

Molecular cloning and characterisation of the δ -aminolaevulinate
synthase gene (δ -ALAS) in *Aspergillus nidulans*.

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Molecular cloning and characterisation of the δ -aminolaevulinate synthase gene (δ -ALAS) in *Aspergillus nidulans*.

Simon W.C. Dixon

Abstract.

δ -Aminolaevulinate synthase (δ -ALAS) is the first enzyme in the haem biosynthetic pathway, catalysing the condensation of succinyl CoA and glycine to form δ -aminolaevulinic acid (δ -ALA). The enzyme δ -ALAS has been studied in a number of species including yeast, a facultative anaerobe, and multicellular eukaryotes. The primary structure of the protein from a number of species exhibit regions of highly conserved amino acids, and a nucleotide probe based on one region was designed and used to clone the structural gene for δ -ALAS from the filamentous fungus *Aspergillus nidulans*.

The cloned gene, *hemA*, was sequenced and found to possess a single intron, of 64bp, located between 355 and 419nt in the gene; between amino acids 119 and 120 of the protein. The deduced protein sequence, of 648 amino acids, shows 64% identity over the carboxyl domain of the protein derived from the yeast *HEM1* gene. The amino-terminal sequence consists of basic amino acids, believed to be involved in mitochondrial targeting, and is consistent with observations on other δ -ALAS proteins.

One copy of the *hemA* gene was disrupted in a diploid strain of *A. nidulans* following the transformation with a suitable disruption vector. The recessive *hemA*⁻ mutant strain was isolated by mitotic haploidisation and shown to have an absolute requirement for δ -ALA, not replaceable by haem supplied in the medium. Genetic analysis by mitotic haploidisation of a heterozygous diploid constructed between a *hemA*⁻ mutant and a suitable mapping strain, located the *hemA* gene to chromosome VII of *A. nidulans*.

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Abbreviations

δ-ALA	δ-Aminolaevulinic acid
δ-ALAS	δ-Aminolaevulinate synthase
ATP	Adenosine 5' triphosphate
bp	base pair
BSA	bovine serum albumin
CAIP	Calf intestinal alkaline phosphatase
Ci	Curies
cpm	Counts per minute
CTP	Cytosine 5' triphosphate
DEPC	Dimethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Any 2' deoxynucleoside 5' triphosphate
ddNTP	Any 2' dideoxynucleoside 5' triphosphate
EDTA	Diaminoethanetetra acetic acid
GTP	Guanosine 5' triphosphate
hrs	Hours
IPTG	Isopropyl-β-D-galactosidase
ITP	Inosine 5' triphosphate
kb	kilobase pair
kD	kilodalton
MCS	Multiple cloning sites
mins	Minutes
MOPS	3-(N-morpholino)-propanesulphonic acid
nt	Nucleotide(s)
OD	Optical density
PEG	Polyethylene glycol
psi	Pounds per square inch
RF	Replicative form
rpm	Revolutions per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
mRNA	messenger Ribonucleic acid
SDS	Sodium dodecyl sulphate

ssDNA	single stranded Deoxyribonucleic acid
TEMED	N,N,N',N'-tetramethylethylenediamide
Tris	Tris (hydroxymethyl)aminomethane
TTP	Thymidine 5' triphosphate
UV	Ultra violet
v/v	Volume for volume
w/v	Weight for volume
X-gal	5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Haem: A generic term to identify ferro protoporphyrin IX

Haemin: A generic term to identify ferri protoporphyrin IX, usually isolated with a counterion.

CHAPTER 1.

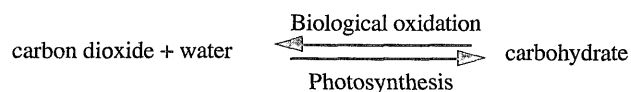
INTRODUCTION.

The diversity of metabolic activities exhibited by the filamentous fungi has made them useful tools for genetic, biochemical and molecular studies. Filamentous fungi are used in the food and beverage industries, namely in traditional Chinese and Japanese food production (reviewed by Sakaguchi *et al.*, 1992). Their ability to produce heterologous proteins, such as Chymosin further warrants their use in commercial enterprises (reviewed by Dunn-Coleman *et al.*, 1992). Physiological and biochemical investigation of this group of fungi have been carried out with a limited number of species amongst which *Aspergillus nidulans* has proved perhaps the best and most versatile model in the broad *Penicillium-Aspergillus* group.

Aspergillus nidulans is an aerobic ascomycete which grows rapidly producing abundant uninucleate conidiospores. The organism exhibits both haploid and diploid growth phases, making it an attractive system for genetic analysis (Pontecorvo *et al.*, 1953). Classic studies in the biochemical genetics of this lower eukaryote has provided an increased awareness of the differences between this organism as an obligate aerobe and the facultative anaerobe, the yeast *Saccharomyces cerevisiae*.

The haem molecule is common to all living systems and has been the subject of numerous investigation in eukaryotes including *S. cerevisiae*. However there is very little information regarding the biosynthesis of haem and its involvement in oxygen regulation in an obligate aerobe such as *A. nidulans*. Such investigations may provide an insight into fundamental differences between facultative anaerobes and obligate aerobes.

Haem belongs to a group of compounds known as the porphyrins. These are highly reactive compounds which biological systems have capitalised to their advantage. These compounds are present in all living organisms where they play a central role in energy entrapment and utilisation through the processes of biological oxidation and photosynthesis.



In a biological capacity, haem is associated with protein molecules where it forms the prosthetic group of haem-proteins such as haemoglobin, catalase and the cytochromes. The haem-proteins occur ubiquitously throughout both the plant and animal kingdoms where they are involved in a vast array of biological processes. A central role of the haem-proteins is in respiration where the cytochromes form the basis of the electron transport chain. Table 1.1 gives examples of some of the important biological activities in which haem-proteins are involved. The production of functional haem-proteins requires the synthesis of haem as a cofactor and its subsequent assembly with specific proteins. Haem is also central to oxidative processes of the cell, exhibiting an active role in oxygen mediated regulation of gene expression.

Table 1.1. Biological processes in which Haemproteins form an integral component.

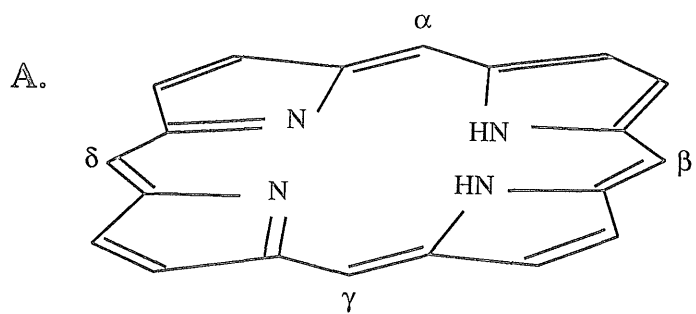
Biological Processes	Haemprotein
Transport and storage of oxygen	Haemoglobin, Myoglobin.
Mitochondrial respiration	Cytochromes <i>b</i> , <i>c</i> ₁ , <i>c</i> , <i>a</i> , <i>a</i> ₃ .
Oxidative metabolism of certain drugs and lipophilic chemical monooxygenase	Xenobiotic inducible cytochrome P450.
Steroid biosynthesis	Steroidogenic cytochrome P450 enzymes.
Desaturation of fatty acids	Microsomal cytochrome <i>b</i> ₅ .
Tryptophan catabolism	Tryptophan oxygenase.
Enzymic destruction of peroxides	Catalase and peroxidase.

1.1. Structure of haem.

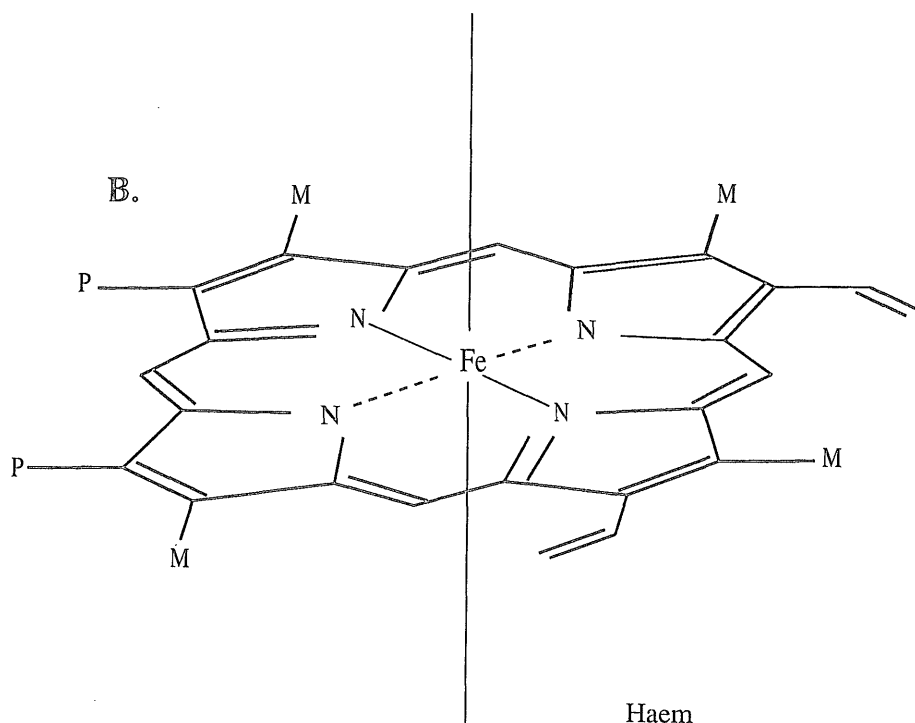
Haem is derived from the parent tetrapyrrole porphyrin (Figure 1.1 Panel A). The basic nucleus of these compounds consists of a macrocycle of four pyrrole rings linked by four methene bridges. The physical characteristics of porphyrin is dependent upon the nature of the side-chain substituents. Protoporphyrin is the most abundant porphyrin containing four methyl groups, two vinyl groups and two propionic acid groups. Of the many isomeric forms possible with protoporphyrin, only one form, protoporphyrin IX, is produced in nature. An important feature of protoporphyrin is its ability to form quadridentate chelate complexes with iron, magnesium, zinc, nickel, cobalt, and copper ions whereby the metal is held by four coordinate bonds. A chelate complex with magnesium forms chlorophyll and with iron, haem (Figure 1.1 Panel B). In haem the four ligand groups of the porphyrin forms a square planar complex with the iron, while the remaining fifth and sixth coordinate positions of the iron are perpendicular to the plane of the porphyrin. These fifth and sixth positions can be occupied by the R-group of specific amino acids, particularly basic amino acids residues, thus binding a specific protein to form an active haem-protein. In other haem-proteins, additional covalent linkages occur between the side chains of the porphyrin ring and the amino acid residues of the protein, as in the case of cytochrome *c* where the linkage are thioethers formed by the addition of the sulphydryl groups of two cysteine residues to the vinyl groups of haem.

Figure 1.1. The structures of porphyrin and haem.

The structure of the basic porphyrin (Panel A) showing the the four pyrrole rings (a, b, c and d) being linked by four methene bridges (α , β , γ and δ). Haem, ferroprotoporphyrin IX (Panel B), is the iron-containing complex derived from the basic porphyrin molecule and contain two coordinate bonds to which protein molecules can bind through specific amino acids to from haemproteins.



Porphyrin



Haem

Ferroporphyrin IX

1.2. The haem biosynthetic pathway.

The tetrapyrrole biosynthetic pathway is similar in all living systems and begins with the production of the highly reactive aminoketone, δ -aminolaevulinic acid (δ -ALA). This compound is produced by two distinct routes, one method utilises the carbon skeleton of glutamate whilst the other method is a condensation reaction involving glycine and succinyl CoA, derived from the tricarboxylic acid pathway. Organisms utilising glutamate include plants, algae and some anaerobic bacteria whereas the condensation reaction is carried out mainly by some aerobic bacteria, fungi and animals. There is some evidence that both pathways may function in some photosynthetic organisms and higher plants (Weinstein and Beale, 1983).

The formation of the tetrapyrrole macrocycle from δ -ALA occurs in three stages. The first step involves the condensation of two molecules of δ -ALA to form the basic pyrrole building block, porphobilinogen. The second step involves polymerisation of four molecules of porphobilinogen to generate the unstable hydroxymethylbilane, also known as preuroporphyrinogen. For the third step, hydroxymethylbilane is circularised by the rearrangement of one of the pyrrole rings, the "d" ring, to yield the intermediate uroporphyrinogen III. This is the universal precursor from which all porphyrins, haems, corrins, chlorophylls and other tetrapyrroles are derived. Uroporphyrinogen III may either be converted to protoporphyrin IX, which can proceed to the formation of haem, chlorophyll or bacteriochlorophyll, or it may be methylated to form sirohaem, vitamin B₁₂, or the nickel cofactor F₄₃₀.

Synthesis of haem involves uroporphyrinogen III being first decarboxylated to yield coproporphyrinogen III, this is followed by a another decarboxylation reaction to form protoporphyrin IX. Sirohaem is involved in a number of

biological processes, including sulphite reduction where the sirohaem-containing sulfite reductase (Yoshimoto and Sato 1971), catalyses an early step in the methionine biosynthetic pathway.

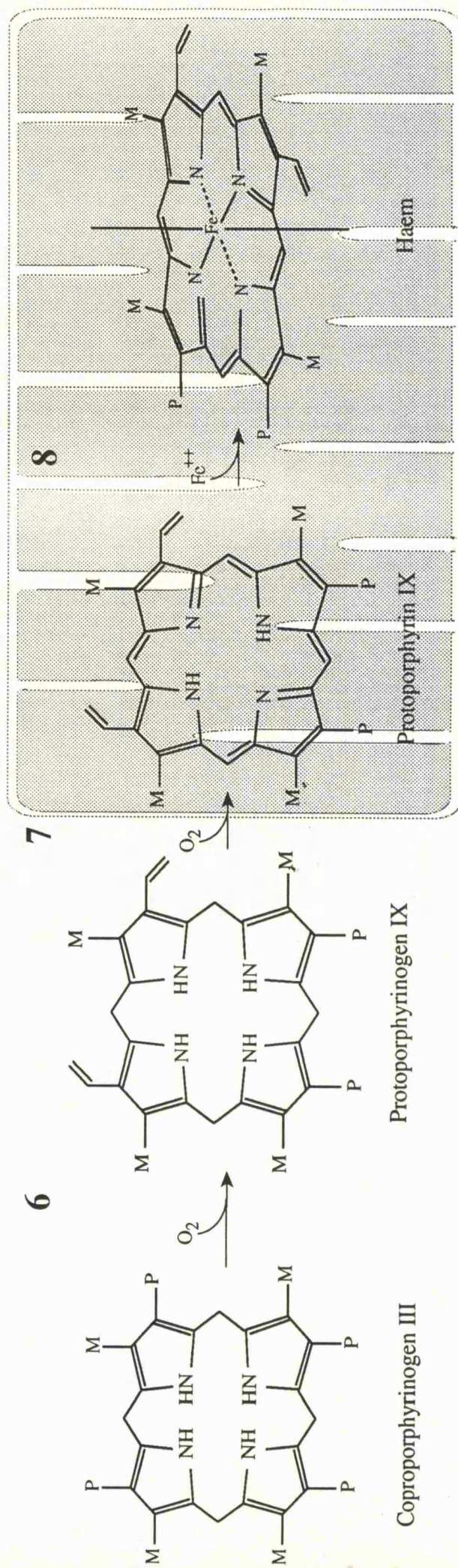
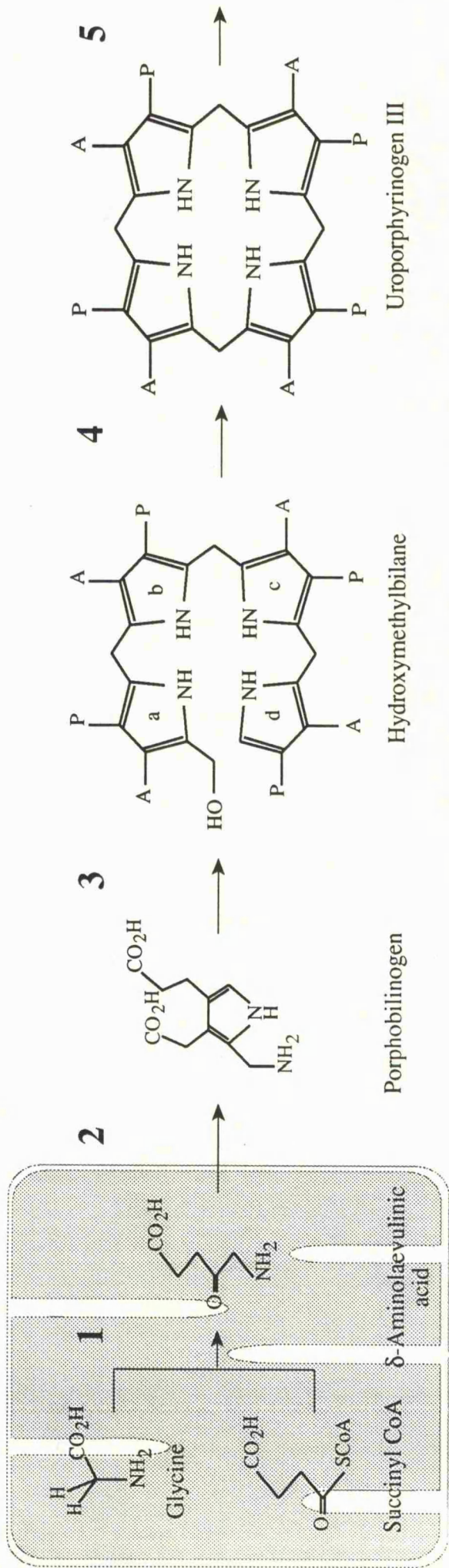
The porphyrin biosynthetic pathway has been fully analysed and extensively studied in the yeast, *S. cerevisiae*, due to the ease with which the organism can be manipulated using the techniques of genetics and molecular biology. The general similarities of biosynthetic mechanisms amongst unicellular eukaryotes would indicate the likelihood of a common route for the production of haem in yeast and *Aspergillus*. The following section describes the biosynthesis of haem in yeast cells (Figure 1.2).

Figure 1.2 The haem biosynthetic pathway.

The haem biosynthetic pathway showing the eight enzymic steps. One of the intermediate porphyrin compound, uroporphyrinogen III, is used to form the porphyrin compound, including L-methionine.

The shaded structures depict mitochondria and indicates the enzymatic reactions taking place within the mitochondrial compartment.

