequently involved in regulation. It is interesting that the 5' UTR introns in *P. paxilli* and *N. lolii* Lp19 *hmg* are the only splicing feature that

Figure 4.1 *Heterogeneity in the 5' UTR of filamentous fungal hmg genes*

In both figures, 5' intron splicing site (GT) are shown as yellow bars and are identified numerically. The 3' intron splice sites (AG) are represented as blue bars and are labelled alphabetically. Lariat consensus sequences (NACTAAPy) are shown as horizontal purple lines. The mRNA products isolated by 5' RACE or cDNA cloning are represented as a solid black line below the genomic sequence. Coding ORFs are shown as white blocks. Genomic sequence, which has not been isolated from a cDNA clone or RACE product, is shown as a broken line. Stop codons are only shown when they are in frame with an upstream ATG start codon.

A) *P. paxilli hmg* 5' UTR showing the 77 bp intron removed between GT site 5 and AG site b.

B) *N. lolii* Lp19 *hmg* 5' UTR showing the 282 bp intron removed between GT site 9 and AG site d.


A
B
Transcription start site
mRNA
mRNA
ATG
STOP
77 bp
282 bp
hmgeco
hmg2eco

is in common with *hmg* genes from higher eukaryotes, although the exact position of the intron is slightly different in the two filamentous fungi species. An optional untranslated exon in the 5' UTR of the HMG CoA synthase gene, is highly conserved between humans and Chinese hamsters, and even appears to be present in the HMG CoA reductase gene from Chinese hamster as well (Gil *et al.*, 1987). Even though the intervening intronic sequences are much longer in the higher eukaryotes, the 5'UTR exons in *P. paxilli* and *N. lolii* Lp19 *hmg* are similar in size to those found in the 5' UTR of Chinese hamster and human HMG CoA synthase. The heterogeneity present in the 5' UTR of the Chinese hamster *hmg* has been extensively characterised. Primer extension showed that 16 different transcripts are synthesised, using a combination of multiple transcription start sites and alternative splicing of the 5' UTR intron. Multiple sites of initiation of translation are also present (Reynolds *et al.*, 1985). Ratios of these multiple transcripts were affected by lovastatin (an inhibitor of HMGR), mevalonate and 25-hydroxycholesterol. Transcripts with shorter 5' UTR (40-100 nt) were isolated from a polysome cellular fraction, while transcripts with longer 5' UTR (300-400 nt) were found in the monosome fraction (Gayen & Peffley, 1995). One of the *Arabidopsis thaliana hmg* genes, HMG1, is also transcribed from two different promoters, one of which includes an in-frame alternative translation start site. Proteins translated from the upstream translation start site have 50 additional residues in the N-terminus, and have been isolated from microsomes (Lumbreras *et al.*, 1995). Therefore, 5' UTR heterogeneity could have a role in targeting HMGR to different subcellular compartments. There is a second ATG upstream of the major translational start site, but this ORF is quickly terminated by a stop codon. In human *hmg*, alternative splicing of the 5' UTR intron does not occur, although several different transcripts are produced, presumably from multiple transcription start sites (Luskey, 1987). Figure 4.1 highlights the potential for alternate splicing and translation initiation in *hmg* genes from *P. paxilli* and *N. lolii* Lp19, including some short ORFs, that are in frame with the major translational start site. Several mechanisms are already known to regulate the mevalonate pathway, including feedback repression of transcription by the end products of cholesterol biosynthesis (Goldstein & Brown, 1990) and phosphorylation of the HMG Co A reductase enzyme (Kennelly & Rodwell, 1985). In *Saccharomyces cerevisiae* and *A. thaliana*, two copies of the HMG CoA reductase gene are differentially expressed to fulfil regulatory requirements (Enjuto *et al.*, 1994; Hampton *et al.*, 1996a). Given the biological importance of the primary metabolic products

produced from mevalonate and the need for regulation of their levels, it is not surprising that multiple regulatory mechanisms exist. Whether the regulatory requirements of the secondary metabolic pathways that use mevalonate as a substrate, such as the paxilline biosynthetic pathway in *P. paxilli*, are met by these primary mechanisms is unclear at present. The complexity seen in the 5' UTR of *P. paxilli* and Lp19 *hmg* genes provides an interesting alternative regulatory mechanism, worthy of further investigation.

4.1.3.4 Putative promoter elements

In higher eukaryotes, the TATA box is frequently found 10-30 bp upstream from the site of transcription initiation (Stryer, 1995) and is essential for assembly of the transcriptional machinery. In filamentous fungi, the TATAAA motif or simply an AT-rich tract is frequently present (Gurr *et al.*, 1987), although the position and exact sequence of the motif is more variable in filamentous fungi (Ballance, 1986). In *P. paxilli hmg*, the motif 'TTAATT' is found 22 bp upstream from the proposed transcription start site and may fulfil the role of this core promoter element. A number of pyrimidine-rich tracts are present in the *P. paxilli hmg* 5' UTR. These motifs are frequently a feature of highly expressed genes (Gurr *et al.*, 1987), although the pyrimidine rich tracts in *hmg* do not directly precede the transcription start site determined by 5' RACE as is usually the case for filamentous fungi (Ballance, 1986). Tomato (*Lycopersicon esculentum*), like many plants, contains two copies of the *hmg* gene. Expression of HMG2 is correlated to the synthesis of carotenoid during ripening. Interestingly, while the TATA motif, upstream of the transcription start site, was not required for expression, a pyrimidine-rich tract in the 5' UTR was (Daraselia *et al.*, 1996). In Chinese hamster, the *hmg* gene also lacks a TATA box, although the gene for HMG CoA synthase does have a TATA box approximately 30 bp upstream from the major transcription start (Goldstein & Brown, 1990). The core promoter element CAAT is generally found 70-90 bp upstream of the transcription start site, however nothing resembling this sequence is found in the *P. paxilli hmg* promoter at this position. Several CAAT motifs exist between the proposed transcription and translation start sites.

Many sterol-regulated genes have the binding motifs for Nuclear Factor Y and Sterol-regulated element binding proteins SREBPs in their promoter. Ericsson *et al.* (1996b) showed that both NF-Y and SREB-3 were required for sterol regulation and the spacing between the two binding sites was critical for the correct protein-protein interaction. The sequence motif for NF-Y binding is an inverted CCAAT box (5'-ATTGG-3'), although mutation of a 5'-CCAAT-3' motif in the Human squalene synthase gene (Inv-Y-Box) resulted in diminished promoter activity (Guan *et al.*, 1998). This motif is located downstream of the transcription start site and is therefore distinct from the CAAT box discussed above. There are two 5'-CCAAT-3' boxes in the *P. paxilli hmg* gene at position -932 bp and -984 bp (Figure 3.7). Examples of the sterol responsive elements (SRE-1, -2 and -3), known to regulate the transcription of cholesterogenic genes are shown in Table 4.1. A putative SRE-1 motif (5'-CGCCCCAC-3') was identified in the 5' UTR of *P. paxilli hmg* at position -509 bp (Figure 3.7), 73 bp upstream from the confirmed transcription start site. Three other putative SRE-1 sites, each containing 2 mismatches (5' CACACCAA-3'), occur in a mini repeat sequence at position -642 bp. Six different sites with homology to SRE-2 were identified at positions, -566 bp, -522 bp, -446 bp, -194 bp, -188 bp and -57 bp respectively. The motif at position -194 bp is inverted and is listed with the other putative SRE-2 motifs in Table 4.1. Two putative SRE-3 sites were identified in the *P. paxilli hmg* 5' UTR, although one of these, at position -508 bp (Figure 3.7), contains 5 mismatches (5'-CTCTCCACCG-3'). However, a second inverted SRE-3 motif (5'-CTCCCACGAG-3'), at position -227 bp (Figure 3.7), has only a single mismatch. In the human squalene synthase gene, a functional, inverted SRE-3 element was also identified. Mutants generated with variations in this site showed decreased sterol activation (Guan *et al.*, 1998). Therefore, even amongst higher eukaryotes, there is some divergence between the sequence motifs involved in sterol regulation. Similar motifs in filamentous fungi may need to be identified experimentally.