1. δ -aminolaevulinate synthase;
2. porphobilinogen synthase;
3. porphobilinogen deaminase;
4. uroporphyrinogen III synthase;
5. uroporphyrinogen decarboxylase;
6. coproporphyrinogen III oxidase;
7. protoporphyrinogen oxidase;
8. ferrochelatase.



1.2.1. The biosynthesis of haem in *Saccharomyces cerevisiae*.

The first step in the haem biosynthetic pathway is the production of δ -aminolaevulinic acid (δ -ALA). The two starting reagents, succinyl CoA and glycine, undergo a condensation reaction to yield δ -ALA, coenzyme A and carbon dioxide (De-Barreiro, 1967). The reaction is catalysed by δ -aminolaevulinic synthase (δ -ALAS) in the mitochondria where there is an abundance of succinyl CoA, produced by the tricarboxylic acid pathway (Jayaraman *et al*, 1971; Porra *et al*, 1972; Volland and Felix, 1984). Furthermore, since the reaction steps following the formation of δ -ALA are energetically favourable, as a result of aromatic formation, deamination, decarboxylation or oxidation, the activated substrate succinyl CoA provides the major source of energy for the entire porphyrin biosynthetic pathway.

The δ -ALA produced is released into the cytosol where the enzyme δ -aminolaevulinic acid dehydratase catalyses its conversion into porphobilinogen (De-Barreiro, 1967). This reaction involves two molecules of δ -ALA undergoing an aldol condensation, the formation of a C-N bond, the loss of two molecules of water and finally a tautomeric shift. Four molecules of porphobilinogen then undergo a transformation to produce uroporphyrinogen III by the action of two enzymes porphobilinogen deaminase and uroporphyrinogen III synthase (Labbe-Bois and Volland, 1977; Jordan and Berry, 1980). The former enzyme carries out a tetrapolymerisation reaction on the pyrrole, porphobilinogen, to yield hydroxymethylbilane. This involves a stepwise addition of four porphobilinogen molecules to the enzyme with the loss of each amino group. The enzyme bound tetrapyrrole is then released into solution as hydroxymethylbilane and becomes the substrate for the second enzyme, uroporphyrinogen III synthase, which rearranges the fourth ring, the

"d" ring, and circularises the molecule to give uroporphyrinogen III. This is the key porphyrin from which all specific porphyrins are produced.

In yeast, uroporphyrinogen III undergoes a decarboxylation reaction, catalysed by uroporphyrinogen III decarboxylase, to form coproporphyrinogen III (Rytka *et al.*, 1984). The enzyme brings about a decarboxylation of all four acetic acid side chains of uroporphyrinogen III to form methyl groups. Coproporphyrinogen III is oxidised to protoporphyrinogen IX in a reaction catalysed by coproporphyrinogen oxidase (Miyake and Sigimura, 1968). This involves the conversion of two propionate groups to two vinyl groups with the elimination of two molecules of carbon dioxide. The reaction requires molecular oxygen as an electron acceptor (Porra and Falk, 1964). In the absence of oxygen pathway terminates at this point and no haem is present the cell. The penultimate step in the pathway involves the oxidation of protoporphyrinogen IX to protoporphyrin IX, catalysed by protoporphyrinogen IX oxidase (Porra and Falk, 1961; Poulson and Polglase, 1975). Protoporphyrinogen IX enters the mitochondria where the mitochondrial membrane bound enzyme, protoporphyrinogen IX oxidase carries out its catalysis using molecular oxygen as an electron acceptor (Porra and Falk, 1964). The product of this reaction, porphyrinogen IX is finally converted to haem (ferroprotoporphyrin IX) by the mitochondrial bound enzyme, ferrochelatase, which incorporates iron into the protoporphyrin IX molecule.

1.3. Regulation of the haem biosynthetic pathway.

The regulation of haem biosynthesis has been extensively studied in animals and reveals the presence of two distinct regulatory mechanisms depending on the cell type, namely that found in erythroid or in hepatic tissues. The mode of regulation in non-differentiating, hepatic cells appear to involve mainly feedback regulation by the product of the pathway, haem, acting on the first enzyme of the haem biosynthetic pathway δ -aminolaevulinate synthase (δ -ALAS) (reviewed by Andrew *et al.*, 1990,). In differentiating erythroid cells regulation appears to occur at a number of different steps in the pathway, however the details have not been resolved.

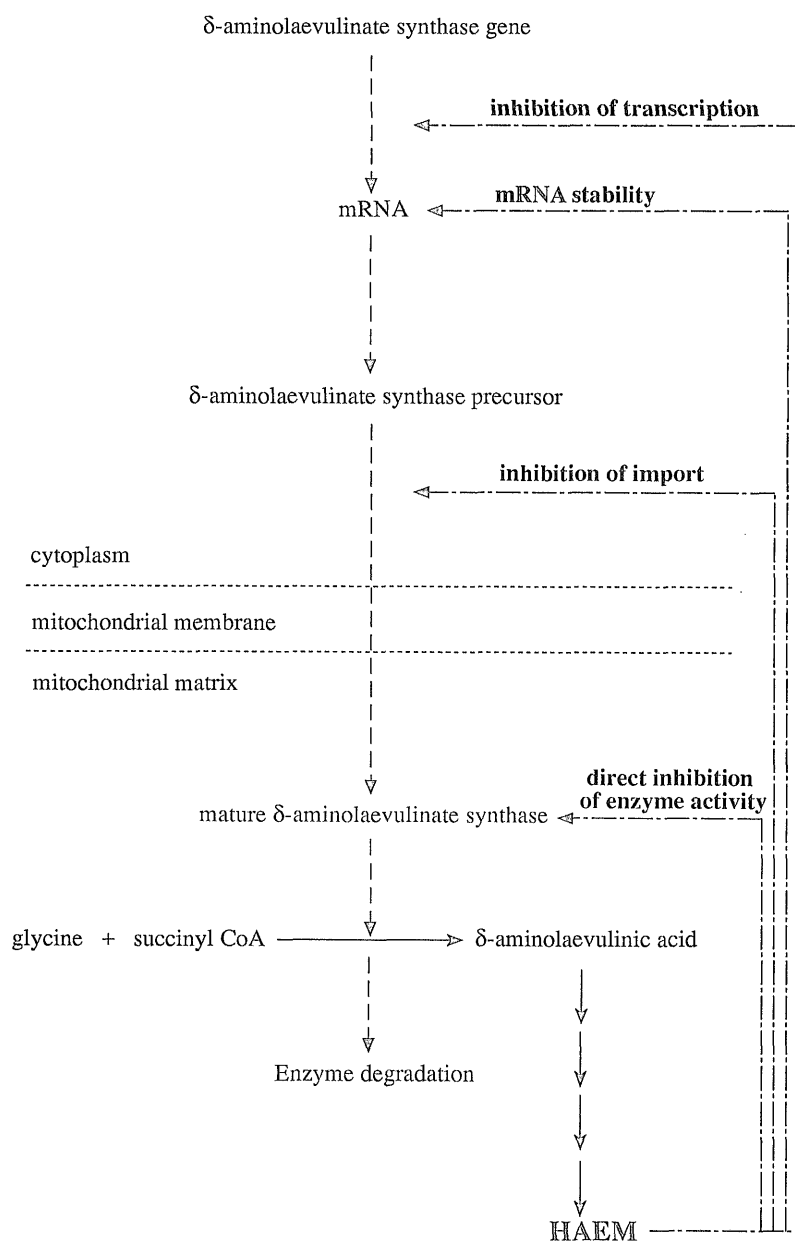
Higher eukaryotes have been shown to possess two different types of enzyme for the first step of the pathway, one proposed as the "housekeeping" δ -ALAS enzyme and the second being an erythroid specific δ -ALAS protein. The "housekeeping" δ -ALAS gene is expressed in all tissues and cells that have been examined, whilst the erythroid specific δ -ALAS gene appears to be expressed in relation to δ -ALA production during the terminal stages of erythroid cell differentiation. The two enzymes exhibit differences in the inhibition of δ -ALAS activity by haem (Pirola *et al.*, 1984; Scholnick *et al.*, 1972).

1.3.1. Hepatic cells.

The first enzyme of the haem biosynthetic pathway, δ -ALAS, has been the focus of attention in the study of the regulatory mechanisms affecting the pathway. Figure 1.3 shows a model for the regulation of haem biosynthesis in hepatic cells. Investigations in the rat liver and chicken embryo liver systems show that δ -ALAS specific mRNA has a short half life which strongly suggests δ -ALAS being a key step in regulation (Gayathri *et al.*, 1973; Sassa and Granick, 1970).

Figure 1.3. Model for the regulation of haem biosynthesis in hepatic cells.

The basis for the model for the regulation of haem biosynthesis in hepatic cells is the control of δ -aminolaevulinate synthase production, location, and activity by the end product of the pathway, haem.



- - - -> Enzyme synthesis route
- . - . -> Regulatory route
- > Enzymatic reaction

In non-erythroid cells δ -ALAS is present at low levels whilst the other enzymes and intermediates of the pathway are present in non-rate limiting amounts, further indicating δ -ALAS as the rate limiting enzyme in the haem biosynthetic pathway (Sassa and Kappas, 1981; Kappas *et al.*, 1983; May *et al.*, 1986).

It is postulated that regulation of the haem biosynthetic pathway is effected by a small cytoplasmic pool of "free" haem (Yannoni and Robinson, 1975; Grandchamp *et al.*, 1981). Investigations using porphyrinogenic drugs have established a strong correlation between dosage of drug and the regulation δ -ALAS at the molecular level. The drugs cause a decrease in the levels of the intracellular haem pool due to an increased requirement for haem for the biosynthesis of cytochrome P450 enzymes involved in detoxification (Jordan, 1991). The source of this pool is haem molecules in transit from the mitochondria to haemoproteins (Schmid, 1973), and molecules loosely associated with apoproteins, such as apocytochrome P450, or in exchange with other free and loosely bound haems (Schmid, 1973; Bissell and Hammaker, 1976).

In hepatic tissues, the regulation of δ -ALAS by haem occurs by several mechanisms extending from the regulation of the transcription of the δ -ALAS gene to a direct effect on the enzymatic activity. The mechanism by which haem regulates the transcription of the δ -ALAS gene is still unclear. Early observations have indicated that the regulation of the production of δ -ALAS by haem occur at the level of transcription (Srivastava *et al.*, 1988). Evidence indicating the repression of transcription of the δ -ALAS gene by haem comes from experiments where induction by porphyrinogenic drugs of δ -ALAS to high levels is suppressed by the presence of haem (Hayashi *et al.*, 1968; Hayashi *et al.*, 1972; Srivastava *et al.*, 1980). For example, using a reticulocyte lysate system to monitor mRNA, the level of δ -ALAS specific mRNA was reduced in

liver polysomes prepared from drug induced rats which were also treated with haemin (Yamamoto *et al.*, 1982). When transcription rates of δ -ALAS mRNA was measured in drug induced rats, further treatment with δ -ALA or haem similarly indicated regulation of transcription (Srivastava *et al.*, 1988; Srivastava *et al.*, 1990). These experiments however, used large doses of haem and δ -ALA in comparison to their physiological levels (Hayashi *et al.*, 1972) and therefore raises questions as to the validity of these findings. This view was supported in experiments where treatment with haem at doses comparable to physiological levels indicated that expression of the δ -ALAS gene was not regulated at the level of transcription (Hamilton *et al.*, 1991).

Studies by a number of groups have indicated that δ -ALAS mRNA has a very short half life (Marver *et al.*, 1966; Sassa and Granick, 1970; Gayathri *et al.*, 1973; Drew and Ades, 1986) and this is considered important in the control of the haem biosynthetic pathway at the first step (Hamilton *et al.*, 1991). For example, repression of the δ -ALAS gene by haem in rat liver tissues results in the rapid disappearance of δ -ALAS mRNA (Yamamoto *et al.*, 1988). Similarly, when the levels of δ -ALAS mRNA were measured in drug induced rat liver tissues, inhibition of RNA synthesis followed by treatment with haem resulted in the rapid disappearance of δ -ALAS mRNA (Hamilton *et al.*, 1991). Cycloheximide was shown to prevent the action of haem to decrease the half life of the δ -ALAS mRNA which suggests that the effect is possibly mediated by a labile protein (Hamilton *et al.*, 1991).

Translational regulation is thought to occur at the level of δ -ALAS synthesis on the mRNA as a result of the interaction of haem (Granick *et al.*, 1975; Sinclair and Granick 1975; Sassa and Granick 1976). Haem has been shown to exert a similar effect on induction of δ -ALAS as the protein synthesis inhibitor, cycloheximide (Strand *et al.*, 1972; Tomita *et al.*, 1974).

The enzyme δ -ALAS is synthesised in the cytoplasm as a precursor protein which is then transported into the mitochondrion and proteolytically processed to the mature form. Haem may prevent the import of the protein into the mitochondrion in rat and chicken embryo liver (Ohashi and Kikuchi 1972; Srivastava *et al.*, 1983; Hayashi *et al.*, 1983). Additionally, the processing of the δ -ALAS precursor within the mitochondrion may be blocked by haemin (Ades, 1983). The precise mechanisms by which post-translation regulation of δ -ALAS occurs is not known but it is possibly that haem may bind with the δ -ALAS precursor directly or via a haem binding factor, forming a pre- δ -ALAS-haem complex.

Once located to the mitochondrion δ -ALAS activity is shown to be subjected to a direct feedback inhibition by haem, though this may not be of physiological importance. (Whiting and Granick 1976; Pirola *et al.*, 1984; Bloomer and Straka 1988). Although purified δ -ALAS enzyme was not inhibited by haem (Pirola *et al.*, 1984) the partially purified enzyme preparation was shown to be inhibited (Scholnick *et al.*, 1972). Additionally intact liver mitochondrion δ -ALAS activity was not affected by haem concentrations (Wolfson *et al.*, 1979). The half-life of the enzyme is less than that of many other mitochondrial enzymes which indicates that feedback control of δ -ALAS may also be a function of the stability of the enzyme (Hayashi *et al.*, 1980).

1.3.2. Erythroid cells.

The biosynthesis of haem in erythroid tissues does not appear to be controlled solely by the amount and activity of δ -ALAS activity, as is the case in non-erythroid tissues. As well as being under a different control mechanism to that exhibited in the liver cells, regulation of haem biosynthesis in erythroid

tissues appears to be more complex and furthermore the mechanism of regulation needs to adjust to the cells' constantly changing requirements for haem. Different effects of haem has been reported during cell differentiation where haem appears to inhibit its own synthesis by direct repression of δ -ALAS activity, (Ibrahim *et al.*, 1978), as well as acting as a translation inhibitor of protein synthesis (Burns and London, 1965) and by preventing the uptake of transferrin bound iron into cells (Ponka and Neuwirt, 1969).

The process of erythropoiesis and haemoglobin synthesis in erythroid tissues has been investigated by *in vivo* studies using anemic mutant mice (Sassa and Bernstein, 1977), foetal mouse (Freshney and Paul, 1971) and chick yolk sac (Wainright and Wainright, 1970). Analysis of the enzyme activities during haem biosynthesis and erythropoiesis indicate that the activities of δ -ALAS, uroporphobilinogen deaminase, the enzyme which converts uroporphobilinogen to hydroxymethylbilane, and ferrochelatase, the enzyme catalysing the insertion of iron into haem, are sequentially induced in erythroid tissues (Cole *et al.*, 1968; Sassa *et al.*, 1978). These results are supported by *in vitro* studies of erythropoiesis in erythroid precursor cells (Levere and Granick, 1965). The current investigations have been carried out mainly in Friend virus transformed murine erythroleukemia (MEL) cells (Sassa, 1983) and in bone marrow tissues (Sassa, 1980; Sassa, 1983; Beru and Goldwasser, 1985)

MEL cell infected with the Friend virus complex can be grown as continuous cell cultures. When treated with dimethyl sulphoxide, these cells undergo differentiation and develop characteristics similar to that of a fully differentiated erythroid cell (Friend *et al.*, 1971). Investigation of cell differentiation indicate that coproporphyrinogen oxidase is also involved in the sequential induction of erythropoiesis (Fadigan and Dailey, 1987). This has led to a model for the induction of haem synthesis where an initial induction of

δ -ALAS (Fraser and Curtis, 1987), along with a decrease in coproporphyrinogen oxidase, which prevents haem production whilst increasing the production of δ -ALA. The mechanism of δ -ALAS induction is unknown but is shown to occur as a result of induction of erythroid δ -ALAS gene transcription in response to a developmental signal (Dierks, 1990).

Since porphobilinogen deaminase is both unstable and inactive in the absence of the substrate, porphobilinogen, and the flux through the haem biosynthetic pathway is inhibited by the decrease in the activity of coproporphyrinogen oxidase, porphobilinogen will accumulate. Furthermore, as haem biosynthesis is blocked, intracellular iron level increases causing a further induction of δ -ALAS through iron responsive elements. Erythroid δ -ALAS mRNAs are shown to possess sequences similar to the cis-acting iron responsive elements (IRE) within its 5' untranslated region (Schoehaut and Curtis, 1986; Casey *et al.*, 1988). These IRE also allow coordination of heavy and light subunits of ferritin, the iron storage protein, at the level of translation (Aziz and Munro, 1987; Hentz *et al.*, 1987), and the synthesis of transferrin receptors with the availability of iron (Casey *et al.*, 1988). It is proposed the low iron concentrations causes a cytoplasmic trans-acting protein iron responsive elements (the IRE-BP) to bind to the IRE preventing translation, this is reversed when iron enters the cell (Casey *et al.*, 1988; Klausner *et al.*, 1993). The presence of such elements in erythroid δ -ALAS mRNAs may allow the activity of the enzyme to be regulated at the translation level by iron.

Following the increase in porphobilinogen concentration, porphobilinogen deaminase activity increases, followed by the induction of coproporphyrinogen oxidase as a result of developmental responses in the later stages of differentiation. The use of ferrochelatase enzyme inhibitors, such as divalent cations results in the inhibition of haem synthesis indicating that the regulatory

mechanism of haem biosynthesis in the early stages of differentiation occurs as a result of inhibition of the enzyme rather than by iron availability (Fadigan and Dailey, 1987). Ferrochelatase activity also increases in the later stages of differentiation and no longer becomes limiting.

1.3.3. Regulation of δ -ALAS in the yeast *Saccharomyces cerevisiae*.

Glucose and oxygen are the major regulatory factors affecting the amount of different haem-proteins produced by the yeast *S. cerevisiae*. Cells in a derepressed state, that is when grown aerobically with a non-fermentable carbon source (such as lactate), with a low concentration of glucose or with a slowly fermentable sugar (such as galactose), produce high concentrations of respiratory proteins. Conversely, when the cells are in a repressed state, that is when grown in the presence of high glucose concentrations, mitochondrial development and mitochondrial cytochrome synthesis are absent. Under these conditions the haem is largely contained in cytochrome P450 enzymes which are abundant within the cell. These enzymes are at low concentrations when the cells are derepressed.