Nitrogen repression often regulates genes involved in many aspects of fungal growth and development, including secondary metabolism and pathogenicity. Major nitrogen regulatory proteins from fungi include NRE from *P. chrysogenum* (Haas *et al.*, 1995a), NIT2 from *N. crassa* (Fu & Marzluf, 1987), AREA from *A. nidulans* (Caddix *et al.*, 1986), NRR1 from *Metarhizium anisopliae* (Screen *et al.*, 1998). AREA-GF from

Table 4.1 Sterol regulatory elements

Motif	Organism	Gene	Features	Sequence	Reference
SRE-1	Human	LDL receptor		5'-CACCCCAC-3'	(Osborne, 1991)
		Squalene Syn.		5'-CACCCCAC-3'	(Guan *et al.*, 1998)
		HMGS		5'-CACCCCAC-3'	(Osborne, 1991)
				5'-CACCCTAC-3'	(Osborne, 1991)
		HMGR	inverted	5'-CACCGCAC-3'	(Osborne, 1991)
	P. paxilli	HMGR	3 repeats	5'-CACACCAA-3'	(This study)
				5'-CGCCCCAC-3'	(This study)
HSS-SRE-1	Human	Squalene syn.		5'-ATCACGCCAG-3'	(Guan *et al.*, 1998)
SRE-2	Ch. hamster	HMGS		5'-CGTCCCGC-3'	(Goldstein & Brown, 1990)
	P. paxilli	HMGR		5'-CGTCTCTC-3'	(This study)
				5'-GGTCCCTC-3'	(This study)
				5'-GGACCCGC-3'	(This study)
			inverted	5'-GCTCCCGC-3'	(This study)
				5'-CGGCCCTC-3'	(This study)
				5'-CCTCCCCC-3'	(This study)
SRE-3	Rat	FPP synthase		5'-CTCACACGAG-3'	(Ericsson *et al.*, 1996a)
	Human	Squalene syn.	inverted	5'-CTCACACTAG-3'	(Guan *et al.*, 1998)
	P. paxilli	HMGR		5'-CTCTCCACCG-3'	(This study)
			inverted	5'-CTCCCACGAG-3'	(This study)
NF-Y	Human	Squalene syn.		5'-ATTGG-3'	(Guan *et al.*, 1998)
			inverted	5'-ATTGG-3'	(Guan *et al.*, 1998)
	Ch. hamster	HMGR		5'-ATTGG-3'	(Goldstein & Brown, 1990)
		HMGS		5'-ATTGG-3'	(Dooley *et al.*, 1998)
	P. paxilli	HMGR	inverted	5'-ATTGG-3'	(This study)
			inverted	5'-ATTGG-3	(This study)

G. fujikuroi (Tudzynski *et al.*, 1999) and NUT1 from *Magnaporthe grisea.* (Froeliger & Carpenter, 1996). These all belong to the GATA family of transcription factors which have a Cys_2/Cys_2 zinc finger motif, and bind the GATA motif in the promoters of nitrogen regulated genes (Wilson & Arst Jr., 1998). In *N. crassa*, nitrate utilisation genes have pairs of 5'-ATAG-3' or 5'-GATA-3' motifs separated by a variable number of nucleotides (Marzluf, 1993). There are two 5'-GATA-3' motifs in the *P. paxilli hmg* promoter, one 27 bp upstream of the transcription start site and a second 358 bp upstream. There are also two pairs of ATAG motifs, one pair at position -955 bp separated by 11 bp, and a second pair at position -875 bp separated by 3 bp (Figure 3.7).

Carbon catabolite repression (CCR) can also regulate expression of secondary metabolic genes. The negative-acting, regulatory protein CREA binds the consensus 5'-(G/C)YGGRG-3' in *A. nidulans* (Panozzo *et al.*, 1998). Two binding sites, matching this consensus, were detected in the *P. paxilli hmg* 5' UTR at positions -603 bp (5'-CTGGAG-3') and -440 bp (5'-GCGGAG-3'). This second sequence overlaps the transcription start determined by 5' RACE. Two CCAAT motifs, which bind the positive-acting Hap2/3/4/5 complex in yeast, appear in the *P. paxilli hmg* promoter 548 bp and 496 bp upstream of the transcription start site. Several CG triplets are present next to the transcription start site, but there is no consensus for the number of nucleotides that separate them. GC triplets are involved in binding the positive acting proteins Gal4 and Mal63, responsible for specific regulation of galactose and maltose utilisation genes in yeast (Gancedo, 1998).

The regulatory element PacC, has been shown to be involved in the pH dependent regulation of the isopenicillin N synthetase gene of *A. nidulans* (Espeso & Peñalva, 1996). PacC is a Cys_2/His_2 type zinc finger protein that binds the consensus 5'-GCCARG-3'. There are no binding sites for PacC in the *P. paxilli hmg* promoter, although two motifs were found approximately 60 bp downstream of the ATG start codon.

Two final features in the promoter region of *P. paxilli hmg*, are mini repeat sequences at position -972 bp and -642 bp. The first repeat has four copies of the sequence 5'-TACA-3', although the third repeat is imperfect. The second repeat has three copies of the sequence 5'-CACACCAA-3' and has some homology to the SRE-1 sequence

motif discussed above. There are no experimental data to confirm that any of these putative promoter elements are functional, except for the intron splicing sites that were confirmed by cDNA sequencing.

4.1.4 *P. paxilli hmg* 3' sequence analysis

Filamentous fungal genes are frequently transcribed beyond the signal sequence for polyadenylation (Gurr *et al.*, 1987) and this may certainly be the case for *P. paxilli hmg*. Using 3' RACE, the 3' UTR was shown to be 563 bp long and, in contrast to the 5' UTR and coding sequence, the 3' UTR has a GC content of only 49.7%. In higher eukaryotes, the sequence 5'-AATAAA-3' is the signal for polyadenylation in 90% of mRNA (Colgan & Manley, 1997). Adherence to this sequence varies in filamentous fungi and often the exact site for polyadenylation is difficult to locate. However, there is a tendency towards AT nucleotides in the 3' UTR (Ballance, 1986), that may serve the same function. In *P. paxilli hmg*, the sequence 'ATAAAA" is found 438 bp downstream from the translation stop codon. S1 nuclease mapping of the 3' UTR of Chinese hamster *hmg* showed three functional polyadenylation sites, although 5 signal sequences for polyadenylation were present (Reynolds *et al.*, 1984). A microsatellite, present in the 3' UTR of *N. lolii* Lp19 *hmg,* was not seen in the *P. paxilli hmg* gene.

4.1.5 *P. paxilli hmg* polypeptide sequence

Translation is initiated at the ATG codon in the majority of eukaryotic genes. Sequence surrounding the preferred translation start sites was determined by Kozak (1984) and shown to be, 5'-CC(A/G)CC**ATG**GC-3'. The consensus sequence proposed for filamentous fungal genes was 5'-TCA(C/A)(A/C)**ATG**(G/T)C-3' (Ballance, 1986). Translation of *P. paxilli hmg* is assumed to initiate at an ATG surrounded by the sequence 5'-TCACC**ATG**GC-3' which supports the filamentous fungal consensus sequence. Translation is assumed to terminate at a TAA codon, which is the established trend in filamentous fungal genes (Gurr *et al.*, 1987).

The peptide sequence of *P. paxilli hmg* is most similar to *hmg* genes from *G. fujikuroi* (74% similarity), *N. lolii* Lp19 (74%), *A. terreus* (69% similarity) and *S. pombe* (64% similarity). An alignment of HMGR protein sequence from a number of species was performed with the PILEUP programme of the GCG package, using a gap weight penalty of 2 and a gap length penalty of 1 (Figure 3.8). The N-terminal domain is most similar to *N. lolii* Lp19 *hmg*. The published sequence for *G. fujikuroi hmg* has a truncated transmembrane domain (Woitek *et al.*, 1997), although evidence presented by Dobson (1997) predicts that the start of this gene is further upstream than that proposed by Woitek *et al.* (1997). Upstream of the *G. fujikuroi* translation codon, an ORF was identified with similarity to the N-terminal domain of both *N. lolii* Lp19 and *P. paxilli hmg*.

A model for the structure of the HMGR protein has been discussed by Liscum *et al.* (1985). Proteolysis experiments on the Chinese hamster HMGR protein (Chin *et al.*, 1984) showed a membrane bound N-terminal domain and a cytosolic, catalytically active C-terminal domain, with a less conserved linker domain separating the two. The structure of the N-terminal domain is less conserved amongst disparate species. In plants, two or more genes are present, each contains two membrane spanning domains in the N-terminus (Campos & Boronat, 1995; Denbow *et al.*, 1996; Enjuto *et al.*, 1994). In *S. cerevisiae*, two genes are present, but the N-terminal domains contain 7 membrane-spanning domains (Basson *et al.*, 1986). In mammals, single genes are present, that contain 7 or 8 transmembrane domains (Chin *et al.*, 1984; Hampton *et al.*, 1996a; Liscum *et al.*, 1985; Roitelman *et al.*, 1992). Thus far, in filamentous fungi, the number of *hmg* genes and organisation in the N-terminal domain most closely resembles mammals. A single *hmg* gene is present in *N. lolii* Lp19 (Dobson, 1997), *G. fujikuroi*, *Sphaceloma manihoticola* (Woitek *et al.*, 1997) and *P. paxilli*. However, Southern analysis of two zygomycetes, *Absidia glauca* and *Blakeslea trispora*, predicts two copies of *hmg* (Burmester & Czempinski, 1994). Hydropathy analysis predicts 7 transmembrane domains in the HMGR of *N. lolii* Lp19 and *G. fujikuroi*. Using the TMPRED programme (Hofmann & Stoffel, 1993) at the website of the ISREC Bioinformatics group, the predicted N-terminal domain of *P. paxilli hmg* was analysed using the method of Kyte & Doolittle (1982) and predicted to contain 7 transmembrane domains in the N-terminal portion of the protein.

The C-terminal catalytic domain has been subjected to mutational analysis, and the catalytically active residues defined. Changes to the histidine residue in position 381 of the HMGR from *Pseudomonas mevalonii*, resulted in an inactive form of the enzyme. The consensus sequence surrounding this residue is 'Leu - Val - Lys - Ser - **His** - Met - Xaa - Xaa - Asn - Arg - Ser' (Damay *et al.*, 1992). The underlined serine is proposed to be involved in regulatory phosphorylation of the HMGR enzyme discussed later in this section. In *P. paxilli* HMGR, the sequence motif 'Leu - Val - Lys - Ala - **His** - Met - Ala - His - Asn - Arg - Ser', highlighted in Figure 3.7, has the catalytically active His residue at position 1099. In *P. paxilli* HMGR, a conserved Asp residue was identified in position 1003, 97 residues upstream of the conserved His^{1099}. The spacing between the conserved histidine and aspartic acid residues in Syrian hamster HMGR is 99 residues. The crystal structure of *P. mevalonii* HMGR was determined by Lawrence *et al.* (1995) and substrate binding sites in the catalytic domain were identified. They are summarised below in Table 4.2, which is derived from Table 2 in the paper by Lawrence *et al.* (1995) and includes the analogous sites in *P. paxilli* HMGR. The E83 loop contains the catalytically active glutamate residue.

Table 4.2 Catalytically active sites in HMGR

Sequence	*Putative Function*	*P. paxilli HMGR Sequence*
E- X_3 -G- X_4 - P	HMG-CoA binding	E-N-V-I-G-Y-L-P-L-P
E83 loop	Catalysis and HMG-CoA binding	E-G-V-L-V-A
D-A-M-G-X-N	NAD(H) binding	D-A-M-G-M-N
G-X_2 -G-G-X-T	NAD(H) binding	G-T-I-G-G-G-T

Regulation of HMGR activity is also controlled by AMP-activated protein kinases, that phosphorylate a conserved serine residue, six residues downstream of the conserved histidine residue (Darnay *et al.*, 1992). In *P. paxilli*, a serine residue is present six amino acids downstream of the conserved His^{1099} residue, as discussed above.

4.2 Isolation and Characterisation of *P. paxilli tub-2*

4.2.1 *P. paxilli tub-2*

The gene for β-tubulin is highly conserved, and together with α-tubulin, the gene products form the microtubules essential for chromosome separation during mitosis (Stryer, 1995). Expression of *tub-2* is an excellent control for studying gene expression in most organisms. A unique *tub-2* gene was isolated from *P. paxilli* and the pattern of gene expression used as a control for the experiments discussed in section 4.4. Due to the high sequence conservation, this gene was isolated from a *P. paxilli* genomic library by heterologous probing with the *N. crassa tub-2* gene. The *N. crassa* probe used for Southern analysis (Figure 3.18), confirmed a single copy of *tub-2* is present in the genome. A single copy of *tub-2* is found in *N. crassa* (Orbach *et al.*, 1986), *Epichloë typhina* (Byrd *et al.*, 1990) and the homobasidiomycete *Schizophyllum commune*, although there is some evidence for a highly divergent second copy in this species (Russo *et al.*, 1992). *A. nidulans* contains two β-tubulin genes (*benA* and *tubC*) which show significant sequence divergence (May *et al.*, 1987). CHEF analysis showed that the *tub-2* gene is located on chromosome IV in *P. paxilli* (Figure 3.24).

4.2.2 *P. paxilli tub-2* nucleotide sequence

The coding sequence of *tub-2* is separated by 6 introns, five of which are clustered in the 5' end of the gene. The sizes of the six introns are 107 bp, 120 bp, 68 bp, 66 bp, 71 bp, and 62 bp respectively. This is within the range (48-240 bp) reported by Ballance (1986). As discussed in section 4.1.2, the GT/AG rule for intron splicing is conserved, with the sequence 'ANGT' following the GT 5' splice junction. The PyAG consensus is conserved at all 3' splice junctions. Introns 3-6 were confirmed by sequencing cDNA clones, and the splice junctions for introns 1 and 2 were predicted by sequence similarity to other *tub-2* genes. The number of introns in *tub-2* genes varies amongst filamentous fungal species. In *A. nidulans*, *benA* has 8 introns and *tubC* 5 introns (May *et al.*, 1987) *E. typhina tub2* contains 4 introns (Byrd *et al.*, 1990), the

N. crassa β-tubulin gene contains 6 introns (Orbach *et al.*, 1986) and *S. commune tub-2* has 8 introns (Russo *et al.*, 1992).

Codon bias in *P. paxilli tub-2* is shown in Table 3.6, and again follows the trends for filamentous fungal genes (Ballance, 1986; Gurr *et al.*, 1987). A cytosine nucleotide was seen in the third position in 51% of all codons and adenosine nucleotides were seen in the third position in only 1% of all codons. The coding sequence of the *tub-2,* from translation initiation to termination, including introns, has a GC content of 55.3%.

4.2.3 *P. paxilli tub-2* polypeptide sequence

The *tub-2* sequence isolated from *P. paxilli* predicts a 447 amino acid protein comprised of 7 ORF. The protein sequence is most similar to β-tubulin genes from *P. digitatum, A. flavus* and *A. parasiticus* (all 93% similarity) and *Emericella nidulans* (92% similarity).

4.3 Physiology of *P. paxilli* in liquid culture

4.3.1 Development of cultures with homogeneous morphology and reproducible induction of paxilline

There are numerous techniques for inoculating filamentous fungi into liquid culture, including spore suspensions, seed cultures, and macerated mycelia fragments. Two of these techniques (spore suspension and seed culture) were compared for their ability to induce i) a homogeneous morphology and ii) paxilline biosynthesis in a reproducible manner. The growth rate was determined by dry weight, which reflects biomass accumulation rather than cellular division. Paxilline content was determined by HPLC (section 2.16) and reflects paxilline production per unit of biomass. Accumulation of biomass and paxilline showed considerable variation in cultures inoculated from a spore suspension (Figure 3.25 A). Distinguishing experimental differences from the inherent variability in experiments performed with this inoculation protocol would have proved difficult.

A number of papers describe the use of a seed cultures to inoculate replica flasks (Ibba *et al.*, 1987; Kaiser *et al.*, 1994; Tkacz *et al.*, 1993) so several variations of this method were tried with *P. paxilli.* Seed culture volumes of 25 mL to 100 mL were examined after 24 h growth and all cultures were found to have produced the large pellet morphology, even when grown at 350 rpm. When a sample of each seed culture was subsequently transferred to a larger culture volume, both the size and number of pellets remained constant. Growth experiments performed with mycelia grown in the pellet morphology were not desired for two reasons. Firstly, sample volumes removed from *P. paxilli* cultures with the pellet morphology are not representative of the entire culture. This is because large pellets sediment rapidly once a culture is removed from the shaker. The loose homogeneous morphology was also preferred during labelling experiments to determine the origin of the paxilline indole moiety in *P. paxilli* (Laws & Mantle, 1989). Loose mycelia fragments do not sediment and samples taken from these cultures are representative of the entire culture. Secondly, individual hyphae in a culture grown with the loose mycelia morphology have equal access to nutrients and oxygen and are therefore in a uniform physiological state. It was important to develop an inoculation protocol that decreased the inherent variability seen in Figure 3.25 A, as

well as maintaining the preferred loose mycelial morphology. A paper by Tkazc *et al.* 1993 recommended the use of oat flour in the seed culture to inhibit the formation of pellets. However pellets were still present when this method was tested on *P. paxilli.* The main problem appeared to be that once pellets had formed, they remained, irrespective of culture volume or shake speed. To solve this problem, the seed culture was sub-cultured before the gross morphology became fixed. After 12 h, viable spores have germinated, and germination pegs are clearly visible under a light microscope (Figure 3.29, photograph A and B). No pellets are visible, although clumps of 20 or more spores are frequently seen at this time and may represent the start of a pellet. The large 300 mL cultures inoculated from a 12 h seed culture showed an almost ideal morphology. The majority of the culture grew as loose mycelia fragments with fine micropellets dispersed throughout. The nutrient and oxygen gradients that exist in larger pellets are less marked in smaller pellets. The biomass accumulation and paxilline content of cultures inoculated from a 12 h seed culture is shown in Figure 3.25 B. Using this protocol, the growth rates and period of paxilline induction are reproducible (Figure 3.27, Figure 3.28).

4.3.2 Wild-type *P. paxilli* in paxilline-inducing media

Production of secondary metabolites in filamentous fungal liquid cultures has been discussed in a number of papers. In particular, the induction of alkaloid biosynthesis in liquid culture can be extremely difficult to achieve. In *Penicillium nigricans* liquid cultures, biosynthesis of the tremorgenic mycotoxin penitrem, was stimulated by calcium induced sporulation (Mantle *et al.*, 1984), while production of lolitrem B in *N. lolii* liquid cultures has not been achieved (Weedon & Mantle, 1987). Conditions for the production of paxilline by *P. paxilli* in liquid culture were determined by Ibba *et al.* (1987). The aim of this thesis was to determine the rate of growth and paxilline production in liquid cultures of wild-type *P. paxilli.* These growth patterns, once determined, would serve as controls for experiments where the growth conditions were varied or when *P. paxilli* mutants were analysed. Two separate growth experiments were performed using the seed culture inoculum protocol. The first experiment identified time points that required more detailed examination, hence the inclusion of additional time points in Experiment 2. The second experiment examined many

parameters of culture physiology, including culture morphology, and paxilline content of the culture supernatant.