When cells are grown anaerobically only a small number of different cytochromes are detected, and these are in minute concentrations. This occurs as a result of a decrease in the "free" haem pool, since there is an absolute requirement for oxygen in haem biosynthesis. On exposure to oxygen the cells resume the synthesis of haem and hence the production of cytochromes is at normal levels.

The unicellular facultative aerobic organism, *S. cerevisiae* provides a powerful tool for the isolation of respiratory deficient mutants defective in haem biosynthesis. Haem deficient mutants in *S. cerevisiae* have been isolated for

seven of the eight steps of the haem biosynthetic pathway shown in Table 1.2 (Gollub *et al.*, 1977; Urban-Grimal and Labbe-Bois, 1981). These mutants grow on semisynthetic medium supplemented with haem. The essential requirement for haem within the cell includes its use as a precursor for enzymes of the respiratory pathway, its use as a precursor for enzymes involved in steroid biosynthesis and its involvement in microsomal cytochromes involved in the desaturation of fatty acids. Since *S. cerevisiae* is a facultative anaerobe it is able to grow in the absence of respiratory enzymes. Therefore, on supplementing with ergosterol and Tween 80, as a source of desaturated fatty acids, *S. cerevisiae* is able to grow in the absence of haem (Gollub *et al.*, 1977; Urban-Grimal and Labbe-Bois, 1981).

Mutant strains, blocked in steps 1, 2 and 3 of haem biosynthesis (Figure 1.2), lack the ability to synthesise uroporphyrinogen III, and are deficient in siroheme, a component of sulphite reductase necessary for the synthesis of methionine. These strains require L-methionine in addition to the above supplements. Haem requiring mutants have been used to investigate the regulation of the haem biosynthetic pathway, leading to the view that haem synthesis is controlled at the level of certain individual enzymes in the pathway (Camadro *et al.*, 1982; Labbe-Bois *et al.*, 1980; Urban-Grimal and Labbe-Bois, 1981).

When the enzymatic activity of δ -ALAS was measured under steady state conditions in haem requiring mutants of *S. cerevisiae* growing in medium supplemented with defined concentrations of Tween 80, ergosterol and L-methionine, there were no obvious correlation between the δ -ALAS activities observed in mutant strains blocked in different steps of the pathway (Bilinski *et al.*, 1981; Gollub *et al.*, 1977; Labbe-Bois and Volland, 1977; Rytka *et al.*, 1984). In similar experiments it was observed that intracellular levels of δ -ALA increased

Table 1.2. Characteristics of the *Saccharomyces cerevisiae* haem deficient mutants.

A series of haem deficient mutants of *Saccharomyces cerevisiae* have been isolated and characterised (Urban-Grimal and Labbe-Bois, 1981; Gollub *et al.*, 1977). Mutants were isolated for seven of the eight enzymatic steps. All of the mutants could be grown on semisynthetic medium supplemented with haem or ergosterol plus Tween 80. However, strains blocked in steps 1, 2 and 3 of haem biosynthesis, lacking the ability to synthesise uroporphyrinogen III, are deficient in siroheme, a component of sulphite reductase necessary for the synthesis of methionine. These strains require L-methionine in addition to haem.

Table 1.2. Characteristics of the *Saccharomyces cerevisiae* haem deficient mutants.

Enzymatic step [†]	Enzyme	Gene	Mutant strains	Growth supplement
1	δ-aminolaevulinate synthase	HEM1	hem1-5	Haem + L-methionine. or Tween 80 + ergosterol +L-methionine
2	porphobilinogen synthase	HEM2	hem10-2	Haem + L-methionine or Tween 80 + ergosterol +L-methionine
3	prophobilinogen deaminase	HEM3	hem11-1	Haem + L-methionine or Tween 80 + ergosterol +L-methionine
4	uroporphyrinogen III synthase	-	-	Not known
5	uroporphyrinogen decarboxylase	HEM12	hem12-1	Haem or Tween 80 + ergosterol
6	coproporphyrinogen III oxidase	HEM13	hem13-1	Haem or Tween 80 + ergosterol
7	protoporphyrinogen oxidase	HEM14	hem14-1	Haem or Tween 80 + ergosterol
8	ferrochelatase	HEM15	hem15-1	Haem or Tween 80 + ergosterol

[†] The enzymatic steps are depicted in Figure 1.2.

in mutants blocked in ferrochelatase activity (step 8), the enzyme catalysing the insertion of iron into haem, indicating that the activity of δ -ALAS is to some extent under feedback control by haem rather than its precursors (Malamud *et al.*, 1983; Rytka *et al.*, 1984).

Two observations strongly suggest that the rate limiting step in haem biosynthesis in *S. cerevisiae* is the conversion of δ -ALA to porphobilinogen (step 2). The activity of porphobilinogen synthase is considerably less than that of δ -ALAS and the haem requiring mutant strains of *S. cerevisiae* accumulate δ -ALA (Urban-Grimal and Labbe-Bois, 1981). Thus the situation in *S. cerevisiae* stands in contrast to that in multicellular eukaryotes where δ -ALAS is the rate limiting enzyme in liver cells.

The activity of coproporphyrinogen III oxidase, the enzyme catalysing the conversion of coproporphyrinogen III to protoporphyrinogen IX (step 6), was found to increase in haem requiring mutants in general grown in medium supplemented with Tween 80, ergosterol and L-methionine (Camadro *et al.*, 1986). However this increase was partially reversed by the addition of haem to the medium (Zagorec and Labbe-Bois, 1986), indicating that the production of coproporphyrinogen III oxidase is controlled by haem (Miyake and Sigimura, 1968). These observations confirm repression of coproporphyrinogen III oxidase synthesis by haem (Labbe-Bois *et al.*, 1980; Rytka *et al.*, 1984; Urban-Grimal and Labbe-Bois, 1981), since it had been shown earlier that haem acts as a transcriptional repressor of the coproporphyrinogen III oxidase gene, *HEM13* (Miyake and Sigimura, 1968; Labbe-Bois and Labbe, 1990). Transcriptional regulation by haem, and also by oxygen, was demonstrated by changes in the level of coproporphyrinogen III oxidase specific mRNA in yeast mutants deficient in haem biosynthesis (Yoshimoto and Sato, 1970; Zagorec and Labbe-Bois, 1986).

When *S. cerevisiae* is grown anaerobically, all of the enzymes for haem biosynthesis are present within the cell in an active state, and the pathway requires only the presence of oxygen to function. The cells respond to low oxygen concentration by increasing the amount of coproporphyrinogen III oxidase (step 6), and the absence of oxygen blocks the pathway since it is an essential component of the enzymatic reaction of coproporphyrinogen III oxidase (Zagorec *et al.*, 1988).

1.4. Genes encoding δ -aminolaevulinate synthase.

The study of genes encoding δ -ALAS in a range of organisms has led to a description of the primary structure of the enzyme and insights into the nature of the domains required for mitochondrial targeting, protein maturation and catalytic activity. A combination of molecular techniques which include cDNA sequencing, *in vitro* translation of mRNA, selected by hybridisation with the cloned δ -ALAS gene, or of total RNA followed by immunoprecipitation using antibodies against δ -ALAS, have shown that the enzymes in yeast and mammalian tissues are synthesised as larger precursor proteins (Grandchamp and Nordmann, 1988; Urban-Grimal *et al.*, 1986).

The processes of mitochondrial targeting of the precursor proteins are reviewed in Hay *et al.* (1984). In general, precursor proteins contain aminoterminal (N-terminal) extensions in the region of 20 to 70 amino acids which are necessary for mitochondrial targeting and intramitochondrial sorting. Once inside the mitochondrion the pre-sequence is generally removed by a metalloprotease. The δ -ALAS enzyme is synthesised in the cytosol as a precursor protein with the N-terminal protein sequence being proteolytically cleaved upon transport of the protein into the mitochondria, where it is attached to the matrix surface of the inner mitochondrial membrane (May *et al.*, 1986).

The sequence of δ -ALAS cDNA is known for the housekeeping enzymes of man (Bawden *et al.*, 1987), the rat (Srivastava *et al.*, 1988; Yamamoto *et al.*, 1988) and chicken (Borthwick *et al.*, 1985); and the erythroid isozymes in man (Cox *et al.*, 1991), mouse (Schoenhaut and Curtis, 1986) and chicken (Riddle *et al.*, 1989). Amongst microorganisms the complete nucleotide sequence of δ -ALAS gene has been determined in HEM1 in *Saccharomyces cerevisiae*, (Urban-Grimal *et al.*,

1986), the *hemA* gene of *Bradyrhizobium japonicum*, (McClung *et al.*, 1987) and the partial sequence of the *Rhizobium meliloti* gene is also known (Leong *et al.*, 1985). The deduced amino acid sequences show highly conserved regions which suggests that the genes have arisen from a common source in evolution.

Amino acid sequence comparisons using the LFASTA algorithm (Pearson and Lipman, 1988) indicates that the mammalian and yeast enzymes consist of a carboxy-terminal (C-terminal), which represents the core of the enzyme, and an amino-terminal (N-terminal) (Cox *et al.*, 1991). The C-terminal domain of all the proteins displays a high degree of sequence conservation which is less evident in the N-terminal domain. Hydropathy plots indicate that the N-terminal domain of these enzymes contain a highly hydrophilic region and is likely to be a suitable mitochondrial targeting signal (Dierks, 1990). The N-terminal signal sequence which is cleaved upon entry into the mitochondrion, possess no significant sequence homology. The cleavage site occurs between two glutamine residues in animals, at positions 61-62 in chicken, rat and human.

In the yeast, *Saccharomyces cerevisiae*, the enzyme δ -ALAS is located in the mitochondrion, using the substrates succinyl CoA and glycine in the production of δ -aminolaevulinic acid (δ -ALA) in a reaction requiring the cofactor, pyridoxal-phosphate. The enzyme is a homodimer having a specific activity of 39 μ mol of δ -ALA synthesised per hour and per milligram of protein at 30°C. The optimal pH of the enzyme is 7.5 (Volland and Felix, 1984).

The *HEM1* gene encoding δ -ALAS has been isolated (Arrese *et al.*, 1983; Bard and Ignolia, 1984; Urban-Grimal *et al.*, 1984) and the coding and flanking

regions sequenced (Urban-Grimal *et al.*, 1986; Keng *et al.*, 1986; Keng and Guarente, 1987). The gene codes a protein of 548 amino acids, with a molecular weight of 59kD. The deduced amino acid sequence shows extensive homologies with that of other δ -ALAS genes sequenced. The enzyme is synthesised in the cytosol as a precursor and transported into the mitochondria where it is cleaved to produce the mature enzyme (Urban-Grimal *et al.*, 1986).

In contrast to the regulation in hepatic tissues where δ -ALAS appears to be controlled by haem, the activity of δ -ALAS in yeast is expressed constitutively. However, analysis of the expression of the *S. cerevisiae* *HEM1* gene by fusion of its promoter to the *E. coli* β -galactosidase reporter gene identified a sequence similar to the upstream activator sites (UAS), for the haem activator proteins, *HAP2/3/4* (Keng and Guarente, 1987). The *HAP2/3/4* heterotrimeric protein is a 'CCAAT' binding transcriptional activation complex (Olesen and Guarente, 1990), which binds to UAS within the promoter region of regulated genes in a haem dependent manner (Zitomer and Lowry, 1992). When these sites are deleted from the promoter region of the *HEM1* gene expression is reduced (Keng and Guarente, 1987) indicating the role of the activation site in the constitutive expression of δ -ALAS. In addition to the positive *HAP2/3/4* activation site within the *HEM1* promoter region, a negative regulatory site is present which decreases the expression of *HEM1* gene under conditions of normal derepression (Keng and Guarente, 1987). Therefore the *HEM1* gene is constitutively expressed due to a composite effect of activation and repression of transcription (Keng and Guarente, 1987). The physiological signals and the protein factors involved in the regulation are still unknown, however it is possible that they operate to maintain a saturating supply of δ -ALA for the next enzyme in the pathway, porphobilinogen synthase.

Mutants strains of yeast lacking δ -ALAS activity (*hem1*⁻) require exogenous δ -ALA for growth (Keng and Guarente, 1987) showing that δ -ALAS is the sole source of δ -ALA in the cell. However recent reports have suggested that an alternative pathway for the biosynthesis of δ -ALA, the dioxovalerate transamination pathway previously reported only in mammals, occurs in three strains of *S. cerevisiae* (Hoare and Datta, 1990). One of the enzymes present this pathway, L-alanine:4,5-dioxovaleric acid transaminase, has been shown to be regulated by the intracellular "free haem pool" and it is possible that the pathway plays a role in controlling the biosynthesis of δ -ALA.

1.5. Aims and objectives.

The study of oxygen regulation in *Aspergillus nidulans* with particular regards to control of the biosynthesis of haem, was an area of active interest when I joined Dr Tim Pillar's group (October, 1990). There were no reports concerning δ -ALAS in filamentous fungi, and the isolation and sequencing of the gene in *A. nidulans* would clearly be a valuable contribution. The point has been made earlier that the general similarities of yeast and *Aspergillus* with the class of Ascomycetes strongly suggest that the nature of the haem biosynthetic pathway are most likely to be closely similar in the two organisms.

The principal objective is to clone and sequence the gene encoding the δ -aminolaevulinate synthase (δ -ALAS) of *A. nidulans*. An *A. nidulans* genomic library in the phage vector λ EMBL3 was available and will be screened using a DNA probe designed from knowledge of a strongly conserved amino acid sequence found in other δ -ALAS proteins. Positive clones will be mapped by restriction analysis, and sequenced using the dideoxy chain termination method. Southern blot analysis will enable characterisation of the gene locus and establish its copy number in the genome. Northern blot analysis will establish the size and abundance of the δ -ALAS specific mRNA. Primer extension analysis and S1 nuclease protection studies may be used to map both the 5' and 3' transcription termini respectively.

There are no haem requiring auxotrophic mutant strains of *A. nidulans* and consequently the regulation of the pathway remains unresolved in the filamentous fungi. A second objective is therefore to isolate, or to generate mutants in the haem biosynthetic pathway.

CHAPTER 2.

MATERIALS AND METHODS.

2.1. Genetic Materials and Methods associated with *Aspergillus nidulans*.

2.1.1. Strains.

The following were provided by Dr. Clive F. Roberts, Department of Genetics, University of Leicester.

<i>Aspergillus nidulans</i> R153	<i>wA3 pyroA4</i>	Armitt <i>et al.</i> , (1976).
D10 Diploid	<i>pyrG89/pyrG89, pabaA1/+</i> , <i>yA/+; +/wA3; bgaA4/+</i> ; <i>+ /pyroA4; qutE208/+</i> , <i>+ /fwA</i>	Streatfield <i>et al.</i> , (1992).
WA55	<i>pyrG89, pabaA1; qutE208</i>	(Kafer, 1958)
"MS" R65	<i>suadE, yA, adE20; wA3; galA1</i> ; <i>pyroA4; facA303; sB3; nicB8</i> ; <i>riboB2</i>	(Kafer, 1958)

2.1.2. Growth media.

All growth media were prepared in Q-water, distilled water passed through the Millipore Standard Milli Q System, which comprised of a Milligrade filter, two

ion exchange columns and a 22µm. Media was sterilised by autoclaving at 15 psi for 15 minutes with subsequent addition of (filtered) carbon source after cooling.

Defined minimal medium was made up according to Pontecorvo *et al.*, (1953) with modification as described by Roberts (1963). A 10x stock salt solution was prepared, containing trace elements, nitrogen and sulphur source. Minimal medium was prepared as a 1x solution and following autoclaving a carbon source was added aseptically from a sterile stock solution (Table 2.1) along with sterile 1M MgSO₄ to a final concentration of 10mM (Armitt *et al.*, 1976).

10x Stock salt solution

<u>Compound</u>	<u>Amount per litre</u>
NaNO ₃	60.0 g
KCl	5.2 g
KH ₂ PO ₄	15.2 g
Trace elements solution	10.0 ml

The pH of the stock salt solution was adjusted to pH 6.5 by the addition of solid sodium hydroxide pellets and dilute sodium hydroxide solution.

Trace element solution

<u>Compound</u>	<u>Amount per 100ml</u>
FeSO ₄ .7H ₂ O	0.100 g
ZnSO ₄ .7H ₂ O	0.880 g
CuSO ₄ .5H ₂ O	0.040 g
MnSO ₄ .4H ₂ O	0.015 g

$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$	0.010 g
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.005 g

Minimal agar (MA) medium for plates was solidified with 1.5% (w/v) agar.

Liquid culture contained the wetting agent Tween 80 at 10^{-5} (v/v).

Malt extract agar is a complex medium commonly used for fast growth and when good conidiation was required.

Malt Extract medium

<u>Nutrient</u>	<u>Amount per litre</u>
Malt extract (Difco)	20.0g
Bacto-peptone (Difco)	1.0g
D-Glucose	20.0g
Agar	15.0g

Nutritional supplements were added to both minimal and malt extract media as required (Table 2.2). The D10 diploid strain used in the knockout experiments (Chapter 6) required the additional uracil at 2.2g per litre of medium.

2.1.3. Growth and storage of strains.

All cultures were grown at 37°C. Stock cultures of strains were maintained on MEA slants. Long term storage of strains was on silica gels (Roberts 1969).

Table 2.1 **Concentrations of carbon sources.**

Carbon source	Stock solution	Final concentration
Acetate	2M	0.10M
Galactose	1M	0.02M
Glucose	1M	0.02M
Glycerol	2M	0.05M
Quinic acid	20% w/v	1% w/v

Stock solutions of 1M glucose, 2M glycerol and 2M acetate pH 6.5 were prepared in distilled water and sterilised by autoclaving at 15 psi for 10 minutes.

Table 2.2 **Concentrations of nutritional supplements.**

Supplement	Stock solution g.100ml ⁻¹	Final concentration µg.ml ⁻¹
p-Aminobenzoic acid	0.10	1.0
L-Methionine	0.50	50.0
Nicotinic acid	0.20	2.0
Pyrodoxine hydrochloride	0.05	0.5
Riboflavin	0.025	2.5
Sodium thiosulphate	4.0	40.0

Stock solutions of each supplement were prepared in distilled water and sterilised by autoclaving at 15 psi for 15 minutes. When required, uracil was added to the media at a concentration of 2.2g solid.litre⁻¹ prior to autoclaving.

2.1.4. Preparation of conidial spore suspension.

From a single fungal spore colony, a dilute suspension of the conidiospores were spread onto the surface of MEA plates and incubated for two to three days to provide a well conidiating confluent growth. The conidia were harvested by flooding the plates with 20ml Tween-saline solution (0.8% (w/v) NaCl; 0.025% (v/v) Tween-80) and drawing a glass spreader across the surface of the plates. The suspension was collected in sterile universal bottles, vortexed to disrupt any conidial chain and filtered through sterile a nylon filter cloth to remove mycelium. The suspension was centrifuged at 4000 rpm for 5 minutes in a Haraeus Christ centrifuge and the conidiospores washed by repeated centrifugation in saline solution (0.8% (w/v) NaCl) and finally in neutral phosphate buffer solution (20mM KH_2PO_4 ; 50mM Na_2HPO_4 ; 50mM NaCl; 0.4mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ final pH 7.2) in which the final suspension was stored at 4°C.

2.1.5. Preparation of heterozygous diploids.

Heterokaryons were synthesised by mixing conidia of two strains carrying complementary nutritional and colour markers on MEA plates containing appropriate supplements and incubating overnight. Small blocks of agar containing mycelium were then transferred to unsupplemented MA plates and incubated for 3 to 4 days. Heterokaryons, identified as rapidly growing sectors, were then subcultured on a fresh MA plate by repeated transfer of agar blocks containing mycelium taken from the edge of the growing colonies. Heterozygous diploid strains were selected by plating diluted suspensions of conidiospores, harvested from heterokaryons, on MA for single colony isolation (Roper, 1952).

Diploid strains were haploidised by stab inoculating conidial suspensions onto MEA plates containing benomyl ($0.7\mu\text{g.ml}^{-1}$) and supplemented with the nutritional requirements of the component haploid strains from which the diploid was constructed (Hastie, 1970). Haploid segregants were purified by streaking conidiospores onto MEA plates and then tested on appropriate media for the segregation of the genetic markers in the diploid.

2.1.6. Preparation of *A. nidulans* cell-free extracts for enzyme assays.

Liquid glucose minimal medium was inoculated with a conidiospore suspension to yield 10^6 spores per ml, in a 400ml baffled flasks and incubated at 37°C in a rotary incubator for 18 hours. Mycelium was harvested by filtration on Whatman No.1 filters, and washed with water and then with the extraction buffer (100mM sodium potassium phosphate buffer pH 7.6, 0.5mM pyridoxal phosphate, 3mM MgSO_4 2mM dithiothreitol). The mycelium was immersed in liquid nitrogen in a precooled mortar and ground to a fine powder with a cold pestle. The frozen powdered mycelium was resuspended in 1ml extraction buffer, allowed to thaw and extracted by gently shaking the slurry in a polypropylene tube on ice for 30 minutes, after which, the cell debris was removed by centrifugation for 10 minutes in an Eppendorf microfuge at 4°C . The supernatant was decanted, stored on ice and prepared to a 1:10 dilution. The enzyme activity was assayed within a maximum of 5 hours after extraction.

2.2. Genetic materials and methods associated with *Escherichia coli*.

2.2.1. Strains.

The following were provided by The Leicester Biocentre, University of Leicester.

NM522	$\lambda(lac-proAB), hisd15(r_k^-, m_k^+),$ $thi^-, supE, F'proAB, lacIQZ\Delta M15$	Gough and Murray (1983).
K12 5K	$thi^-, thr-1, leuB6, lacY1, tonA21,$ $supE44, r_k^-, m_k^+$	Leicester Biocentre strain
Q359	$r_k^-, m_k^+, supE44, tonA21$	Leicester Biocentre strain

2.2.2. Growth media.

Bacterial growth media were similar to those described in Miller (1972) and were used as liquid broth or as solid media when supplemented with Difco agar to a final concentration of 2.0% w/v. All media were prepared in Q-water and sterilised as described in section 2.1.2. The antibiotic ampicillin was added to appropriate media as required, to a final concentration of $25\mu\text{g.ml}^{-1}$.

Luria (LB) Media.

10g.l^{-1} Tryptone, 5g.l^{-1} Yeast extract, 5g.l^{-1} NaCl.

pH was adjusted to 7.2 with 5M NaOH.

2x YT Media.

16g.l⁻¹ Tryptone, 10g.l⁻¹ Yeast extract, 5g.l⁻¹ NaCl.

pH was adjusted to 7.2 with 5M NaOH.

M9 Media.

Prepared by diluting sterile 10x M9 salts and 100x M9 additive to 1x in sterile Q-water. For growth of *E. coli* NM522 the media is supplemented with 4% lactose, 1% thiamine B1 and 50ml.l⁻¹ amino acid mix.

10x M9 salts.

<u>Compound</u>	<u>Amount g.l⁻¹</u>
Na ₂ HPO ₄	70.0
KH ₂ PO ₄	30.0
NH ₄ Cl	10.0

100x M9 additives.

<u>Compound</u>	<u>Amount g.l⁻¹</u>
MgSO ₄	24.6
CaCl ₂ .6H ₂ O	2.2

Baltimore Biological Laboratories (BBL) Media.

10g.l⁻¹ BBL Trypticase, 5g.l⁻¹ NaCl, 10g.l⁻¹ Agar

For M13 overlays, BBL-Soft media was prepared as above but using 6.5g.l⁻¹ agar.

2.2.3. Growth and storage of *E. coli*.

All cultures were grown at 37°C. For short-term storage the organism was streaked on solid M9 media. A single colony was used to restreak fresh solid M9 media at three month intervals. Long-term storage was carried out as described in Maniatis *et al.* (1982). A single colony was used to inoculate 5ml of LB and grown to stationary phase by incubation with shaking at 37°C overnight. The cells were pelleted by centrifugation at 5000 rpm for 5 minutes in a Heraeus Christ centrifuge at 4°C and the pellets resuspended in 5ml M9 media. The cells were pelleted as before, resuspended in 1.5ml M9 media and 0.4ml aliquots mixed with 2.5ml sterile 50% w/v glycerol in 2ml screw cap tubes. The mixture was rapidly frozen at -80°C in a dry ice/ethanol bath and stored at -80°C. The cells were revived by transferring a small sample from the surface of the frozen aliquot to a plate of solid M9 media.

2.2.4. Plasmids and phage.

The origin of plasmids used for the construction of an *A. nidulans hemA* gene disruption vector is given below.

Plasmid	Reference
pDJB1	Ballance <i>et al.</i> , 1985
pIC 19R	Marsh <i>et al.</i> , 1984
pBS-M13+	Leicester Biocentre

2.3. Transformation.

2.3.1. DNA mediated transformation of *A. nidulans*.

The method used was based on that described by Ballance *et al.*, 1983.

Circular cellophane discs, the size of a 9cm petri dish, were sterilised by autoclaving and placed onto the surface of 20 appropriately supplemented MEA glucose plates. Approximately 10^6 conidiospores were evenly dispersed over each plate using a sterile glass spreader and the cultures were incubated for 15 hours at 37°C. The cellophane discs bearing young mycelium were divided equally between 4 clean petri dishes containing a 15ml solution of the cell wall dissolving enzyme complex Novozym 234 (5mg.ml^{-1}) in an osmotic stabiliser, 0.6M KCl, and placed at 30°C on a slow rotary shaker for 90 minutes. The cellophane discs were then removed from the resulting protoplast suspension and rinsed with 15ml of 0.6M KCl to remove adhering protoplasts. The suspension was passed through a nylon filter (Gallempkamp GMX-500-V) and a sintered glass filter (porosity 1) to remove all cell debris. The protoplasts were pelleted by centrifugation at room temperature at 1500 rpm for 5 minutes in a Hereaus Christ centrifuge followed by two washes in 0.6M KCl and one wash in 0.6M KCl, 50mM CaCl_2 . The protoplasts were resuspended in 500 μl of 0.6M KCl, 50mM CaCl_2 , and the concentration was estimated by counting the protoplasts present using a haemocytometer examined under a light microscope. The volume of the suspension was then adjusted to give $1\text{-}5 \times 10^7$ protoplast per ml. Approximately 10 μg of DNA in 20 μl of TE buffer pH 8.0 was added to 200 μl aliquots of the protoplast suspension in 20ml sterile disposable universal tubes, followed by the addition of 50 μl of PEG solution (25% PEG 6000, 50mM CaCl_2 , 10mM Tris.HCL, pH 7.5). The mixture was incubated on ice for 20 minutes after which a further 2 ml of the PEG solution

was added and the mixture left at room temperature for 5 minutes. Following the addition of 4ml 0.6M KCl, 50mM CaCl₂, the total transformation mixture was added to 100ml of appropriately supplemented molten minimal medium containing 0.6M KCl and 2% (w/v) agar held at 48°C. This molten agar was poured as a top layer onto similar supplemented minimal medium plates containing 0.6M KCl and 1.5% (w/v) agar. The plates were incubated at 37°C for 3 days.

The efficiency of protoplast regeneration was assessed by adding 100µl aliquots of 10⁻², 10⁻³ and 10⁻⁴ dilution of the final transformation mixture (including the PEG solution and the KCL/CaCl₂ solution) to molten complete medium containing 0.6M KCl and 2% (w/v) agar, pouring as a top layer onto similar complete medium plates and comparing the number of colonies obtained after 48 hours incubation at 37°C with the previous visual estimation of the number of protoplasts present.

All transformants obtained were replated by spreading dilute suspensions of conidiospores for single colony isolation on selective medium. This procedure was generally repeated at least twice, and the strains isolated stored by transferring conidiospores from a single colony to selective media slants to guard against loss of transforming DNA integrated into the genome.

2.3.2. DNA mediated transformation of *E. coli*.

Transformations were carried out with the *E. coli* strain NM522 based on the method described by Maniatis *et al.*, (1982).

A 1.5ml overnight stationary phase LB culture of *E. coli* was used to inoculate 200ml of LB prewarmed to 37°C, which was subsequently shaken vigorously at

37°C until reaching an OD of 0.45 - 0.6 at a wavelength of 600nm. Approximately 10ml of the culture was chilled on ice for 15 minutes and the cells were pelleted by spinning at 2500 rpm for 10 minutes in a Heraeus Christ centrifuge at 4°C. The cells were resuspended in 10ml of ice-cold 0.1M CaCl₂ and incubated on ice for 10 minutes. The cells were pelleted again before resuspending in 1ml ice-cold 0.1M CaCl₂. The cells were incubated on ice for 60 minutes upon which time they were competent for transformation.

Frozen stocks of competent cells were prepared by adding 1ml of competent cells to 333µl of glycerol/CaCl₂. The mixture was dispensed as 500 µl aliquots into Eppendorf tubes and quickly frozen by transferring to a dry ice/ethanol bath and stored at -80°C.

DNA to be transformed was mixed with 200µl of competent cells (either freshly prepared or thawed slowly from stock aliquots) and incubated on ice, with occasional agitation, for 50 minutes. The mixture was heat-shocked by incubation at 42°C for 2 minutes and returned to ice for 20 minutes. To each aliquot of transformed cells 10ml of LB prewarmed at 37°C was added and the mixture incubated at 37°C for 90 minutes. Aliquots of the transformation mixture (100 µl) or of appropriate dilutions in LB, were plated onto suitable selective media and the plates incubated overnight at 30°C.

2.4. Isolation of nucleic acids from bacteria and fungi.

2.4.1. Small scale rapid preparation of plasmid DNA from *E. coli* using Alkaline lysis.

The small-scale preparation of bacterial plasmid DNA was carried out according to the procedure of Birnboim and Doly (1979). This method was used for analysis of plasmids.

Single bacterial colonies were inoculated using sterile toothpicks into 2.0ml aliquots of LB containing an appropriate antibiotic selection condition and incubated overnight at 37°C with vigorous shaking. Approximately 1.5ml of the culture was transferred to an Eppendorf tube and the cells harvested by centrifugation for 2 minutes in a MSE Microcentaur centrifuge. The supernatant was removed by aspiration and the pellet was resuspended in 100µl of an ice cold TEG solution (50mM glucose, 10mM EDTA, 25mM Tris.HCl, pH 8.0). The sample was incubated at room temperature for 5 minutes and 200µl of a freshly prepared solution of 0.2M NaOH, 0.1% (w/v) SDS was added. The tube was mixed gently followed by incubation on ice for 5 minutes. 150µl of an ice cold solution of potassium acetate (60ml of 5M potassium acetate, 11.5ml of glacial acetic acid, 28.5ml of Q-water, pH 4.8) was added to the tube and mixed by gentle vortexing followed by incubation on ice for 5 minutes. The tube was centrifuged in an MSE Microcentaur centrifuge for 5 minutes to yield a clear supernatant which was transferred to a fresh tube. 400µl of phenol/chloroform was added, the tube was vortexed followed by centrifuging in an MSE Microcentaur centrifuge for 2 minutes after which the supernatant was transferred to a fresh tube.

To the supernatant 800µl of ethanol was added to precipitate nucleic acids. The samples were mixed well and incubated at room temperature for 2 minutes. The precipitate was collected by centrifugation for 5 minutes and the supernatant discarded. The pellet was resuspended in ice cold 70% ethanol and recentrifuged for a further 5 minutes. The supernatant was discarded and the nucleic acids dried *in vacuo* for 5-10 minutes. The nucleic acids were resuspended in 50µl TE buffer pH 8.0 containing 1µl RNase A (10mg.ml⁻¹) which had been boiled for 10 minutes to remove DNase activity. Plasmid DNA prepared from this method were stored at -20°C.

2.4.2. Large-scale preparation by caesium chloride buoyant density centrifugation.

A single colony of transformed *E. coli* NM522 was inoculated into 400ml of LB containing an appropriate selection and incubated at 37°C overnight with vigorous shaking. The cells were pelleted by centrifugation at 8000 rpm for 10 minutes in a Sorvall GSA rotor at 10°C. The cells were resuspended in 20ml of ice-cold SUT solution (25% w/v sucrose, 50mM Tris.HCl, pH 8.0) and recentrifuged at 10000 rpm for 10 minutes in a Sorvall SS34 rotor at 4°C. The supernatant was discarded and the cells were resuspended in 8ml TES solution (50mM Tris.HCl, pH 8.0, 5mM EDTA, 50mM NaCl) and transferred to a 200ml Erlenmeyer flask. 2ml of 5mg.ml⁻¹ lysozyme made up in TES solution was added to the flask and the suspension mixed by vortexing followed by incubation on ice for 10 minutes. 300µl 0.5M EDTA was mixed into the suspension followed by a 10 minute incubation on ice. With gentle swirling 10ml Lysis Buffer (0.2% Triton, 100mM EDTA, pH 8.0, 20mM Tris.HCl, pH 8.0) was added and the mixture incubated on ice for a further 10 minutes.

The lysate was ultracentrifuged at 35000 rpm for 35 minutes in a Sorvall T-865 ultracentrifuge rotor at 4°C. The supernatant was decanted into a fresh 100ml flask, 3ml 1M Tris.HCl pH 8.0 and 10ml phenol were added, the suspension mixed and incubated at room temperature for 5 minutes. Phases were separated by centrifugation at 8000 rpm for 10 minutes in a Sorvall HB-4 rotor at 4°C in Corex tubes. The aqueous layer was transferred to a clean flask and extraction repeated with 12ml phenol/chloroform.

The cleared lysate was mixed with 2.0g ammonium acetate and an equal volume of iso-propanol and the nucleic acid precipitated for 30 minutes. The precipitate was pelleted by centrifugation at 8000 rpm for 10 minute in a Sorvall GSA rotor at 4°C. The supernatant was discarded and the pellets resuspended in 5ml TE buffer into which 50µl RNase A (10mg.ml⁻¹) was added and the mixture incubated at room temperature for 30 minutes. A further 16.0ml of TE buffer was added along with 200µl 0.5M EDTA, pH 8.0, followed by 21.2g solid caesium chloride. The suspension was decanted into a Sorvall VTi-850 polyallomer centrifuge tube and filled with paraffin oil. 700µl of ethidium bromide was added and the tubes sealed. The tubes were centrifuged under vacuum at 40000 rpm for 18 hours at 20°C in a Sorvall VTi-850 rotor. After centrifugation DNA was visualised using short-wave UV light and the lower, plasmid, band removed using a 5ml syringe and a 19 gauge hypodermic needle.

The plasmid-containing fraction was vortexed for 1 minute with a 1/2 volume of butanol, the phases were allowed to separate and the aqueous layer removed to a clean tube. This procedure was repeated until no ethidium bromide was visible within the plasmid-containing phase. The nucleic acid was precipitated by mixing the plasmid-containing phase with 100% ethanol and incubating at room temperature for 10 minutes. The mixture was centrifuged at 9000 rpm for

10 minutes in a Sorvall HB-4 rotor at 20°C, the supernatant discarded and the pellet washed in 70% ethanol and recentrifuged.

The nucleic acids were redissolved in 600µl sterile Q-water and absorbance at 260nm determined using a Shimadzu spectrophotometer. Yields varied between 500µg and 5mg of plasmid DNA.

2.4.3. Small scale preparation of chromosomal DNA from *A. nidulans*.

Chromosomal DNA was prepared from a dense conidiospore suspension by a method from Morris (1978) which was adapted by Beri *et al.*, (1987). Conidiospores were harvested from the surface of a single MEA plate as described above (Section 2.1.4). The conidiospores were washed twice by centrifugation in ice-cold spermidine lysis buffer (5mM spermidine, 100mM KCl, 10mM EDTA, 10mM Tris.HCl pH 7.5, 250mM sucrose) and were finally resuspended in 0.8ml of the same buffer. The conidiospore suspension was transferred to a glass MacCartney bottle containing 3g sterile acid washed 0.45mm glass beads (Section 2.4.4) and vigorously agitated by vortexing for 5 minutes. A further 0.8ml of spermidine lysis buffer was added to the slurry and the supernatant distributed between two Eppendorf tubes. To each tubes 67µl of a 10% (w/v) solution of SDS was added followed by incubation at 60°C for 60 minutes. After incubation 222µl of 4M sodium acetate pH 6.0 was added to each tube, gently mixed and incubated on ice for 30 minutes. The tubes were centrifuged for 5 minutes in a microcentrifuge and the supernatant transferred to fresh tubes. To each supernatant proteinase K, at a final concentration of 200µg.ml⁻¹, and RNase A, at a final concentration of 100µg.ml⁻¹, were added followed by incubation at 37°C for 60 minutes. The DNA was precipitated by the addition of 90µl 4M sodium acetate pH6.0 and 540µl isopropanol. The tubes were centrifuged for 5 minutes in a microcentrifuge and the nucleic acids

pellet were rinsed with 70% ethanol, dried *in vacuo* and pooled by resuspending in 200µl TE buffer. The solution was extracted twice with phenol/chloroform and then with chloroform, after which the nucleic acid was recovered by the addition of 0.1 volume 4M sodium acetate and 0.6M isopropanol followed by washing in 70% ethanol, drying as above and finally resuspending in 50µl TE buffer. DNA preparations were stored at 4°C.