4.3.2.1 Biomass accumulation

The majority of biomass accumulation occurs during the primary growth or trophophase. Growth at this stage is essentially exponential (Prosser & Tough, 1991). However, additional biomass, in the form of storage products can still accumulate after the cessation of exponential cellular division. In some cases, the biomass is maintained at a steady level through balanced autolysis and regeneration (Prosser & Tough, 1991). In *P. paxilli*, the primary growth phase and accumulation of biomass ceased between 48 and 60 h after seed culture inoculation (Figure 3.27 A and 3.28 A), with an average biomass accumulation of 20 g/L. During the late secondary phase, the biomass begins to drop dramatically rather than remaining at a constant level. The rate of autolysis is presumably greater than the rate of regenerative growth at this time. Possibly, the biomass is being broken down to provide carbon for the production of paxilline. A dramatic decrease in biomass and viscosity was observed in *P. paxilli* cultures after 144 h of growth. This final phase is known as the death phase (Prosser & Tough, 1991). It is at this time that paxilline was first detected in the culture broth, presumably as a result of increased autolysis during the death phase. Microscopy showed that increased autolysis was occurring after 100 h (Figure 3.29, photographs M and N), but only trace amounts of paxilline were detected between 84 h and 120 h. The amount of autolysis must increase drastically after 120 h to account for the presence of broth paxilline and the decreased culture viscosity.

4.3.2.2 Culture pH

P. paxilli liquid cultures exhibited two distinct changes in pH, which were correlated with distinct phases of growth (Figure 3.27 B and 3.28 B). During the initial exponential growth phase, culture pH increases in parallel with biomass accumulation. The pH then begins to slowly decline, until 96 h, when a dramatic decrease was

recorded in Experiment 2. Whether the pH drop is a cause or consequence of the transition from primary growth phase to secondary growth phase is unclear. An initial experiment to investigate this question is discussed in section 4.3.5.

4.3.2.3 Paxilline

Paxilline isolated from mycelia was detected after 72 h, and continued to accumulate for the duration of the experiment. Paxilline was not detected in the broth until 144 h, although an extremely small peak was observed from 84 h onwards, that corresponded with the elution time of paxilline. The size of this peak did not change until 144 h, when a dramatic increase was observed; indicating that paxilline is not actively secreted into the media. The rapid release of paxilline could result from massive cellular lysis after the culture enters the death phase. Interestingly, a steady decrease in biomass was recorded from 60 h onwards, with no equivalent increase in broth paxilline. Paxilline contained within mycelia undergoing autolysis at earlier time points, does not appear to be released into the culture supernatant until large scale autolysis at 144 h.

4.3.2.4 Microscopy

Microscopic analysis of *P. paxilli* liquid cultures revealed information relevant to the biosynthesis of paxilline. Firstly, sporulation does occur in the paxilline-inducing media CDYE. However, the appearance of spore-bearing structures is less apparent after 76 h, when paxilline is first detected in mycelia. Spore production in the paxilline-inducing media was also significantly lower than that recorded in culture media containing high concentrations of the sporulation-inducing supplement, calcium. Secondly, despite the decrease in overall biomass, the majority of hyphae showed no sign of vacuolation or autolysis, until 100 h. Vacuoles were observed in hyphae after 76 h, although limited vacuolation and autolysis might have gone undetected in earlier samples. Vacuolation is a feature of hyphal age, and therefore increases over time (Cui *et al.*, 1998). The decrease in biomass could reflect the increasing size of vacuoles and decreasing amount of cytoplasm and storage products in individual hyphae.

4.3.3 Effect of sporulation on paxilline production

In contrast to many filamentous fungi (Guzmán-de-Peña & Ruiz-Herrera, 1996; Mantle *et al.*, 1984), *P. paxilli* production of paxilline is not correlated with sporulation, as documented in a paper by Ibba *et al.* (1987). Microscopic analysis of cultures showed that minimal sporulation does occur in the paxilline-inducing media, however in comparison to the spore-inducing media, this is significantly reduced. The effect of increased sporulation on paxilline production is even more pronounced. Paxilline was not detected in cultures supplemented with 2% $CaCl_2$ (Table 3.8, Figure 3.30) and only trace amounts of paxilline were detected in mycelia grown in PD media for 120 h. There is also a significant difference in biomass accumulation between CDYE based media and PD media. Biomass accumulation was not affected during calcium induced sporulation in CDYE media, in comparison to the approximately 50% decrease that was observed in PD cultures (Table 3.8). This result confirms that the secondary production of paxilline is a distinctly separate process from sporulation. In cases where sporulation and secondary metabolism are temporally related, similarities are frequently seen in the general structure of spore pigments and the particular secondary metabolite. In *Aspergillus,* spore pigments are similar in structure to the polyketide-based metabolite aflatoxin. Non-sporulating mutants of *A. parasiticus* were unable to make aflatoxins (Guzmán-de-Peña & Ruiz-Herrera, 1996). In another *Penicillium* species, two spore pigments belonging to the melanin family were identified; a green chromoprotein and an insoluble black pigment (Ha-Huy-Ke & Luckner, 1979). Paxilline, an indole-diterpenoid, is not structurally related to the *Penicillium* spore pigments, and there is no apparent link between sporulation and secondary metabolite production in this case. Mutants of *P. paxilli*, that produced brown spores, were also shown to synthesis wild-type levels of paxilline (Itoh & Scott, 1994b). The opposite scenario is observed in *P. nigricans*, where synthesis of penitrem, also an indole-diterpenoid, was shown to be sporulation-dependent (Mantle *et al.*, 1984).

4.3.4 Effect of yeast extract supplement on paxilline production

The indole moiety that is part of the basic paxilline structure is proposed to be derived from tryptophan (Laws & Mantle, 1989). In *C. purpurea*, the preferred source of

nitrogen during production of clavine-alkaloids was asparagine. In response to asparagine, both alkaloid production and concentrations of intracellular tryptophan were increased (Rehacek *et al.*, 1977). *P. paxilli* cultures, supplemented with yeast extract (YE), have access to a large pool of amino acids, which may enhance paxilline production. Cultures grown in CD only, or supplemented with tryptophan, exhibited diminished paxilline production (Figure 3.30, lane 4-5). However, the growth of *P. paxilli* in these media was also severely reduced (Table 3.8), such that the entire culture volume was needed for paxilline analysis. Therefore, the paxilline detected in Figure 3.30, lanes 4 and 5, represents 10 times the culture volume analysed in lane 2 and is still considerably less than the paxilline detected in the control culture (lane 2-3). The biomass accumulation in the non-paxilline-inducing media PD is also reduced, although a small amount of paxilline was detected. It appears that the addition of YE affects biomass accumulation more than the paxilline production. Presumably the lowered paxilline content is due to the decreased biomass of these cultures.

4.3.5 Effect of pH on paxilline production

Changes in ambient pH affect secondary metabolism in a number of fungal species. PacC, a pH-dependent transcription factor, binds the promoters of penicillin biosynthetic genes in *P. chrysogenum* (Suarez & Peñalva, 1996), and *A. nidulans* (Espeso & Peñalva, 1996; Then Bergh & Brakhage, 1998). This pH regulation was shown to be independent of mechanisms that control glucose repression of penicillin biosynthesis in *P. chrysogenum* (Gutierrez *et al.*, 1999). Therefore, regulation of switching from primary to secondary metabolism can involve multiple environmental triggers. One of the genes (*paxA*) isolated from the paxilline biosynthetic gene cluster, showed significant homology to a pH responsive gene. There is also a significant decrease in pH, during cessation of biomass accumulation (Figure 3.27 B and 3.28 B). These two factors prompted an additional experiment to examine the effect of a biological buffer on the production of paxilline. The decreases in pH, observed in a control CDYE culture and a culture supplemented with MOPS, were significantly different between 60 h and 120 h (Table 3.8) with a greater decrease being observed in CDYE. Increased biomass accumulation was also observed in the culture supplemented with MOPS, however, paxilline accumulation was decreased. Paxilline was not

detected after 60 h, and after 120 h, the level of paxilline was comparable to that seen in the control culture at 60 h (Figure 3.30, lanes 2-3 and 10-11). Although only two time points were analysed, preliminary results indicate that addition of a biological buffer to liquid cultures of *P. paxilli* has two effects. Firstly, biomass accumulation is increased, and secondly, paxilline production is decreased. A similar result would be seen, if the switch from primary growth phase to secondary growth phase were delayed. Changes to ambient pH may act as an environmental trigger for phase switching in *P. paxilli.*