2.4.4. Extraction of total RNA from *A. nidulans*.

The method used for the extraction of total RNA from *A. nidulans* was as described by Bradshaw and Pillar (1992).

A density of 10^6 spores.ml⁻¹ (section 2.1.4) were inoculated into siliconised flasks containing 400ml *Aspergillus* minimal medium with the appropriate supplements. The cultures were grown at 37°C with shaking at 200 rpm for 18 hours then chilled on ice for 10 minutes before harvesting by vacuum filtration using sterile Whatman filter paper.

Mycelium were washed with ice-cold water (vacuum filtration) and quickly transferred to a 30ml siliconized glass Corex tube (DuPont) containing ice-cold sorbitol lysis buffer (700mM sorbitol, 50mM Tris.HCl pH 7.5, 10mM EDTA) such that a final concentration of 1.5g wet weight of mycelium to 1.5ml buffer is achieved. To this, 50µl β-mercaptoethanol (Sigma) and 3g weight of cold glass beads (B. Braun, Melsungen AG. Diameter 0.45 - 0.50mm) were added, the mixture was vortexed for 60 seconds then rapidly frozen in a dry ice/ethanol bath. To the frozen pellet 2.5ml water, 300µl 1M Tris.HCl pH 8.0, and 500µl 10% (w/v) SDS was added and the mixture boiled for 3 minutes in a 105°C PEG 6000 bath followed by a 20 second vortex.

Glass beads: Glass beads were washed in 1M HCl overnight and rinsed thoroughly to pH 7.0. They were then dried and baked at 150°C for a minimum of 4 hours.

Phenol:chloroform:IAA (25:24:1) extractions were performed and repeated until the interface was cleared after which a final chloroform:IAA (24:1) extraction was carried out. Nucleic acids were precipitated with a 0.3M solution of ammonium acetate in ethanol for 10 minutes, on ice and pelleted by centrifuging in a MSE microcentrifuge. The pellets were dried and resuspended in TE and the quantity of nucleic acids estimated by measuring the absorbance at 260nm in a Shimadzu UV240 spectrophotometer and assuming an OD₂₆₀ of 1.0 is equivalent to 37µg single stranded RNA.ml⁻¹ (Maniatis *et al.*, 1982).

2.5. DNA manipulation techniques.

All methods were based upon those described by Maniatis *et al.*, (1982) but modified where recommended by suppliers.

2.5.1. Restriction endonuclease digestion of DNA.

Typically, the required quantity of DNA was digested in a total volume of between 10 μ l and 100 μ l. The quantity of enzyme used and the time of incubation were normally equivalent to a ten fold excess digestion. All restriction endonuclease enzymes were incubated with DNA at temperatures and in buffering conditions as recommended by the suppliers. All restriction endonucleases were purchased from BRL. All restriction enzyme buffers (BRL REact buffers) were supplied by the manufacturers at 10x concentration and stored at 4°C.

2.5.2. Preparation of DNA restriction fragment for further modification.

Restriction containing 0.5 μ g to 1.0 μ g of the DNA were diluted to a volume of 100 μ l with sterile Q-water and vortexed for 2 minutes with 100 μ l of phenol. Phases were separated by centrifugation at high speed in a microcentrifuge for 10 minutes. The aqueous layer was removed and the extraction was repeated using 100 μ l phenol/chloroform.

The aqueous layer was then mixed with 10 μ l 3M sodium acetate pH 5.5 and 250 μ l absolute ethanol. The mixture was incubated at -70°C for 15 minutes, then the DNA was pelleted by spinning for 10 minutes in a microcentrifuge. The pellet was drained, rinsed in 500 μ l of 70% v/v ethanol, repelleted, drained,

vacuum dried and resuspended in TE buffer. DNA fragments were stored at -20°C until required for further restriction.

2.5.3. Agarose gel electrophoresis of DNA.

Agarose was dissolved at concentrations ranging from 0.4% to 1.5% (w/v), in 1x TAE buffer by boiling. The agarose was allowed to cool before casting on to a perspex gel bed with a well former. Gels were submerged in 1x TAE buffer with 1.0µg.ml⁻¹ ethidium bromide. Between 100ng and 5µg of DNA were mixed with 0.1 volume of 10x loading buffer (2M urea, 50% glycerol, 50mM TAE buffer, 0.4% xylene cyanol, 0.4% bromophenol blue, 0.4% Orange G) and the mixture loaded into the wells. Electrophoresis was carried out at a constant voltage between 20V and 90V and completed when sufficient resolution was obtained in a range of molecular weights.

DNA were visualised in agarose gels by irradiation under short-wave UV light from a Fotodyne transilluminator. Results were photographed using a Polaroid MP4 camera with type 57 or 55 film.

2.5.4. Recovery of DNA fragments from agarose gel.

Two methods were used to isolate DNA fragments from agarose gels.

2.5.4.1. GeneClean.

This method was originally described by Vogelstein and Gillespie (1979). The procedure and reagents are supplied commercially by BIO 101 as their GeneClean Kit.

A quantity of plasmid DNA as described in Section 2.5.1 was digested to release a known amount of the required restriction fragment. The digested reaction mix was then loaded onto a 0.8% - 1.0% (w/v) low gelling temperature agarose gel (FMC Sea Plaque agarose) and electrophoresed until suitable separation of the fragments was achieved. The section of agarose containing the required fragment was removed using a scalpel and transferred to a preweighed Eppendorf tube and the tube reweighed to determine the mass of the gel slice. The gel slice was then macerated and a volume of saturated NaI (supplied with the GeneClean Kit) equivalent to 1 - 2.5x the mass of the gel slice in mg was added. The agarose was melted by incubation at 55°C for 8 minutes. 5µl of an aqueous solution of glass powder (Glassmilk, as supplied with the GeneClean Kit) was then added with a 5 minute incubation on ice to allow binding of DNA to the glass surface. The Glassmilk was pelleted by a 5 second spin at high speed in a microcentrifuge, the supernatant was discarded and the pellet washed three times in 500 µl of NEW buffer (50% (20mM Tris.HCl pH 7.2, 0.2M NaCl, 2mM EDTA), 50% ethanol).

The washed pellet was then resuspended in 20µl sterile TE buffer and incubated at 55°C for 3 minutes to elute the DNA from the Glassmilk. The glass was pelleted by a 30 second spin at high speed in a microcentrifuge and the supernatant containing the DNA transferred to a fresh Eppendorf tube. This procedure was repeated with two 15µl aliquot of sterile TE buffer to give a final volume for the eluted DNA solution of 50µl. 5µl of this solution was electrophoresed with known amounts of lambda bacteriophage DNA to estimate the recovery of fragment DNA. Recovery with this method ranged between 80% - 100%.

2.5.4.2. Electroelution.

A known quantity of the DNA fragment to be isolated was electrophoresed on a 1% (w/v) medium gelling temperature agarose (FMC SeaKem) until suitable separation of restriction fragment was achieved. The section of agarose containing the required fragment was removed with a scalpel and transferred to a dialysis bag which was previously sterilised in 1x TAE buffer. 500µl of 1x TAE buffer was added to the bag which was then sealed. The bag was placed, with the agarose gel in the same orientation to the original electrophoresis, in an electrophoresis tank containing 1x TAE buffer and electrophoresed at 90V for 60 minutes followed by a reverse run for 30 seconds. The solution in the dialysis bag containing the nucleic acid was placed in an Eppendorf tube. The DNA was then purified by phenol/chloroform extraction and ethanol precipitation as described in Section 2.5.2.

2.5.5. Fill-in of recessed ends of DNA fragments.

Filling in of 3' recessed ends of DNA fragments generated by restriction endonuclease digestion was carried out by incubating the recovered fragment in 16µl sterile Q-water, 2µl 10x "fill in" buffer (500mM Tris.HCl pH 7.2, 100mM MgSO₄, 10mM DTT, 500µg.ml⁻¹ BSA), 2µl Chase mix (10mM Tris.HCl pH 8.0, 0.1mM EDTA, 0.25mM dATP, 0.25mM dGTP, 0.25mM dCTP, 0.25mM dTTP) and 1 unit of the Klenow fragment of DNA polymerase I at 37°C for 30 minutes. The reaction mixture was then extracted with phenol/chloroform, and the DNA precipitated with ethanol and resuspended in the required volume of TE buffer.

2.5.6. Ligation of DNA fragments.

DNA fragments, isolated as described above, were heated at 65°C for 5 minutes to separate annealed molecules, rapidly cooled on ice and mixed in an approximate ratio of 5 molecules of required insert fragment : 1 molecule of required vector fragment. Typically, 100ng of the vector DNA was used. To the DNA fragment mixture 1µl of 10x Ligation Buffer (0.1M MgCl₂, 0.1M DTT, 10mM ATP, 1mg.ml⁻¹ BSA, 0.5M Tris.HCl pH 7.5) was added and the volume made up to 9.5µl with sterile Q-water. 0.5 µl of 5 units. l⁻¹ BRL T4 DNA ligase was added to the mixture and the reaction incubated overnight at 15°C.

Calf intestinal alkaline phosphatase was also used in some cases to reduce the degree of recircularisation of the vector molecule. The endonuclease digested plasmid DNA molecule recovered from an agarose gel was incubated in dephosphorylation buffer (10mM ZnCl₂, 10mM MgCl₂, 100mM Tris.HCl pH 8.0) with the appropriate amount of calf intestinal phosphatase (manufacturer's conditions) for 30 minutes at 37°C. Subsequently, SDS and EDTA were added to a final concentration of 0.5% and 5mM respectively and proteinase K to a final concentration of 100µg.ml⁻¹. The mixture was incubated for a further 30 minutes at 56°C in order to digest the phosphatase. Prior to conducting ligation reactions, DNA was recovered from the reaction mixture by phenol/chloroform extraction and ethanol precipitation, and was finally resuspended in the appropriate volume of TE buffer.

2.6. Nucleic acid hybridisation analysis.

2.6.1. DNA-DNA (Southern) hybridisation.

Southern blot analysis of bacterial plasmid was performed as described in Maniatis *et al.*, (1982).

2.6.1.1. Southern Transfer.

DNA samples are fractionated by electrophoresis on a 1.5% (w/v) agarose gel as described in Section 2.5.3. After electrophoresis the gels were photographed with a ruler alongside so enabling a graph of log molecular weight verses mobility of the (unlabelled) marker fragments to be drawn. The gels were denatured for 30 minutes in denaturing solution (0.5M NaOH, 1.5M NaCl) and neutralised for 30 minutes in neutralising solution (1.5M NaCl, 0.001M EDTA, 0.5M Tris.HCl pH 8.0).

A wick, cut from Whatman 3MM paper was soaked in 20x SSC buffer and placed across a glass plate. The glass plate and wick were then placed in a plastic tray containing 20x SSC buffer (3M NaCl, 0.3M sodium citrate, pH adjusted to 7.0 with NaOH) such that only the ends of the wick were submerged. A piece of Whatman 3MM paper was cut to a size 1cm larger than the gel to be blotted, soaked in 20x SSC buffer and placed on flat surface of the wick. Two pieces of Whatman 3MM paper were cut to the same size of the gel, soaked in 20x SSC buffer and placed onto the previous sheet of filter paper to act as a pad. The wick and glass plate were covered in SaranWrap and a hole cut with a scalpel around the pad of filter papers to the same size as the gel.

The gel was placed, with wells facing down, onto the pads and air bubbles were removed by rolling a glass pipette along the gel surface. A piece of Hybond-N hybridisation filter (Amersham International) cut to the size of the gel to be blotted was wetted with 20x SSC buffer for 45 minutes prior to transfer and placed on the gel surface. Air bubbles are again removed and five pieces of dry Whatman 3MM paper cut to the size of the gel were placed over the filter. A stack of Kimwipe paper towels 6-7cm thick were placed over the gel and compressed using a suitable weight.

Transfers were carried out overnight at 4°C after which the Hybond-N filter was removed from the gel surface and rinsed in 3x SSC buffer to remove residual agarose. The filter was blotted with Whatman 3MM filter paper and dried further by placing in a 65° C incubator for 10 minutes. DNA was covalently crosslinked to Hybond-N by placing the filter on a single layer of SaranWrap over a Fotodyne UV transilluminator and irradiating for 2 minutes.

2.6.1.2. Preparation of radioactive DNA probes.

DNA fragments used in radiolabelling reactions were prepared as described in Section 2.5.4.

DNA to be radiolabelled was first denatured by boiling in an Eppendorf tube placed in a 105°C PEG 6000 bath for 10 minutes. The DNA was subsequently placed on ice to prevent renaturation of the denatured DNA.

The radiolabelling reaction was carried out by the addition of the following reagents in a total volume of 10µl:

3.0 μ l of sterile Q-water,
2.0 μ l of OLB C,
0.5 μ l BSA (Sigma, 10mg.ml⁻¹),
50ng DNA,
1.0 μ l [α -³²P]dCTP (3000Ci.mMol⁻¹, 10mCi.ml⁻¹),
1.0 μ l Klenow solution (3 μ l water, 1 μ l OLB C, 1 μ l Klenow fragment 6.0units.l⁻¹).

The labelling reaction was incubated at room temperature for 5 hours or overnight.

OLB buffer was prepared as follows:

Solution O: 1.25M Tris.HCl, pH 8.0
0.125M MgCl₂,
pH adjusted to 8.0 and stored at 4°C

Solution A: 1000ml solution O,
18 μ l β -Mercaptoethanol (Sigma),
5ml each of dATP, dTTP, dGTP.

dNTP's: dATP, dTTP, dGTP. Each 0.1M in TE.

Solution B: 2M HEPES-NaOH (pH adjusted to 6.6 with 4M NaOH)

Solution C: Hexadeoxynucleotides. (Pharmacia)
50 OD_{260nm} units dissolved in 550 μ l TE to give a
concentration of 90 OD_{260nm}.ml⁻¹. Stored at -20°C.

OLB C buffer: Mix solutions A:B:C in the ratio 10:25:15.

The reaction was terminated by the addition of 100 μ l of Stop C buffer (20mM NaCl, 20mM Tris.HCl pH 7.5, 2mM EDTA, 0.25% (w/v) SDS, 1 μ M dCTP).

To analyse the efficiency of incorporation 1.0 μ l of the stopped reaction mix was added to 2.5 μ l of salmon sperm DNA (10mg.ml⁻¹), 500 μ l water and 150 μ l 50% TCA. The mixture was filtered through a Whatman GF/C glass microfibre filter paper, washed with 2.0ml 50% TCA followed by 3.0ml 100% ethanol. The filter paper was allowed to dry before placing in a scintillation vial with 5.0ml Optiscint 'T' scintillation fluid (LKB). A further 1 μ l of the stopped reaction mix was added to a Whatman GF/C glass microfibre filter paper, placed into a scintillation vial to which 5.0ml of Optiscint 'T' scintillation fluid (LKB) was added. The radioactive counts per minute present was determined by 32-phosphorus counting in a Beckman LS 6800 liquid scintillation counter. A percentage ratio of the filtered mix against the unfiltered mix revealed the percentage efficiency of incorporation. DNA was routinely labelled to a percentage efficiency of 45% - 80%.

Unincorporated nucleotides were removed by fractionating the stopped reaction mix on a column of medium grade Sephadex G-50 resin. A glass Pasteur pipette was plugged with polyallomer wool and packed with Sephadex G-50, equilibrated with TE, to within 0.5-1.0cm of the top. The column was washed with 2.0ml TE (pH 8.0). The reaction mixture was applied to the top of the column and the first fraction collected in an Eppendorf tube. A further 13 fractions were collected by applying 100 μ l aliquots of TE (pH 8.0) to the column. Radiolabelled DNA fragments appeared in the first 4 - 5 fractions containing radioactivity. These were pooled and the number of radioactive counts per minute present was determined by Cerenkov counting in a Beckman LS 6800 liquid scintillation counter. An estimate of the specific activity of the

radiolabelled DNA fragment was made by calculating the counts per minutes per μg of DNA. DNA was routinely labelled to a specific activity of $0.5 - 1.0 \times 10^9 \text{CPM} \cdot \mu\text{g DNA}^{-1}$. It was assumed that near 100% recovery of the probe DNA fragment occurred in the first four radioactive fractions.

2.6.1.3. Hybridisation of Southern blots with radiolabelled DNA probes.

Southern blots were sealed into plastic bags (Transatlantic Plastics Ltd) with an electric bag sealer (Calor) ready for prehybridisation and hybridisation to radiolabelled probe DNA. The blots were prehybridised by adding 10ml 3x SSC, 0.5% (w/v) SDS, $50 \mu\text{g} \cdot \text{ml}^{-1}$ sonicated salmon sperm DNA (Sigma), 1x Denhardt's solution to the bags.

Salmon sperm DNA: The DNA was previously boiled for 10 minutes in a 105°C PEG 6000 bath.

50x Denhardt's solution is: 1% w/v Ficoll, 1% w/v PVP, 1% w/v BSA.
(All reagents from Sigma).

The hybridisation bags were resealed after removal of any air bubbles and prehybridisation carried out at 65°C with gentle agitation for 2 - 4 hours.

The solution used during hybridisation was the same as that for prehybridisation. Fresh solution was added to the bags. Radiolabelled probe DNA was denatured by boiling for 10 minutes in a 105°C PEG 6000 bath. The probe was placed on ice before injection into the hybridisation bag with a 1.0ml plastic syringe and 1.2mm x 40mm needle. Air bubbles were removed before resealing the bag which was then placed at 65°C with gentle agitation for 16 - 48 hours.

2.6.1.4. Washing of radioactive Southern blots.

After hybridisation excess radiolabelled probe DNA was removed from Southern blots by rinsing four times in 3x SSC, 0.2% (w/v) SDS. Each wash was carried out for 30 minutes in a sealable plastic sandwich box with gentle agitation.

2.6.1.5. Autoradiography of radioactive Southern blots.

After washing radioactive Southern blots were blotted dry on Whatman 3MM paper and covered in SaranWrap. Autoradiography was performed in sealed X-ray cassettes (Genetic Research Instrumentation Ltd) with tungstate intensifying screens at -80°C using Fuji RX or Kodak XAR X-ray film. Autoradiographs were processed in a Agfa-Gevaert Gevomatic-60 film processor.

Analysis of the autoradiographs was carried out by a comparison of the migratory distances of the band sizes on the autoradiograph of Southern blots with those of restriction fragments of known molecular weights.

2.6.1.6. Preparation of radioactive Southern blots for rehybridisation.

Probes bound to the target DNA and any background radioactivity on the Southern blot was removed by washing the filters in 1% (w/v) SDS, 1mM EDTA heated to boiling and then incubated in the same buffer at 65°C for 1 hour in a resealable plastic sandwich box. Efficiency of removal was confirmed by use of a Mini-Monitor. Rehybridisation with radiolabelled probe DNA probe was then carried out as above.

2.6.2. DNA-RNA (Northern) hybridisation.

Northern analysis was performed essentially as described in Bradshaw and Pillar (1992).

2.6.2.1. Denaturation and electrophoresis of RNA.

Agarose (Miles HSB) was dissolved, to a concentration of 1.0% (w/v), in 1x MOPS buffer by boiling in a microwave oven. The molten agarose was allowed to cool to 55° C before pouring onto a flat 12.5cm x 10.0cm glass plate with slot formers whereby, due to surface tension, a gel thickness of 2mm was achieved.

For the electrophoresis of RNA, 20µg RNA, as prepared in Section 2.4.4, was diluted into 200µl DNase buffer (0.1M sodium acetate, 0.5M MgSO₄ pH 5.0) to which 1 unit of RNase free DNase (Mannheim Boehringer) is added and the mixture incubated at 37°C for 15 minutes. To the mixture 100µl of phenol/chloroform was added, vortexed and the phases separated by spinning in a MSE microcentrifuge. The upper phase was removed to a fresh tube and the nucleic acids precipitated with 600µl 0.3M ammonium acetate/ethanol solution by incubating on ice for 5 minutes. The precipitate was pelleted by spinning in a MSE microcentrifuge and the supernatant discarded. The pellet was washed in 70% ethanol, respun, the supernatant discarded and then dried before thoroughly resuspending in 4.0µl sterile Q-water.

In a separate Eppendorf tube 1.5M glyoxal (Sigma), 50% DMSO (Fluka) and 1x MOPS solution (10x MOPS: 0.4M MOPS pH 7.0, 100mM sodium acetate, 10mM EDTA pH 8.0) was mixed of which 16µl was added to the 4.0µl of resuspended

RNA. After thorough vortexing, the RNA suspension was incubated, under a layer of light mineral oil, at 50°C for 60 minutes.

Glyoxal: Glyoxal was deionized on mixed-bed resin (bird, AG 501-X8) and stored in aliquots at -80°C under autoclaved light mineral oil (Sigma).

To the wells in the agarose, 8.0µl RNA was loaded directly from the glyoxalation reaction and a tracker dye was included in one lane. Electrophoresis was carried out at 80V for 3 hours at 4°C. RNA was visualised on gels by staining in a solution of 5µg.ml⁻¹ ethidium bromide. Where Northern transfer was to be performed RNA markers (BRL) were electrophoresed in a lane at the edge of the gel which was removed after electrophoresis and stained separately. This was photographed next to a ruler so that a calibration graph as described in Section 2.6.1.1 was obtained.

2.6.2.2. Northern transfer.

Transfer of RNA from agarose gels to Hybond-N filter was carried out essentially as described in Section 2.6.1.1. Gels containing RNA samples were rinsed briefly in sterile Q-water and set up on the blotting apparatus as detailed. Filters were treated after Northern transfer in the same way as Southern blots (Section 2.6.1.1). Following UV crosslinking, glyoxalation was reversed by baking the filter at 80°C for 2 hours in a vacuum oven.

2.6.2.3. Hybridisation of Northern blots with radiolabelled DNA.

The conditions used during the hybridisation of Northern blots to radiolabelled DNA are based on those recommended for use with Hybond-N by the manufacturers.

Northern blots were sealed in plastic hybridisation bags as detailed in Southern blots and prehybridised in 5x SSPE (20x SSPE: 3M NaCl, 0.2M NaH₂PO₄·2H₂O, 0.02M EDTA, pH adjusted to 7.7 with NaOH), 5x Denhardt's solution, 0.2% (w/v) SDS, 50µg.ml⁻¹ sonicated salmon sperm DNA (Section 2.6.1.3). The bag was sealed and incubated at 45°C for a minimum of 4 hours with gentle agitation.

Hybridisation was carried out after removing the prehybridisation buffer and replacing it with a fresh sample. Denatured radiolabelled DNA (Section 2.6.1.2) was introduced into the hybridisation bag as detailed in Section 2.6.1.3. Hybridisations were carried out at 65°C for 36-48 hours with gentle agitation.

2.6.2.4. Washing, autoradiography and analysis of radioactive Northern blots.

Procedures for the washing, autoradiography, and analysis of Northern blots were carried out essentially as described for Southern blots. Size analysis of bands on autoradiographs of Northern blots hybridised with radiolabelled probe DNA was by comparison with the migration of RNA size markers (BRL).

2.6.2.5. Preparation of radioactive Northern blots for rehybridisation.

Probes bound to the target RNA and any remaining background was removed by washing the filter in 2mM EDTA pH 8.0, 0.1% Denhardt's solution, 5mM Tris HCl pH 8.0 at 65°C for 2 hours in a plastic sandwich box. Efficient removal of the radiolabelled probe DNA was confirmed by use of a Mini-Monitor.

2.7. Primer extension analysis.

2.7.1. Synthesis and purification of oligonucleotides.

Synthetic oligonucleotides were used as radioactively-labelled DNA probes, sequencing primers and primer extension primers. Oligonucleotides were synthesised using an Applied Biosystems 380B DNA synthesiser with the reagents and protocols of the Department of Biochemistry, University of Leicester.

Oligonucleotides were precipitated in 0.1vol 3M sodium acetate, 2vol ethanol and resuspended in 200 μ l Q-water. The concentration of the nucleic acid was determined on the basis that 1unit OD_{260nm} = 20mg DNA (Maniatis *et al.*, 1982). Purification by UV-shadowing was carried out as follows: A 20% denaturing acrylamide gel was prepared which comprised of 42g urea, 50ml 40% stock acrylamide (38:2, acrylamide:bisacrylamide) and 10ml 10x TBE (55g.ml⁻¹ Boric acid, 9.3g.ml⁻¹ EDTA, 108g.l⁻¹ Trizma base) in a total volume of 100ml. Following degassing, polymerisation was initiated by the addition of 600 μ l 10% ammonium persulphate and 50 μ l TEMED and the liquid poured between two narrow siliconised glass plates ensuring that no air bubbles were introduced. After polymerisation the gel was electrophoresed with 1x TBE buffer for 45 minutes at 1000V. The oligonucleotide sample was loaded with TBE marker dyes after boiling for 2 minutes in the presence of 50% formamide and electrophoresed for 2 - 3 hours at 1500V.

The gel was then removed from the apparatus, placed between two sheets of SaranWrap and the oligonucleotide band was visualised under short-wave UV with the aid of a TLC backplate. The main oligonucleotide band was recovered from the gel simply by cutting out the relevant area and removing both strips

of SaranWrap. The acrylamide slice was then transferred to an Eppendorf tube containing 200 μ l Q-water and left to elute overnight. The oligonucleotide was passed down a TE-equilibrated Sephadex G25 column to remove particles of acrylamide. The concentration of DNA in the eluant (300ml) was determined and adjusted to 1mg.ml⁻¹.

2.7.2. Preparation of RNA.

50 μ g of RNA template was precipitated with an equal volume of 6M lithium acetate (brought to pH6.0 with acetic acid) and incubated on ice for 1 hour. The RNA was recovered by pelleting in a microcentrifuge, the pellet was washed with 3M lithium acetate and finally dissolved in Q-water to a final concentration of 10 μ g. μ l⁻¹.

2.7.3. Preparation of kinased primer.

The oligonucleotide primer was end-labelled with [γ -³²P]dATP as follows. The reaction was carried out in a total volume of 25 μ l and consisted of 50ng primer, 2.5 μ l Kinase buffer (0.5M Tris.HCl pH 7.5, 0.1M MgCl₂, 50mM DTT, 1mM spermidine, 1mM EDTA), 5 units T4 Polynucleotide kinase (New England Biolabs) and 50 μ Ci [γ -³²P]dATP. The mixture was incubated at 37°C for 30 minutes before termination by the addition of 2.0 μ l 0.5M EDTA and the volume made up to 100 μ l with Q-water. Unincorporated radioactivity was removed by column fractionation using fine grade G25 Sephadex whereby the sample was applied to the top of the column. Once it was equilibrated by the addition of 5 x 100ml aliquots TE, the column was briefly centrifuged in a Hereaus Christ centrifuge for 1 minute. The eluant was collected and the kinased primer precipitated by the addition of 2 volumes of ethanol, 0.3M sodium acetate and incubating on dry ice for 30 minutes, followed by a 15 minute

microcentrifugation. The kinased primer was finally resuspended in 10 μ l Q-water.

2.7.4. Annealing and extension reaction.

The annealing mixture consisting of 5.0 μ l 5x RT Buffer (0.25M Tris.HCl pH 8.3, 0.25M NaCl, 40mM MgCl₂), 6.0 μ l 100mM DTT, 5.0 μ l total RNA, 5.0 μ l Kinased primer, 37 units RNA-Guard, was made up to a total volume of 25 μ l with Q-water. The RNA and primer were heated at 65°C for 5 minutes prior to being added to the mixture which was subsequently incubated at 45°C for 1 hour. RNA-Guard (Pharmacia) was added to inhibit RNase activity during incubation. Following incubation, 25 μ l of an extension mixture (5 μ l RT buffer, 5 μ l 10mM dNTPs, 0.5 μ l BSA (5mg.ml⁻¹), 10 units Reverse transcriptase in a total volume of 50 μ l) was added and the mixture incubated at 42°C for 2 hours. The reaction was terminated by the addition of 1 μ l 10% (w/v) SDS and 5 μ l 0.5mM EDTA, Excess RNA was degraded by the addition of 20ml 1M NaOH and 30 μ l Q-water and hydrolysed for 5 - 10 minutes followed by neutralisation with 20 μ l 1M HCl.

The extended product was recovered by precipitation with 5 μ g carrier tRNA (0.5 μ g.ml⁻¹) and the addition of 300 μ l ethanol followed by incubation on dry ice for 15 minutes. Following microcentrifugation, the pellet was washed thoroughly with 1ml 70% ethanol and allowed to dry. The pellet was resuspended in 3 μ l TE and 3 μ l formamide dye boiled for 2 minutes before analysis by polyacrylamide gel electrophoresis. The size of the extended product was determined by carrying out sequencing reactions on the cloned DNA template using the same oligonucleotide as the primer and electrophoresing in parallel.

2.8. S1 nuclease analysis.

S1 nuclease analysis was carried out according to the method of Burke (1984) using total cellular RNA and a radioactively labelled probe from an M13 single stranded clone.

2.8.1. Preparation of the probe.

Approximately 800ng of template (Section 2.9.4) was incubated with 8ng of universal primer and 3 μ l of 10x Klenow reaction buffer (100mM Tris.HCl, 50mM MgCl₂, pH 8.5) in a total volume of 10 μ l at 60°C for 30 minutes. The radioactive second strand was synthesised by the addition of 16 μ l dNTP solution (4mM each of dATP, dTTP, dGTP, 1.5mM dCTP, 10mM Tris.HCl pH 7.5, 1mM EDTA), 2 μ l [α -³²P]dCTP (3000Ci.mMol⁻¹, 10mCi.ml⁻¹) and 15units of Klenow fragment of *Escherichia coli* DNA polymerase. The reaction was incubated at 37°C for 15 minutes after which 2.5 μ l of dCTP solution (2mM dCTP, 10mM Tris.HCl pH 7.5, 1mM EDTA) was added with further incubation at 37°C for 15 minutes.

The reaction was inactivated by incubating at 65°C for 5 minutes, the volume was made up to 90 μ l with Q-water. 10 μ l of 10x of the appropriate reaction buffer and 40units of appropriate restriction enzyme were added followed by incubation at 37°C for 90 minutes. Following a phenol/chloroform wash the nucleic acid was precipitated in 600 μ l 3M ammonium acetate/ethanol solution and incubation on dry ice for 15 minutes. The DNA was pelleted, washed in 70% ethanol and resuspended in 18 μ l Q-water and 2 μ l loading dye. The DNA was separated by electrophoresis using a 1% (w/v) agarose gel. The desired radioactively-labelled band was excised and the DNA recovered as described in Section 2.5.4.2. Having precipitated the DNA, the pellet was resuspended in

100µl TE buffer. The incorporation of radioactivity was determined by counting 1ml of the sample in a scintillation counter by Chorenkov counting.

2.8.2. Hybridisation of total RNA to the radioactively-labelled probe.

Approximately 50µg of total RNA was mixed with an equal volume of 6M LiCl and incubated on ice for 15 minutes. The RNA was pelleted, washed with the same volume of 3M LiCl and resuspended in 20µl Q-water. Probe equal to 5×10^4 counts per second was added to the RNA. The volume was made up to 100µl to which 10µl of 3M sodium acetate and 250µl ethanol were added. The nucleic acids were precipitated together at -80°C for 30 minutes and recovered by centrifugation. The DNA pellet was resuspended in 20µl S1 hybridisation buffer (80% deionized formamide, 40mM PIPES pH 6.4, 400mM NaCl, 1mM EDTA pH 8.0) and denatured by incubating at 72°C for 10 minutes. The mixture was immediately transferred to a desirable temperature ranging from 49°C - 60°C and incubated for 3 hours. A control was used whereby yeast tRNA replaced total cellular RNA.

2.8.3. S1 nuclease reaction.

300µl of ice cold S1 nuclease mix, composed of 150µl 2x S1 nuclease buffer (0.56M NaCl, 0.1M sodium acetate pH 4.5, 9mM ZnSO₄), 20µg salmon sperm DNA, 147µl sterile Q-water and 100 - 1000 units S1 nuclease was added to the hybridisation mixture. Following incubation at 37°C for 30 minutes the reaction was terminated by the addition of 80µl S1 stop buffer (4M ammonium acetate 20mM EDTA pH 8.0, 40µg.ml⁻¹ tRNA). The nucleic acids were precipitated using 1ml ethanol with incubation at -70°C for 1 hour. The DNA

pellet was recovered and resuspended in 5 μ l TE buffer and 5 μ l formamide loading buffer. The sample was boiled prior to electrophoresis on an acrylamide gel with a set of sequencing reactions to allow estimation of the size of the fragments protected by S1 nuclease.

2.9. Sequencing Methodology.

The sequencing reaction was based on the chain-termination DNA sequencing method and was carried out essentially as described in Tabor *et al.*, (1987). The protocol was carried in accordance to the Sequenase Kit (USB) which contained all the necessary reagents needed for sequencing. All cloning procedures described within this section used isolates of the *E. coli* strain NM522 maintained on an M9 media plate supplemented with 1% w/v thiamine B1. This was to ensure maintenance of the F' episome and hence susceptibility to M13 phage (BRL) infection.

2.9.1. Transfection of *E. coli* with M13mp-Derived recombinant molecules.

Restriction fragments, and appropriately digested M13mp DNA were ligated as described in Section 2.5.5. The ligation mixture was diluted and mixed with 200µl competent cells of *E. coli* strain NM522. The mixture was incubated on ice for 30 minutes and then heat shocked at 42°C for 2 minutes. The cells were then mixed with 3.5ml BBL-soft agar at 45°C, 200µl of a logarithmic phase culture of NM522, 20µl of 20mg.ml⁻¹ IPTG and 20µl of 20mg.ml⁻¹ X-GAL (dissolved in N,N-Dimethylformamide). The mixture was poured onto a BBL plate (prewarmed at 37°C) and allowed to solidify. The plates were incubated overnight at 37°C to allow for growth of plaques and development of blue colouration from wild-type M13mp plaques.

2.9.2. Analysis of recombinants.

White plaques were picked with a Pasteur pipette, vortexed with 2ml 2x YT broth containing a 50-fold dilution of a logarithmic phase culture of *E. coli* NM522, then vigorously shaken at 37°C for 6 hours.

The cells were pelleted at room temperature by spinning for 5 minutes in a MSE microcentrifuge. The pellets were resuspended in glycerol and stored for later use. 20µl of the supernatant was mixed with 1.0µl 2% (w/v) SDS and 3.0µl loading buffer. The samples were electrophoresed on a 0.7% (w/v) agarose gel at 90V for 3 hours the gel stained with 5µg.ml⁻¹ ethidium bromide and photographed as described (Section 2.5.3). A control using blue, wild-type, plaques which were treated the same as white plaques were also electrophoresed. It was assumed that DNA samples from white plaques which migrated at a slower rate compared to DNA from the blue plaques contain an inserted fragment.

Where opposite orientation were required from a single transfection, with the above supernatants, two were treated as above and in a separate tube 40µl of each supernatant was mixed with 2.0µl of 2% (w/v) SDS, 2.0µl 5M NaCl and incubated at 60°C for 30 minutes after which 3.0µl loading buffer was added. The samples were electrophoresed as above and if hybridisation occurred the DNA band migrated at a slower rate than the individually treated supernatant hence indicating opposite orientation.

2.9.3. Isolating single plaques made by M13mp vectors.

A series of 10⁻¹ dilution of the pellets (Section 2.9.2) were prepared in 2x YT medium. 100µl of each dilution was overlaid onto BBL plates as described (Section 2.9.1) and the plates incubated overnight at 37°C and well isolated white plaques selected.

2.9.4. Preparation of single stranded template DNA.

Well isolated white plaques were picked with a Pasteur pipette, vortexed with 2.0ml of 2x YT broth containing a 50-fold dilution of a logarithmic phase growth of *E. coli* NM522, then shaken at 300 rpm at 37°C for 5 hours.

Cells were pelleted at room temperature by centrifuging for 5 minutes in a MSE microcentrifuge. 1.25ml of the supernatant was poured into a fresh Eppendorf tube the pellets stored by adding 30% glycerol and freezing at -80 °C.

Phage were precipitated by addition of 250µl 30% w/v PEG 8000, 1.6M NaCl. The solution was vortexed, incubated for 15 minutes at room temperature and phage pelleted by centrifugation for 5 minutes at high speeds in a MSE microcentrifuge. The supernatant was removed from the pellet using a Gilson pipette and the rest of the liquid removed in a number of cycles of centrifugation and pipetting.

When the pellet was completely dry, it was resuspended in 200µl TE buffer and 100µl of phenol added. The mixture was vortexed for 60 seconds, allowed to stand for 5 minutes and vortexed for a further 60 seconds. Phases were separated by centrifugation in a MSE microcentrifuge for 5 minutes at room temperature and the aqueous layer removed to a clean tube containing 100µl phenol/chloroform. The extraction was repeated and the aqueous layer removed to a clean tube containing 20µl 3M sodium acetate pH 5.5 and 400µl 100% ethanol. After vortexing the mixture was placed at -80° C for 15 minutes, then precipitated DNA was pelleted at room temperature by spinning in a MSE microcentrifuge for 5 minutes. The pellet was submerged in 1.0ml of 70% (v/v)

ice-cold ethanol, vortexed and centrifuged as above. The pellets were drained, vacuum dried, resuspended in 25µl TE buffer and stored until required for sequencing.