4.3.6 Effect of carbon source on paxilline production

Many species are specialised to utilise a preferred carbon source. Production of metabolites can be affected by the availability of preferred carbon. Production of the antibiotic pnuemocandin A_0 in submerged cultures of *Zalerion arboricola*, was improved when mannitol was used as the major carbon source (Tkacz *et al.*, 1993). The presence of glucose or carbon sources, that give rise to carbon catabolite repression (CCR), can have an inhibitory effect on production of secondary metabolites. CCR, which arises when a preferred carbon source is available, is known to affect penicillin biosynthesis in *A. nidulans* (Espeso & Peñalva, 1992). The standard paxilline-inducing media contains 3% sucrose and it was postulated that alternative carbon sources might affect the rate of paxilline production. Therefore, *P. paxilli* was grown in media containing 3% sucrose, glucose, mannitol or glycerol. The growth rate and biomass accumulation in all carbon sources were not significantly different (Table 3.9). However, paxilline content after 120 h, was significantly higher in 3% glucose, compared to the other carbon sources (Figure 3.31). Interestingly, the paxilline content of the glucose culture was lower than the control at 60 h. This may result from a distinct separation of primary growth phase and secondary growth phase in the presence of a preferred carbon source (Liao *et al.*, 1995). The presence of glucose could exert CCR on genes involved in paxilline biosynthesis. In the presence of non-repressing carbon sources, paxilline biosynthesis would be initiated earlier. A number of additional indole-diterpene moieties, staining green with alcoholic Erhlich's reagent, were seen to increase in cultures grown in glucose. Bands with a greater R_f than paxilline are probably intermediate products, as two of these bands were identified as paspaline and 13-desoxypaxilline (Lisa McMillan pers. Comm.). TLC separates

molecules based on their polarity, therefore products with fewer oxygenated side-groups travel further. Two further indole-diterpene bands are seen below paxilline, indicating they have greater polarity than paxilline. There is evidence for a grid-like biosynthetic pathway during synthesis of paxilline (Munday-Finch *et al.*, 1996a). Therefore, paxilline is not necessarily the final product of biosynthesis and further oxidation steps may occur. Oxidation can also occur during preparation and storage of samples, although steps are taken to limit exposure. Glucose was the optimal carbon source for paxilline production in *P. paxilli*, although during the initial growth stage, paxilline biosynthesis may be affected by CCR.

4.3.7 *Effect of carbon and nitrogen starvation on paxilline production*

Secondary metabolism in many fungal species is triggered by the depletion of preferred carbon and nitrogen source from the media. By prematurely depleting carbon or nitrogen from the media, it was postulated that secondary metabolism and production of paxilline might be initiated sooner. The culture transferred to a media depleted of carbon had a severely reduced rate of growth and no detectable paxilline (Figure 3.32, lanes 3-4 and Table 3.9). A normal level of biomass accumulation and paxilline production was observed in the culture transferred to media depleted of nitrogen. Although carbon (as 3% sucrose) and nitrogen (as 0.2% $NaNO_3$) were removed, YE was still present in the media. Yeast extract appears to compensate for the lack of nitrogen, but not for the lack of carbon.

4.4 Expression of Paxilline Biosynthetic Genes

4.4.1 Development of a gene expression assay

A qualitative technique was devised to detect the steady state level of mRNA from a single gene of interest. The cDNA was reverse transcribed using random hexamer primers, which creates a pool of cDNA, representative of mRNA in the original sample. The technique is extremely sensitive and a single reverse transcription reaction generates sufficient cDNA template for 40 subsequent PCR reactions. In this way, experimental variation due to efficiency of reverse transcription was eliminated. The synthesis of cDNA was standardised by PCR amplification with *tub-2* gene specific primers and PCR conditions for individual genes were optimised. Standard protocols used 30 cycles of amplification, however, genes expressed in low abundance needed up to 40 cycles of amplification before they could be visualised by ethidium bromide staining. PCR saturation can be reached when excess template is present and this results in ethidium bromide staining of a uniform intensity. Each gene specific PCR was performed on serial dilutions of the cDNA template. At the lower dilutions, saturation was avoided and differences could be seen. This also served as an internal control for PCR, with the same expression patterns being recorded from multiple reactions.

4.4.2 Expression of paxilline biosynthetic genes

The paxilline biosynthetic gene cluster is located in a 50 kb region of chromosome Va (Young *et al.*, 1998). Open reading frames from this locus, whose translation products were predicted to function in paxilline biosynthesis, were labelled *pax* genes. Genes, whose predicted function could not be associated with paxilline biosynthesis, were used to define the cluster boundaries. To date, all genes within the cluster have a predicted function that could be associated with the requirements of paxilline biosynthesis, except where no significant homology to any other proteins has yet been identified. Genes with unrelated functions have not appeared within the cluster to date (Young *et al.*, 2000).

One question I hoped to answer through analysis of gene expression, was whether the *pax* genes would show a coregulated pattern of transcription, similar to the pattern seen in the aflatoxin/sterigmatocystin biosynthetic gene clusters found in *Aspergillus* spp. (Brown *et al.*, 1996a). Many secondary metabolite gene clusters have coregulated expression. The paper by Martin & Liras (1989) discusses a number of mechanisms by which this can be achieved. Biosynthesis of secondary metabolites usually occurs during the secondary or stationary growth phase, when nutrients become scarce, but primary metabolites and storage compounds are abundant. Coregulation of gene expression would avoid the synthesis of excess proteins during the secondary growth phase. In the case of the paxilline biosynthetic cluster, the observed patterns of expression, only partially fit this expected model. Coregulation of some genes is apparent and this pattern of expression has been called the Pax pattern. Expression of these genes is first detected after 36 h, some 36 h before paxilline is detectable in the culture, and when biomass is generally still accumulating. Genes that exhibit this expression pattern included *paxG*, *paxP1*, *paxP2* and *paxD*. Gene replacement 'knockout' mutants of *paxG* (Young *et al.*, 2000), *paxP1* (Lisa McMillan pers. comm.) and *paxP2* (Carr, 1999) have a Pax^- phenotype, indicating that these genes are required for the synthesis of paxilline. During the creation of a *paxG* gene replacement mutant, a deletion mutant was created that left *paxG* intact, but deleted *paxM1*, *paxU1* and part of *paxP1*. This mutant, called LM-G130, is also Pax^- (Young *et al.*, 2000). A gene replacement knockout mutant of *paxD* constructed by Carolyn Young, was shown to have a Pax^+ phenotype, indicating it is not essential for paxilline biosynthesis (Carolyn Young Pers. Comm.). However, in wild type *P. paxilli*, *paxD* exhibited the pax pattern of expression. Two genes with variations on the Pax pattern of expression are *paxM1* and *paxU1*. Transcripts from both these genes are present from 24 h onwards, however, a significant increase in expression is seen after 36 h. Evidence from a gene replacement mutant constructed by Lisa McMillan, has shown *paxM1* to be essential for paxilline biosynthesis (Young *et al.*, 2000). A function for *paxU1* has yet to be defined, although there is some homology with prenyl transferases. Two other genes that have shown the Pax pattern of expression are *lip2* and *ara*, located outside the putative boundaries of the paxilline biosynthetic gene cluster. These genes are not predicted to function in paxilline biosynthesis, yet they exhibit the same pattern of expression as genes shown to be essential for paxilline biosynthesis. It is possible that the increase in their expression is due to changes in chromosome conformation that allows the access

of transcriptional machinery to the *pax* genes. However, if this were the case, all the *pax* genes, would be expected to have the Pax pattern of expression. *PaxA*, is located close to the left boundary of the paxilline biosynthetic gene cluster, yet expression of this gene does not change over time. A putative dehydrogenase, *paxH*, also shows no change in expression over time. However, until gene replacement mutants of these genes have been analysed, their role in paxilline biosynthesis can not be confirmed on the basis of gene expression alone.

4.4.3 Expression of a putative pathway regulator

Regulation of secondary metabolite pathways seems to occur at two levels. Global regulators, such as those involved in nitrogen and carbon repression, and ambient pH, are known to interact with the promoters of secondary biosynthetic genes. Secondary metabolite pathways can also be regulated in a pathway-specific manner, usually by a product encoded within the cluster itself (Keller & Hohn, 1997). The best example of this, is the regulation of aflatoxin and sterigmatocystin biosynthesis by *aflR* (Woloshuk *et al.*, 1994). Induced expression of *A. flavus aflR*, has been shown to initiate transcription of sterigmatocystin biosynthetic genes in *A. nidulans* (Yu *et al.*, 1996). In *Streptomyces griseus*, expression of streptomycin biosynthetic genes is controlled by *strR*, which is in turn controlled by A-factor (Ohnishi *et al.*, 1999). The trichothecene biosynthetic gene cluster from *Fusarium sporotrichioides* (Hohn *et al.*, 1993a) is regulated by a zinc finger protein encoded by *Tri6*. Mutants lacking *Tri6*, were unable to synthesise trichothecenes, despite being supplemented with a number of pathway intermediates (Procter *et al.*, 1995). Two genes, *paxR1* and *paxR2*, isolated from within the paxilline biosynthetic gene cluster contain regions with homology to $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motifs. This motif has been found in numerous secondary metabolism pathway regulators (Baumgartner *et al.*, 1999; Cazelle *et al.*, 1998; Panozzo *et al.*, 1998; Todd *et al.*, 1997; Woloshuk *et al.*, 1994). Gene replacement knockout mutants of *paxR1* created by Lisa McMillan were able to synthesise paxilline (Lisa McMillan Pers. Comm.). Similar analysis of *paxR2*, only recently isolated, is in progress. Expression of *paxR1* does not follow the Pax pattern of expression, but peaks at 48 h, then slowly declines. Transcription of a pathway transcription factor would be expected to increase before the subsequent increase in

transcription of enzymatic genes was seen. Alternatively, the gene product of *paxR1* may function as a pathway specific repressor, inhibiting expression of pathway genes at earlier time points, although expression of *paxR1* is low at 24 h.