2.9.5. Sequencing using the USB Sequenase kit.

The USB Sequenase kit involved the use of a modified T7 DNA polymerase, as described by Tabor and Richardson, 1987. The analogue dITP was also available to replace dGTP when problems of GC compressions occurred. Other supplied reagents include dNTP and ddNTP (labelling and termination) mixtures of nucleotides, as follows:

5x dGTP labelling mix:	7.5µM of each dGTP, dCTP, dTTP
5x dITP labelling mix:	7.5µM of each dCTP, dTTP, 15µM dITP
dGTP termination mix:	80µM of each dGTP, dATP, dTTP, dCTP, 50mM NaCl plus 8µM of the appropriate dideoxynucleotide (ddGTP, ddATP, ddTTP, ddCTP for G, A, T, C termination mixtures respectively).

Termination mixes for dITP reactions each contained 160µM dITP, 80µM of each dATP, dTTP, dCTP, 50mM NaCl and 1.6µM ddGTP (G termination mix only) or 8µM of the appropriate dideoxynucleotide (ddATP, ddTTP, ddCTP for G, A, T, C termination mixtures respectively).

Annealing of sequencing primers (M13 universal primer or synthetic oligonucleotides) to templates was carried out as follows: 1µl primer at 0.5pmol.µl⁻¹ and 2µl 5x reaction buffer (200mM Tris pH 7.5, 100mM MgCl₂, 250mM NaCl) was mixed with 7µl (1-2µg) single stranded M13 template

prepared as described above. The reaction was incubated at 65°C in 250ml beaker placed in a water bath, such that after 2 minutes the entire beaker could be removed and the reaction cooled slowly to ambient temperature over a period of 30 minutes.

Dilutions of labelling mixture and duration of labelling times was dependent on the distance from the primer to the sequence of interest. Thus, for sequences within 30bp of the primer, labelling mix was diluted 15 folds and the reactions (labelling and termination) limited to 3 minutes. For standard reactions however, the labelling mix was used at 1x concentration and reactions extended to 5 minutes in order to read up to 300bp from the primer.

The following additions were made to the annealed primer: Template mixture; 1µl 0.1M DDT, 0.5µl [α -³⁵S]dATP (at 650 mCi.mMol⁻¹), 2µl diluted labelling mixture and 2µl sequenase enzyme (diluted 1:8 in ice-cold TE buffer). This labelling reaction proceeded for between 3-5 minutes after which time 3.5µl aliquots were dispensed into four Eppendorf tubes, each containing 2.5µl of the four respective termination mixes. Incubation of these tubes for 3-5 minutes was continued at 37°C and the termination reaction stopped by the addition of 4µl stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) prior to placing the completed reactions on ice. Before loading on denatured acrylamide gel for electrophoretic analysis, samples were heated for 2-5 minutes at 105°C and then rapidly cooled on ice. Generally 2µl of the mixture was sufficient for gel electrophoresis and the remaining samples could be stored at -20°C for up to 2 weeks.

2.9.6. Preparation and electrophoresis of sequencing gels.

Acrylamide was obtained as a 40% solution (Acugel 40: sequencing grade, National Diagnostics) which consisted of acrylamide and bisacrylamide in the ratio 19:1.

Polyacrylamide/urea gels for the resolution of dideoxy-terminated fragments were normally poured between glass plates of 33 x 41cm and 33 x 43 cm. Before assembling the plates were cleaned with sterile Q-water and ethanol. The smaller plate was siliconised by application of approximately 2ml of Sigmacote (Sigma). Plates were separated by 0.4mm thick plasticard side spacers and the bottom edge sealed with tape.

For the preparation of a 6% (w/v) sequencing gel, 27.6g urea (enzyme grade, BRL) was dissolved in 35ml sterile Q-water by gentle warming and stirring. When all the urea had dissolved 6.0ml of 10x TBE buffer, 9.0ml 40% acrylamide solution and 420µl freshly prepared 10% (w/v) ammonium persulphate (dissolved in sterile Q-water) were added and the solution made up to 60ml using sterile Q-water. The mixture was filtered (Whatman filter paper), degassed, cooled on ice and 22.5µl TEMED (Sigma) added. The polymerising acrylamide was poured between the glass sequencing plates and the top edge of the gel formed using two inverted 0.4mm thick shark tooth combs (BRL). The plates were then clamped together with bulldog clips and polymerisation allowed to proceed overnight.

When polymerised, wells were formed using the reverted 0.4mm shark tooth combs and the sequencing gel assembled vertically in BRL electrophoresis sequencing tanks. Top and bottom reservoirs were filled with 1x TBE buffer

and the gel pre-run at 60W constant power for 1 hour before loading the sequencing reaction. Prior to loading the gel was flushed with 1x TBE buffer to prevent accumulation of urea.

Sequencing reactions were divided into two equal aliquots and the first aliquot loaded onto the gel. Electrophoresis was carried out at 60W constant power until the blue dye had ran out of the gel. The second aliquot was then loaded into adjacent wells and electrophoresis carried out as above until the green dye had ran out of the gel. Gels were transferred to a sheet of Whatman 3MM paper, covered with SaranWrap and dried under vacuum at 80°C for 2 hours. After removal of the SaranWrap, gels were exposed to Kodak XAR-5 film at room temperature for 72 hours.

2.10. Production of the λ EMBL3 library.

The methods used for the production, plating and screening of lambda libraries is as depicted in Maniatis *et al.*, 1982.

2.10.1. Amplification of bacteriophage λ EMBL3.

A 100 μ l volume of library stock was transferred to a 5ml test tube with 200 μ l of a late log culture of *E. coli* 5K in 25ml L-broth containing 0.2% (w/v) maltose and 10mM MgCl₂. Phage were allowed to adsorb onto host cells for a period of 20 minutes after which 3ml BBL soft agar, cooled to 45°C, was added to the mixture and poured as an overlay on L-agar plates. Plates were incubated for 18 hours at 37°C after which 2ml L-broth was added to each plates and the soft overlay scraped into a Corex tube. Bacterial cells were destroyed by the addition of 1/100 volume of chloroform, mixing thoroughly and centrifuging at 8000 rpm for 10 minutes. The supernatant was transferred to a screw- capped glass storage bottle and titrated at 2x10⁴ pfu.ml⁻¹.

2.10.2. Screening the λ EMBL3 library.

Bacteriophage lambda that are to be screened were grown to a plating density of 4-6x10³ plaques per 8.5cm plate as described in Section 2.10.1. When the plaques were approximately 0.2mm in diameter, the plates were removed from the incubator and chilled at 4°C for 60 minutes.

To a 10-fold dilution of an overnight culture of the host cell, *E. coli* Q359, sterile nitrocellulose filters were submerged. The filters are then removed and allowed to dry completely on a sterile sheet of Whatman 3MM paper in a laminar flow hood. The filter was placed onto the surface of one of the plates

and the orientation marked using Indian ink. The plates are stored at 4°C for 5 minutes to allow bacteriophage transfer to take place and then transferred to a fresh agar plate with the side in contact with the bacteriophage facing upwards. This procedure was repeated to obtain duplicate sets of plaque lifts. The master plate was stored at 4°C and the replica plates incubated at 37°C for 10 hours.

The filters are removed from the plated and transferred to Whatman 3MM paper, saturated with 0.5M NaOH and 1.5M NaCl, with the plaque side up for 5 minutes. The filter was then transferred to 3MM paper saturated with a solution of 0.5M Tris.HCl pH 8.0, and 1.5M NaCl and then to a piece of 3MM saturated with 2x SSPE. The filters are allowed to dry before baking for 2 hours at 80°C before under vacuum. The filters can be used for *in situ* hybridisation as described in Section 2.6

2.10.3. Isolation of recombinant λ EMBL3 clones.

Liquid lysates of positive plaques were prepared by transferring a plug of agar containing the plaque and surrounding host cells to a polypropylene tube. Phage particles were allowed to diffuse out over a period of 2 hours at room temperature in 1ml λ Tris buffer (6mM Tris pH 8.0, 10mM MgCl₂, 100mM NaCl 0.5mg.ml⁻¹ gelatine). Serial dilutions of the resulting lysate which contains approximately 10⁶ phage particles, were incubated with 200 μ l host cell, *E. coli* Q359 grown to mid-log, and allowed to adsorb for 15 minutes before adding 3ml BBL soft agar to each dilution. Following *in situ* hybridisation with the δ -aminolaevulinate synthase oligonucleotide as a heterologous probe, a well isolated positive clone could be isolated.

2.10.4. Small scale preparation of λ EMBL3 DNA.

The method used is as described in Frischauf *et al.*, 1983. From a plate of well separated lambda purified recombinant plaques, a single agar plug was removed to 400ml L-broth containing 10mM MgCl₂ in a 2L flask and shaken vigorously for 18 hours at 37°C. 4ml chloroform was added and the infected culture shaken for a further 15 minutes. After standing for 10 minutes at room temperature to separate the chloroform, the culture was centrifuge in a GSA rotor at 8000 rpm at 4°C for 10 minutes and the supernatant recovered.

To 50ml supernatant, DNase and RNase was added to a final concentration of 10 μ g.ml⁻¹, and incubated at room temperature for 2 hours. Phage particles were concentrated by the addition of NaCl to a final concentration of 1M and PEG 6000 to a concentration of 10% w/v and incubating on ice for 2 hours. The suspension was centrifuged in a SS34 rotor at 9000 rpm for 10 minutes and the pellets resuspended in 2ml λ Tris buffer. The suspension was recentrifuged at 12000 rpm for 10 minutes and the supernatant extracted with an equal volume of phenol:chloroform for 30 minutes. After centrifuging at 8000 rpm for 5 minutes and repeating the phenol:chloroform wash, the DNA was precipitated by the addition of 2 volumes of ethanol containing 0.3M ammonium acetate. The pellet was washed in 1ml ethanol, dried and resuspended in 1ml TE. The solution was then transferred to an Eppendorf tube and centrifuged for 10 minutes to remove any debris. The supernatant containing phage DNA was then transferred to a fresh tube and used for restriction analysis.

2.11. Determination of protein concentration in cell extracts.

The method was based on that developed by Bradford (1976) in the form of the Biorad protein assay reagent. BSA standards (0, 2.5, 5, 10, 15 and 20 $\mu\text{g} \cdot \text{ml}^{-1}$) were prepared as a dilute series; 800 μl sample (or standard) was added to 200 μl Biorad reagent, mixed by inversion and the absorbance was measured at 595_{nm} after 20 minutes against a water blank. Sample protein concentrations were taken to half the value of the BSA standards since BSA had twice the absorbance of most other proteins in the assay.

CHAPTER 3.

THE ISOLATION OF THE *ASPERGILLUS NIDULANS* GENE FOR δ -AMINOLAEVULINATE SYNTHASE.

3.1. Introduction.

The strategy for isolation of the δ -aminolaevulinate synthase (δ -ALAS) gene using a degenerate oligonucleotide probe for the highly conserved protein sequence 'V W S N D Y L' has been described in the Introduction (Chapter 1). Further details of this conserved δ -ALAS sequenced is shown below (Figure 4.12). This chapter describes the construction of an *Aspergillus nidulans* genomic DNA library using the lambda phage replacement vector λ EMBL3 and the isolation of the δ -ALAS gene.

The genomic library was constructed, in order to isolate genes involved in oxygen regulation in *A. nidulans* and screened for sequences coding for cytochrome *c* and δ -ALAS by Dr. Rosemary Bradshaw. Dr. Rosemary Bradshaw constructed the library and carried out the screening using the 23mer degenerate oligonucleotide probe. She successfully isolated three positive phage clones and following preliminary restriction endonuclease analysis one of these clones was selected for subsequent analysis by myself. Details of the library construction and screening, although not carried out by myself, are included in this chapter for completeness.

3.2. Preparation of the λ EMBL3 vector arms and *A. nidulans* genomic DNA.

The phage vector λ EMBL3 (Murray 1983) was prepared as described in Section 2.10. The phage DNA was digested with *Eco*RI and *Bam*HI to release the non-essential *Eco*RI stuffer fragment. The left and right arms, 19.9 and 9.2kb respectively, possessing *Bam*HI cohesive ends and two small *Bam*HI/*Eco*RI oligonucleotides are also released by this double digest (Figure 3.1). The products of the digestion reaction were extracted with phenol/chloroform to remove proteins followed by three precipitation cycles with 0.1vol 3M sodium acetate and 0.6vol ice cold isopropanol to recover the vector arms and remove the *Bam*HI/*Eco*RI oligonucleotides.

Genomic DNA from a wild type *Aspergillus nidulans* strain R153 genomic DNA was subjected to limited digestion in order to obtain fragments in the range of 10 - 20kb. The restriction enzyme *Sau*3A was used by virtue of its ability to generate cohesive ends compatible with *Bam*HI digested DNA. In order to determine the optimum conditions necessary to produce fragments largely within the 10 - 20kb range, 20 μ g genomic DNA in a total volume of 100 μ l was digested with 5units *Sau*3A at 37°C over a series of times ranging from 0 to 20 minutes. A sample from each timed digestion reaction was analysed by agarose gel electrophoresis as described in Section 2.5.3 (Figure 3.2). Fragments suitable for cloning into the λ EMBL3 vector were produced optimally by incubating 20 μ g genomic DNA for 4 minutes and this partially digested genomic DNA preparation was used to construct of the library.

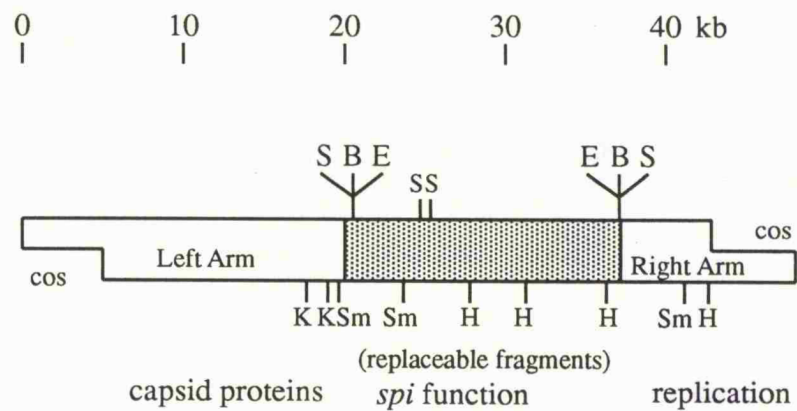
The partially digested genomic DNA preparation was then incubated with calf intestinal alkaline phosphatase (CAIP) to remove 5' phosphate groups in order to prevent re-ligation of non-contiguous segments of genomic DNA in tandem

(Section 2.5.5). It was assumed that with a desired fragment size of 15kb, given that 1µg pBR322 (4.4kb) constitutes 0.69pmol DNA ends, there will be 11.6pmol of DNA ends in 20µg of 15kb DNA. With this evaluation, 0.01U CIAP activity was used per pmol substrate, such that a total of 6µl of a 1:50 dilution of stock enzyme ($1\text{U}\cdot\mu\text{l}^{-1}$) was incubated with the partial digest mixture at 37°C for 30 minutes.

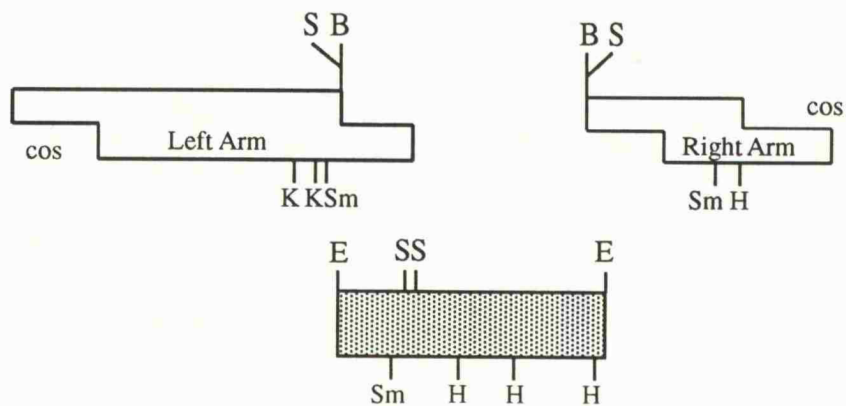
Figure 3.1. Preparation of λ EMBL3 vector arms for cloning.

Digestion of the phage vector with *Eco*RI and *Bam*HI yield two *Bam*HI and cos-ended fragments (19.9 and 9.2kb), a central replaceable *Eco*RI-ended fragment and two *Bam*HI/*Eco*RI oligonucleotides. Before ligation of the vector arms with the genomic DNA the oligonucleotides were removed by isopropanol precipitation.

The restriction sites shown are as follows: B - *Bam*HI; E - *Eco*RI; H - *Hind*III; K - *Kpn*I; S - *Sal*I; Sm *Sma*I.



Digest with *Eco*RI and *Bam*HI

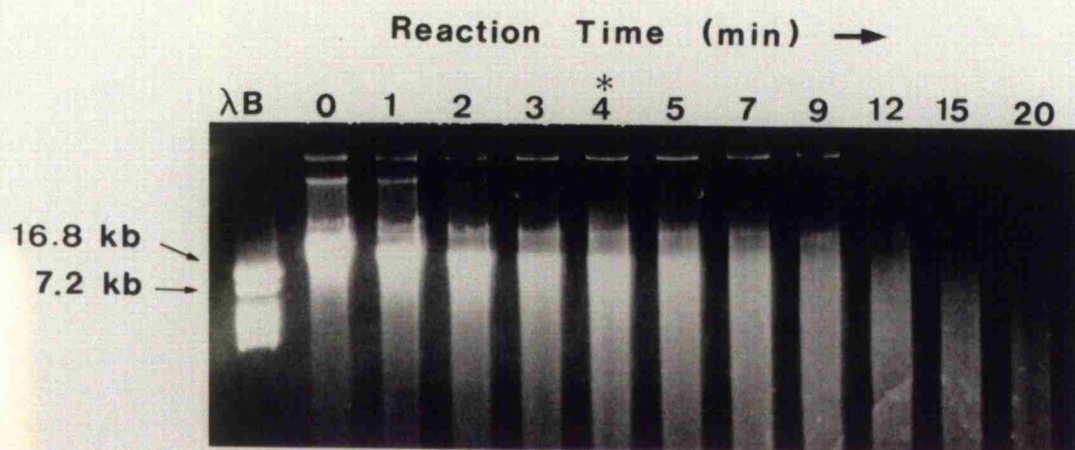


+ *Eco*RI/*Bam*HI oligonucleotides

Figure 3.2. *Sau3A* Partial digests of *A. nidulans* DNA.