4.4.4 Expression of primary metabolic genes

HMGR provides mevalonate, the essential precursor of many essential primary products and of paxilline biosynthesis. Analysis of the *P. paxilli* genome confirmed that a single copy of *hmg* is present. The gene expression pattern of *hmg* reflects this dual role. Transcription of *hmg* is high at 24 h, but then drops at 30 h and increases steadily after 36 h. One of the primary products synthesised from mevalonate is ergosterol, an important component of fungal cell walls. Synthesis of ergosterol would initially be high during the primary growth phase, when biomass accumulation was occurring. Other primary functions, such as the synthesis of prenyl groups, which are attached to some proteins, would require an ongoing source of mevalonate. Regulation of HMGR occurs at many levels, but chiefly by the feed-back inhibition of transcription by sterols and other end-products (Goldstein & Brown, 1990; Hampton *et al.*, 1996a). When paxilline is being synthesised, the majority of the available mevalonate may be channelled towards secondary metabolism. The end-products of primary mevalonate metabolism would not build up to levels capable of inhibiting *hmg* transcription. Therefore, transcription may be increased to compensate for the demand for mevalonate. Alternatively, a regulator, specific for the paxilline biosynthetic pathway, may increase transcription of *hmg* independent of the primary feed-back inhibition mechanism. This could involve the alternative splicing of the *hmg* 5' UTR, and targeting of HMGR to organelles specific for paxilline biosynthesis.

Expression of another primary metabolic gene *ggs-1* was also examined. This gene encodes a second geranylgeranyl pyrophosphate synthase that is proposed to fulfil the primary metabolism requirements for GGPP. GGPPS-1 functions downstream of HMGR in the isoprenoid biosynthetic pathway, of which mevalonate is the primary substrate. Expression of *ggs-1* is highest at 24 h, and then decreases slightly to an almost steady state. It would be interesting to see if expression of *hmg,* in non-paxilline-inducing media, resembled this pattern.

The aim of this analysis was to establish the normal patterns of expression for genes involved in the biosynthesis of paxilline. In the future, experiments involving specific *pax* gene replacement mutants or media supplements could use gene expression patterns as a key indicator of changes in pathway regulation. In particular, the expression of *hmg*, a gene spatially isolated from the paxilline biosynthetic gene cluster could provide information about the way paxilline biosynthesis is regulated.

Appendix 1.0 Vector maps

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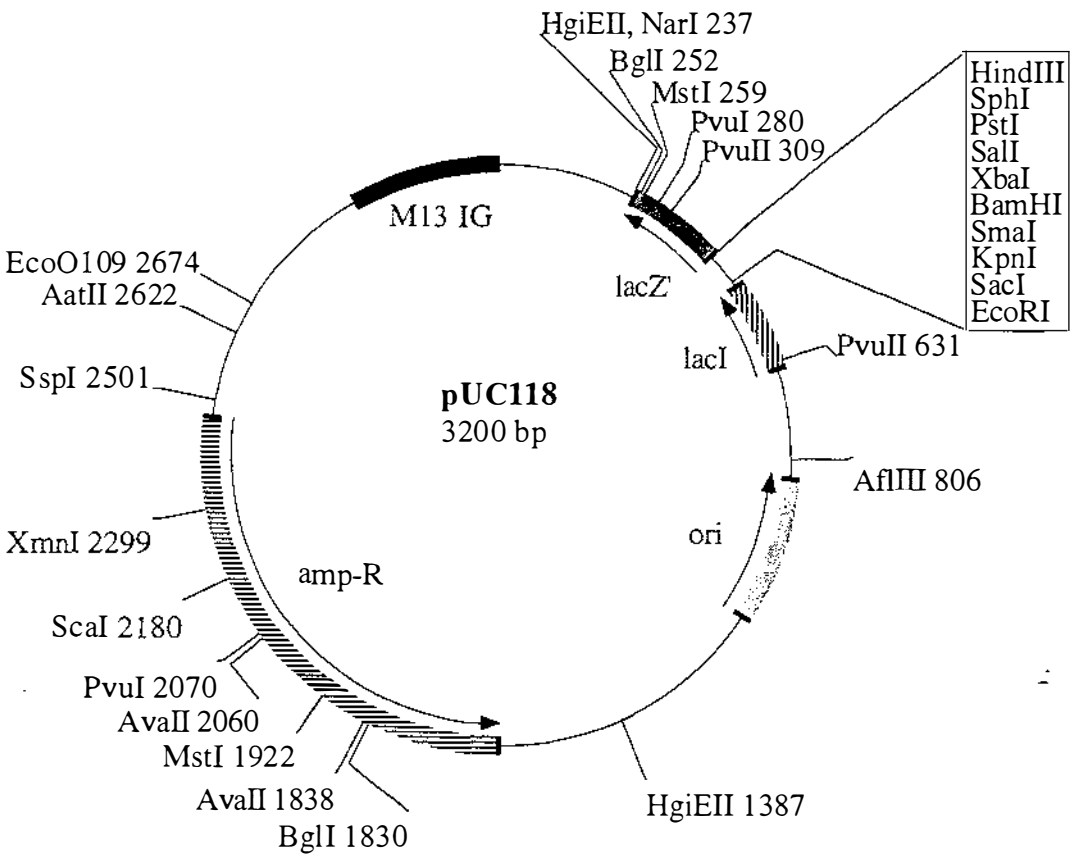

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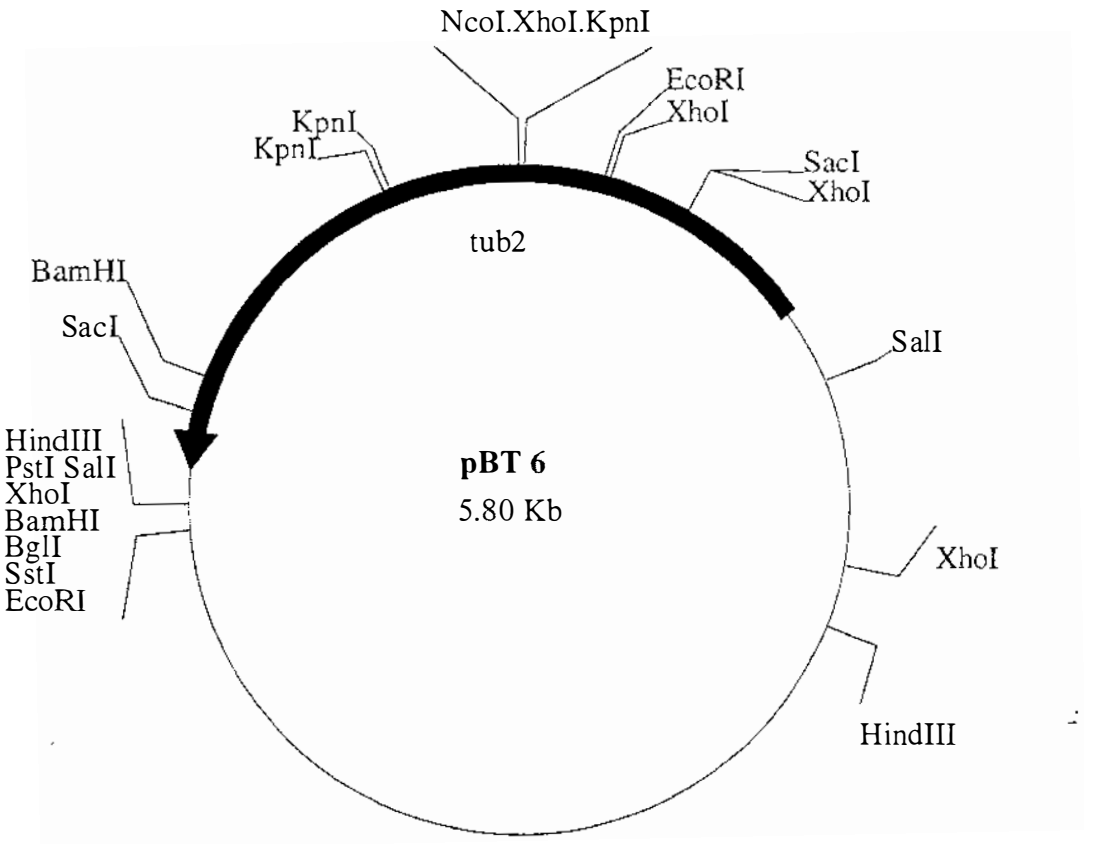
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PstI SalI
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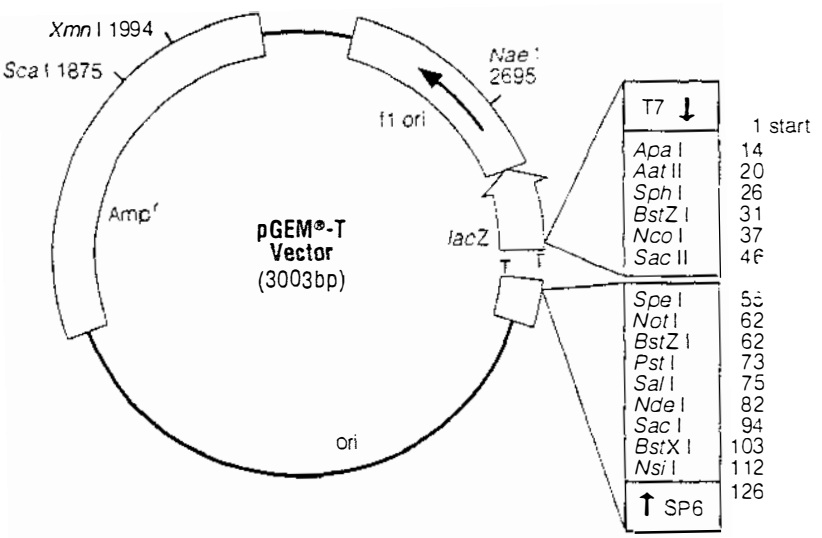
A 1.3 pGEM®-T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Nae I 2695
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Amp'
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ori
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Aat II 20
Sph I 26
BstZ I 31
Nco I 37
Sac II 46
Spe I 55
Not I 62
BstZ I 62
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SP6
1 start

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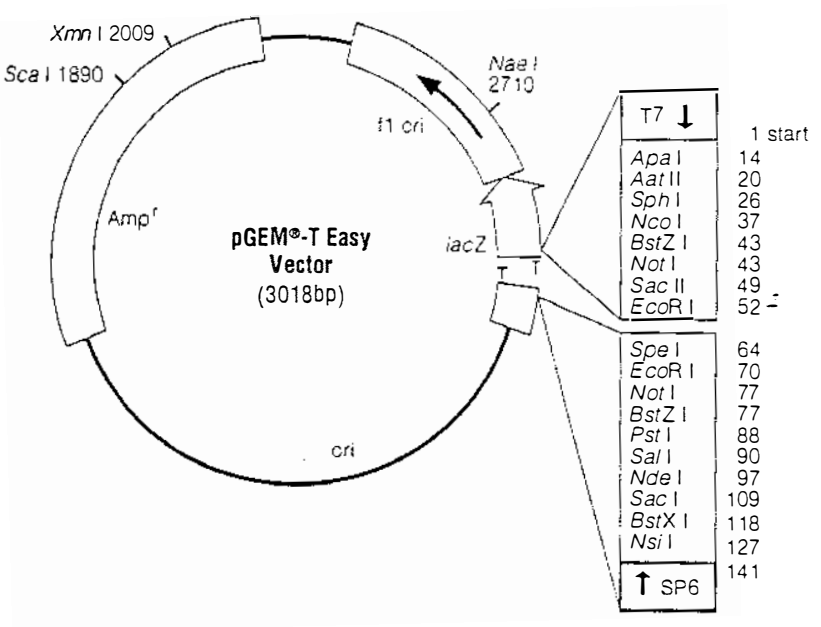

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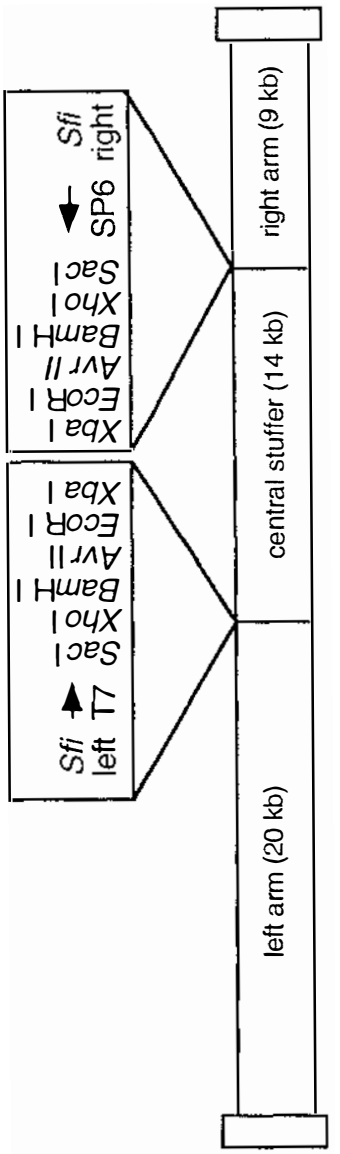

A 2.0 Sequence data

A 2.1 Sequence data from 359 bp product

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     HMG1>                                              ATGTCATGgTCCA 13
     HMG3< ATGAANATgATCTCCAANNGCTGTGaAAAGGCTNtTGATGTCATGGTCCA 50
CONSENSUS> ATGAAnATGATCTCCAAnnGCTGTGAAAAGGCTnTTGATGTCATGGTCCA 50
                   +         +         +         +         +

     HMG1> AGTGAATGCGGTTTTGACGACATGTCTATCATTTCGCTCTCTGGTAATTT 63
     HMG3< AGTGAATgCGGTTTTGACGACATGTCTATCATTTCGCTCTCTGGTAATTT 100
CONSENSUS> AGTGAATGCGGTTTTGACGACATGTCTATCATTTCGCTCTCTGGTAATTT 100
                   +         +         +          +         +

     HMG1> CTGTACCGACAAGAAGTCTGCGGCTATCAACTGGACTGA'TGGTCGTGGAA 113
     HMG3< CTGTACCGACAAGAAGTCTgCGGCTATCAACTGgACTGATGgTCGTgGAA 150
CONSENSUS> CTGTACCGACAAGAAGTCTGCGGCTATCAACTGGACTGATGGTCGTGGAA 150
                   +         +         +         +         +

     HMG1> AGTCCGTCGTGGCTGAAGCGATTATTcCTGGTGATGTCGTTAAGAGTGTC 163
     HMG3< AGTCCGTCgTGgCTgAAgCgATTATTCCTGgTgATGTCGTTAAGAGTGTC 200
CONSENSUS> AGTCCGTCGTGGCTGAAGCGATTATTCCTGGTGATGTCGTTAAGAGTGTC 200
                   +         +         +         +         +

     HMG1> CTGAAGAGTGATGTCAACGCCTTGGTCGAGCTCAATGTGAGCAAGAACTT 213
     HMG3< CTgAAGAgTgATgTCAACGCCTTGGTCgAGCTCAATGTGAGCAAGAACTT 250
CONSENSUS> CTGAAGAGTGATGTCAACGCCTTGGTCGAGCTCAATGTGAGCAAGAACTT 250
                   +         +         +         +         +

     HMG1> GATCGGAAGTGCCATGGCTGGCAGCTTGGGCTGGCTTCAACGCCACGCTC 263
     HMG3< GATCGGAAGTGCCATGGCTGGCAgCTTGGGCTGgCTTCAACGCCACGC   298
CONSENSUS> GATCGGAAGTGCCATGGCTGGCAGCTTGGGCTGGCTTCAACGCCACGCTC 300
                   +         +         +         +         +

     HMG1> GAACATTGTCNCGCATCTTCCTGGCCACTGGCcAAGA              300
CONSENSUS> GAACATTGTCnCGCATCTTCCTGGCCACTGGCCAAGA              337
                   +         +         +         +         +
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A 2.2 Sequence data from 700 bp product

```
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                +         +         +         +         +
HMG1> ATTCTCACACTCATAGTCACTAGTGAACTGTATCTAGTGATACATGATCA 150
                +         +         +         +         +
HMG1> ATCACTTTCCGAATCCAAAAGAGAGTGAATGATACAATTTTTTGATACTG 200
                +         +         +         +         +
HMG1> ATTTCGACTGCTTGAGACTAAAACGGTTGTAGTAGTGTACCTGATGATGT 250
                +         +         +         +         +
HMG1> AATCGCACCCCTTCTTACCGTGGAGTAGTTAATCTGCTAGTACTGATGTA 300
                +         +         +         +         +
HMG1> GACTGTAGGGCAAGCCGCGAAATTCTTGGAGGAAGCAGTATTACAGGACA 350
                +         +         +         +         +
HMG1> CTGGAA                                             356
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HMG3> AGACCGCTTCGTAGGGAGAGCTGGTCAATATCGAGCTCCTCGGACCATGG 50
                +         +         +         +         +
HMG3> TGGCTGATAGGTGGGAGCCGTGAGCTGCCAGGGCCGAGAGGACAATCGTT 100
                +         +         +         +         +
HMG3> GGGTAGAATTGTGACCAGCTTGTATACCGACTACTGTGGAGGTCGTACAG 150
                +         +         +         +         +
HMG3> CCTGCCTTTGCCGAGAACAGCAACAGCAAAACAACTACGCTATAGGGTAG 200
                +         +         +         +         +
HMG3> TATCAGCTACTAAGAGATGCTGGAAATTGACAACAATTCCTAACAACTTT 250
                +         +         +         +         +
HMG3> CGAATCTGATTCGTTCCAGTGTAAACTGTCTACTGGCTTCCACCAAGATT 300
                +         +         +         +         +
HMG3> GCAGGCTGGCCTACAGCT                                  318
```
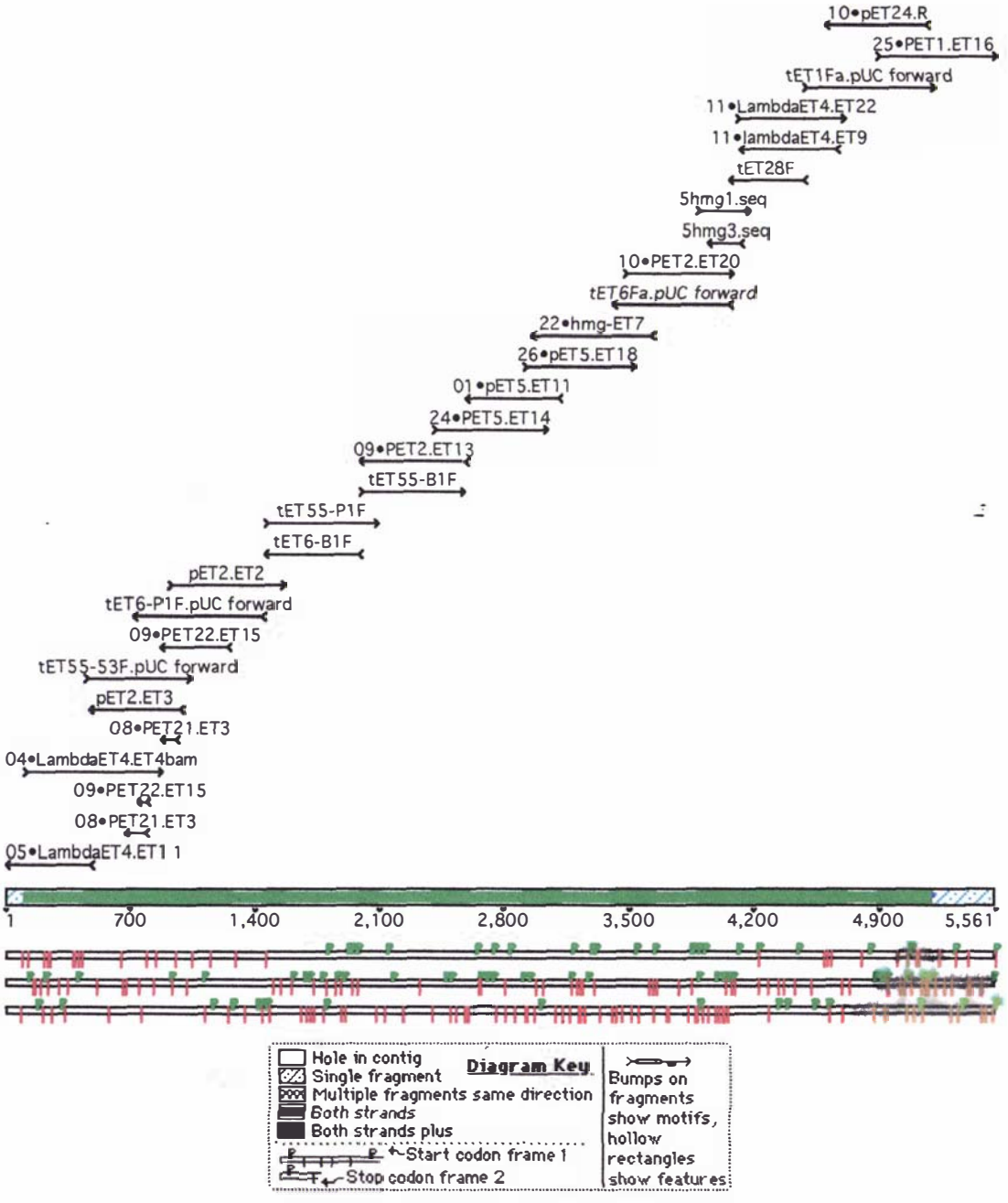
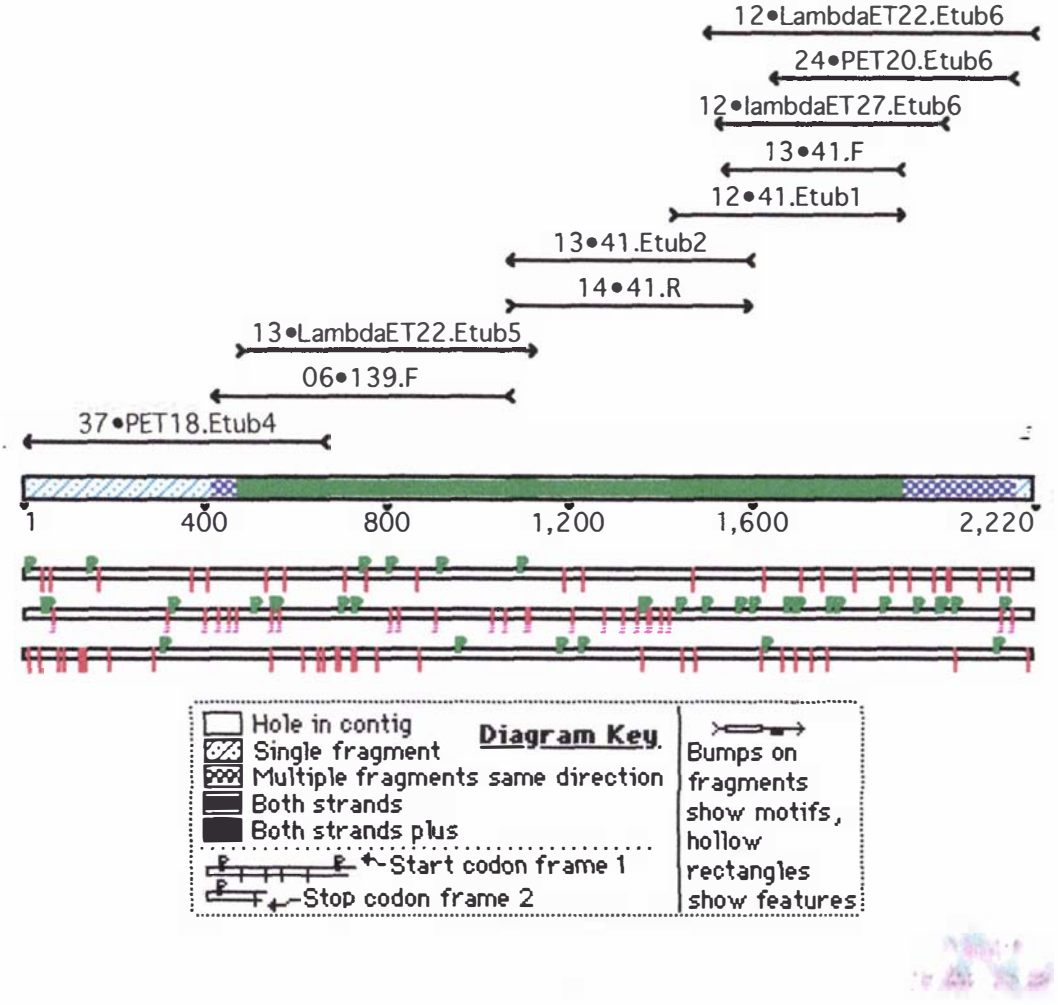

Appendix 2.3 *Individual sequences used to compile the doublestranded P. paxilli hmg sequence*

Sequences were aligned into a contig with the Sequencher™ DNA analysis package and printed using the Sequencher™ formatting. Sizes shown below the contig are in bp.



Appendix 2.4 *Individual sequences used to compile the doublestranded P. paxilli tub-2 sequence*

Sequences were aligned into a contig with the Sequencher™ DNA analysis package and printed using the Sequencher™ formatting. Sizes shown below the contig are in bp.



A 3.0 Calculations

A 3.1 Calculating paxilline content of mycelia

The standard used to calibrate HPLC analysis of paxilline contained 25 μg/mL of authentic paxilline. The standard was injected three times, standard A, B and C.

10 μL injected = 0.25 μg paxilline in 10 μl.

The chromatopac recorder calculated the area for each peak.

Average peak area = (peak area of A + peak area of B + peak area of C) /3
= (I)

The average paxilline /unit area was calculateed.

= 0.25 μg / (I)
= (II)

A 10 μL sample of paxilline, isolated from mycelia, was injected into the HPLC.

Paxilline in 10μL = peak area x (II)
= (III)

The 10μL sample was removed from the 3 mL extract collected from the Sepak column.

Sample paxilline in 3 mL (DCM:ACN) = (III) x 300
= (IV)

The 3 mL filtered extract was isolated from a 1.5 mL aliquot of the 6 mL $CHCl_3$: Methanol extraction, and is therefore equivalent to 1/4 of the original sample volume.

Sample paxilline in 6 mL ($CHCl_3$:Methanol) = (IV) x 4

= (V) (μG)

The paxilline was extracted into $CHCl_3$:methanol from a 5 mL sample of culture. These samples were freeze-dried and the dry weight recorded before paxilline was extracted.

Paxilline (μg) / g of mycelia = (V) /Dry weight (g)

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I'm a bit busy this weekend Barry,
but I'll get onto that first thing
Monday morning.