A total of 20 µg of genomic DNA was digested with 5 units of *Sau3A* for up to 20 minutes at 37°C. Samples were taken at appropriately timed intermediate points. The lane marked * (4 minutes) indicates the chosen time period for incubation of the full scale reaction.

The DNA size marker (λ) was DNA fragment generated by the digestion of phage lambda DNA with *Bam*HI.



3.3. Ligation of the genomic and vector DNA.

Ligations were carried out as described in Section 2.5.5 with the relative concentrations of genomic to vector DNA being 0.4 to 0.1 $\mu\text{g} \cdot \mu\text{l}^{-1}$. This provided that the lambda DNA was sufficiently concentrated in the mixture to promote the formation of long concatemers for subsequent phage encapsidation.

3.4. Packaging ligation products.

Lysates of *Escherichia coli* strain BHB 2690 (sonicated) and BHB 2688 (freeze-thawed) were obtained as packaging extracts (Amersham lambda packaging kit) for *in vitro* encapsidation of lambda recombinant phage. A sample of 2.5 μl of the CAIP treated ligation reaction mixture was mixed with 2 μl of BHB 2690 lysate followed by 3 μl BHB 2688 lysate and held at 20°C for two hours. The volume was then brought to 100 μl with λ Tris buffer and 2 μl of chloroform was added to destroy residual bacterial cells. 10 μl of these packaged reactions mixed with 200 μl of a late log culture of *E. coli* strain 5K and plated onto L-agar plates with 0.7% (w/v) BBL agarose overlay as described in Section 2.10.1. The plates were incubated at 37°C for 18 hours after which plaques were transferred to duplicate nylon membrane discs for *in situ* DNA hybridisation as described in Section 2.10.2.

3.5. Preparation of the δ -aminolaevulinate synthase coding region probe.

A 23mer degenerate oligonucleotide (Figure 3.3) based on a conserved region of a series of amino acids coding for δ -ALAS in bacteria, yeast and chicken (Urban-Grimal *et al.*, 1986; Brothwick *et al.*, 1985; Leong *et al.*, 1985) was provided courtesy of the Department of Biochemistry, University of Leicester

using an Applied Biosystems 380 DNA synthesiser. Following purification the oligonucleotide was labelled with [γ - ^{32}P]dATP (Section 2.7.3) and used to probe the genomic library.

3.6. Isolation of the δ -aminolaevulinate synthase gene.

Nucleic acid hybridisations were carried out at 57°C using the conditions described in Section 2.6. The membranes were finally washed using moderate stringency conditions in a solution of 3x SSC and 0.1% (w/v) SDS at 57°C. The hybridised membranes yielded three positive clones. These clones were purified by replating and repeating the screening procedure. Recombinant phage was isolated and the DNA extracted as described in Section 2.10. Of the three positive clones one, designated ALA2 was selected for further analysis.

A V W C S N D Y L
B 5' GTN TGG TGY TCN AAY GAY TAY CT 3'

Figure 3.3. Conserved region of the δ -ALAS gene.

Analysis of the δ -ALAS sequences from various species revealed a highly conserved amino acid sequence (A) and using this amino acid region a degenerate oligonucleotide was prepared (B).

3.7. Restriction analysis of the cloned fragment.

Further analysis of the λ EMBL3 ALA2 clone was carried out by restriction digestion and Southern blot analysis prior to probing with the 23mer oligonucleotide to locate the δ -ALAS gene. The λ EMBL3 clone was digested with a number of enzymes and following digestion the products were separated on a 1.0% (w/v) agarose gel. The DNA was transferred to Hybond-N as described in Section 2.6 and this was then hybridised with the 23mer oligonucleotide probe.

Single restriction digests with the restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, *Kpn*I and *Sma*I and Southern blot analysis was carried out on the λ EMBL3 clone. These digests yielded a complex pattern of fragments on separation by electrophoresis (Figure 3.4A). Common among all lanes are unresolved bands at approximately 22kb in length and the lack of separation within this region made it difficult to decipher with any accuracy the number of bands present within this region of the gel. The pattern of unresolved bands in this 22kb region may reflect the presence of large DNA fragments from the insert or incomplete DNA digestion. The polylinker of the original λ EMBL3 cloning vector contained sites for the restriction enzymes *Bam*HI, *Eco*RI and *Sal*I, however during the cloning procedure the *Bam*HI and *Eco*RI sites were removed. Digestion of the λ EMBL3 clone with *Sal*I produces bands at 20kb and 10kb which correspond to the left and right vector arms respectively as well as six other well isolated bands (Figure 3.4, Lane 4). Therefore the sum of these six bands resulting from this digestion is equal to the size of the genomic DNA insert and is estimated to be 16kb in length.

Digestion with the restriction enzymes *Bam*HI and *Eco*RI did not yield bands which corresponded to the size of the vector arms since their restriction sites

were removed from the polylinker therefore as 9.2kb is the length of the smaller vector arm those bands which are greater than 9.2kb possibly contain the vector arms attached to genomic DNA (Figure 3.4 A, Lanes 1 and 2). With respect to the other restriction enzymes used for restriction analysis, both the left and right vector arms possess restriction sites for the restriction enzymes *HindIII*, *KpnI* and *SmaI* (Figure 3.1), hence digestion with these enzymes does not yield any bands representing the sizes of the vector arms (Figure 3.4 A, Lanes 3, 5 and 6).

Hybridisation analysis revealed that all lanes yielded bands of various sizes giving positive hybridisation signals when probed with the 23mer oligonucleotide (Figure 3.4B). Digestion with *BamHI*, *HindIII*, *SalI*, and *SmaI*. each yielded a single hybridising band, of the sizes 4.5, 4.5, 3.3 and 3.5kb respectively. Two enzymes, *EcoRI*, and *KpnI*, showed multiple hybridisation signals. In the case of *EcoRI* there are intense bands at 22kb and 15kb, the larger band was taken to represent undigested DNA and the 15kb band to contain actual genomic sequences. Similarly, *KpnI* exhibits a 22kb undigested band with the 9.0kb band taken as the correct hybridisation signal.

Single restriction digests and Southern blot analysis have indicated the *A. nidulans* genomic insert to be approximately 16kb. Hybridisation analysis has revealed that sequences corresponding to the 23mer oligonucleotide probe are located on this 16kb fragment. Further Southern analysis of the λ EMBL3 cloned DNA using double restriction digests will allow the position of the target sequence to be determined more accurately enabling the construction of a map of restriction enzyme sites in the cloned DNA around the putative δ -ALAS gene.

The DNA fragments released by digestion using pairs of restriction enzymes, with consideration given for the buffer and incubation temperature for each enzyme requires, were separated by gel electrophoresis which again resulted in a complex digestion pattern (Figure 3.5A). The gel was blotted and hybridised with the 23mer oligonucleotide probe (Figure 3.5B).

Hybridisation of the probe to single bands was shown with all the combinations of enzymes including *Bam*HI, namely *Bam*HI with *Eco*RI, *Hind*III, *Sal*I or *Kpn*I. The hybridisation pattern following digestion with *Bam*HI and *Hind*III gave a single band at 4.5kb, which is similar in size to the pattern observed when these enzymes were used individually. This result indicates that the restriction sites for these two enzymes are in the same vicinity and flank the region to which the probe hybridises. This conclusion is reinforced by the results of digestion with the combination of *Hind*III with *Sal*I or *Kpn*I. These combinations produce single hybridisation bands of 3.0kb and 1.8kb respectively and are identical to the results obtained with *Bam*HI and *Sal*I and with *Bam*HI and *Kpn*I. Taken together these results further indicate that *Eco*RI, *Sal*I and *Kpn*I restriction sites are present within the 4.5kb *Bam*HI fragment containing the putative δ -ALAS gene.

Digestion with *Eco*RI and *Sal*I yielded a hybridisation pattern of two bands at 3.2kb and 0.4kb. The former is possibly a partially digested *Sal*I fragment and the 0.4kb fragment the true result, indicating that the probe hybridises to a 0.4kb *Eco*RI/*Sal*I region of the clone. Two signals were also shown with *Sal*I/*Kpn*I combination at 3.3kb and 1.4kb, again the 3.3kb signal being the result of a partial digest with the 1.4kb signal being a true result. The 14kb fragment from the *Eco*RI/*Kpn*I combination digest is again the true result as opposed to the 22kb fragment again being a possible partially restricted fragment.

Figure 3.4. Single enzyme restriction analysis and Southern hybridisation of the λ EMBL3 clone.

Panel (A) shows various restriction digests of the λ EMBL3 clone after electrophoresis on 1.0% (w/v) Miles HSB Agarose. The restriction enzymes used and the size of each restriction fragment are shown in the table below.

Panel (B) shows the autoradiograph pattern of hybridisation of the 23mer oligonucleotide probe to the above DNA fragments after transfer to Hybond-N. The hybridisation conditions are described in Section 2.6.1. Bands hybridising to the probe are indicated by bold typescript in the table. Fragment sizes in brackets represents possible vector arms.

The DNA size markers (λ) are DNA fragments generated by the digestion of phage lambda DNA with *Hind*III and *Eco*RI.

Lane	Enzyme	Fragment sizes (kb)
1	<i>Bam</i> HI	(20.0), 12.0, 6.6, 4.5
2	<i>Eco</i> RI	(20.0), 14.0 , 9.0
3	<i>Hind</i> III	(20.0), 7.0, 4.5 , 3.0, 1.8, 1.5, 0.9
4	<i>Sal</i> I	(20.0), (10.0), 3.9, 3.3 , 3.1, 2.9, 2.3
5	<i>Kpn</i> I	(20.0), 9.0 , 2.3, 1.5
6	<i>Sma</i> I	(20.0), 5.5, 4.5, 4.4, 3.5 , 1.4

A.



B.

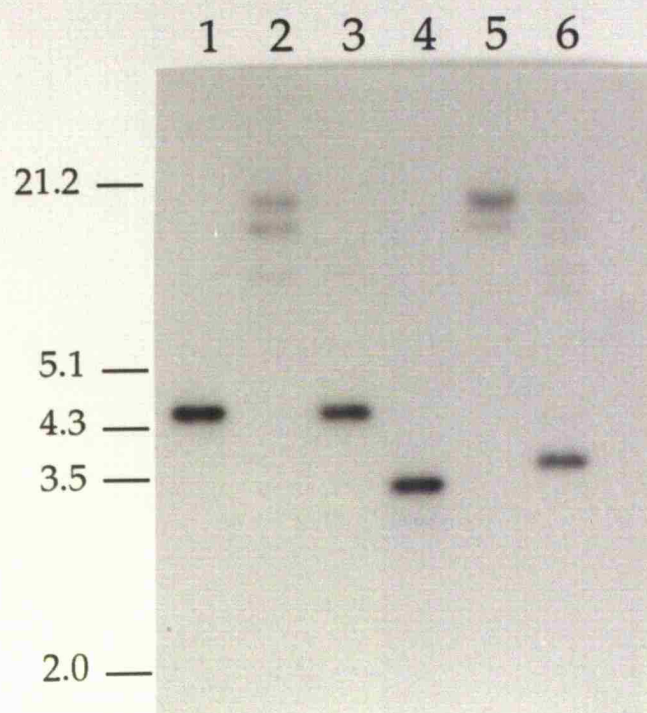


Figure 3.5. Double enzyme restriction analysis and Southern hybridisation of the λ EMBL3 clone.

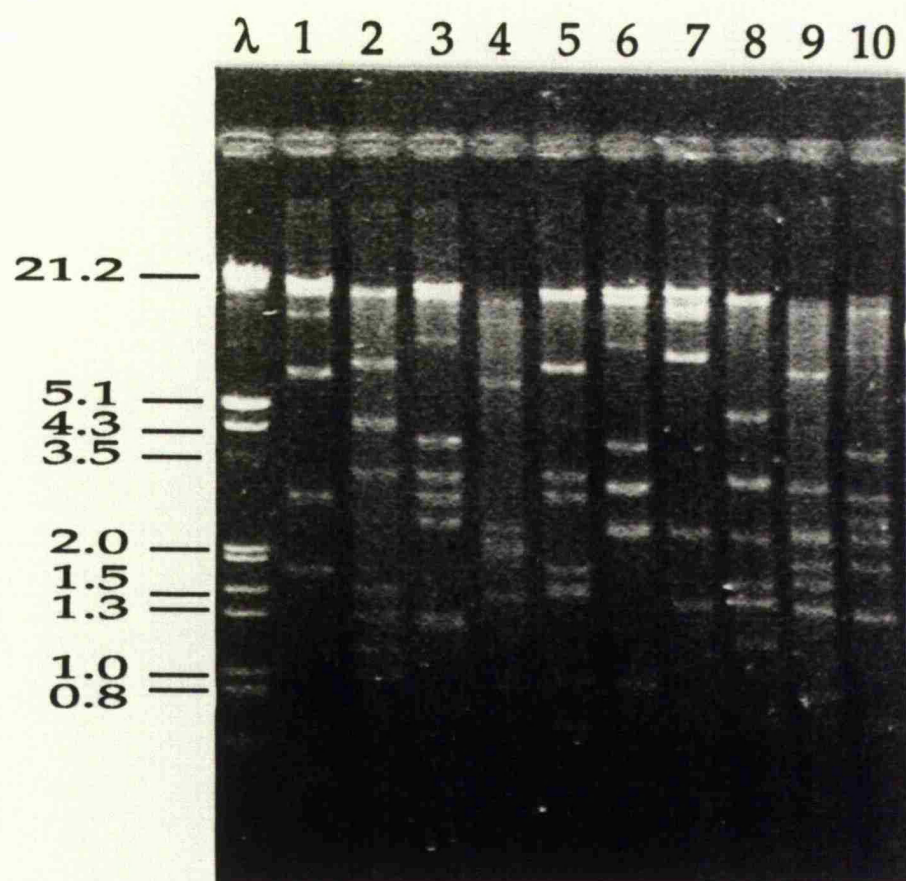
Panel (A) shows various restriction digests of the λ EMBL3 clone after electrophoresis on 1.0% (w/v) Miles HSB Agarose. The restriction enzymes used and the size of each restriction fragment are shown in the table below.

Panel (B) shows the autoradiograph pattern of hybridisation of the 23mer oligonucleotide probe to the DNA fragment after transfer to Hybond-N. The hybridisation conditions are described in Section 2.6.1. Bands hybridising to the probe are indicated by bold typescript in the table.

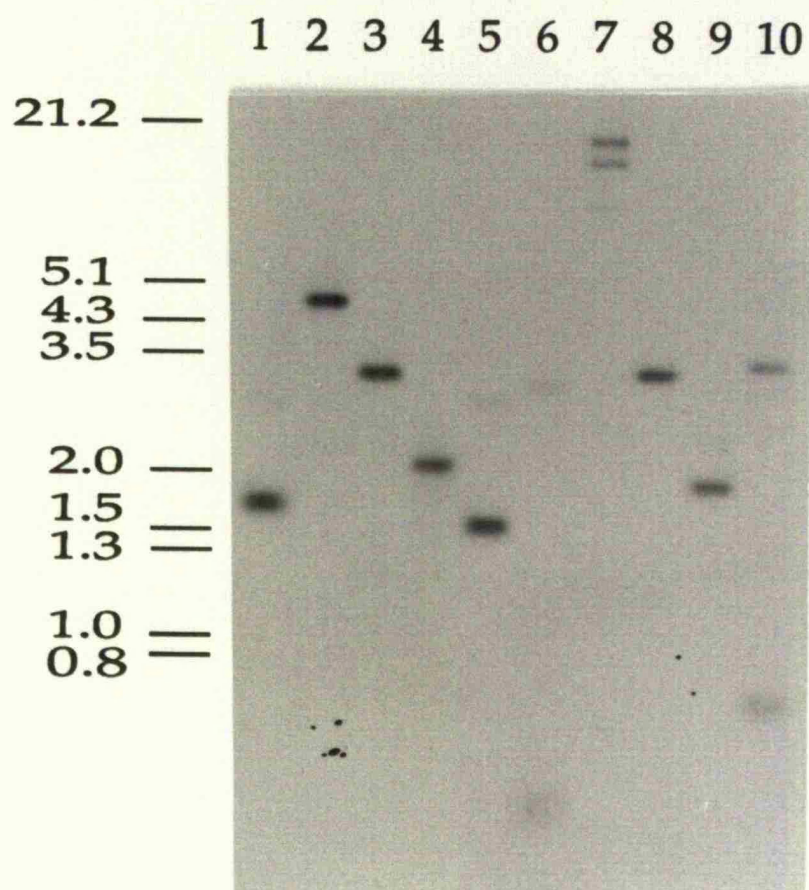
The DNA size markers (λ) are as described in Figure 3.4.

Lane	Enzyme	Fragment sizes
1	<i>Bam</i> HI/ <i>Eco</i> RI	12.0, 6.6, 2.8, 1.7
2	<i>Bam</i> HI/ <i>Hind</i> III	8.0, 4.4 , 3.0, 1.5, 1.4, 1.2, 1.0
3	<i>Bam</i> HI/ <i>Sal</i> I	9.5, 4.0, 3.2 , 2.7, 2.3, 1.4
4	<i>Bam</i> HI/ <i>Kpn</i> I	6.5, 2.3, 2.0 , 1.9, 1.3
5	<i>Eco</i> RI/ <i>Hind</i> III	8.0, 4.4, 3.0, 2.7, 1.7, 1.6 , 1.0, 0.6
6	<i>Eco</i> RI/ <i>Sal</i> I	9.5, 4.0, 2.8, 2.3, 2.2, 1.0, 0.4
7	<i>Eco</i> RI/ <i>Kpn</i> I	15.0, 14.0 , 9.5, 2.3, 1.3, 0.7
8	<i>Hind</i> III/ <i>Sal</i> I	5.0, 4.4, 3.2 , 2.3, 1.7, 1.4, 1.2
9	<i>Hind</i> III/ <i>Kpn</i> I	8.0, 4.5, 3.0, 2.4, 2.3, 2.0, 1.8 , 1.4
10	<i>Sal</i> I/ <i>Kpn</i> I	9.5, 4.0, 3.3, 3.0, 2.8, 2.6, 2.3, 2.0, 1.4, 0.8

A.



B.



3.8. Restriction map and subcloning of the λ EMBL3 clone.

The restriction analysis described above enables the construction of a map of the 16.0kb cloned genomic fragment and the presence of the putative δ -ALAS gene is indicated by the hybridisation probe (Figure 3.6A). These results revealed that the probe hybridised to a position within a 4.5kb *Bam*HI fragment recovered from the library.

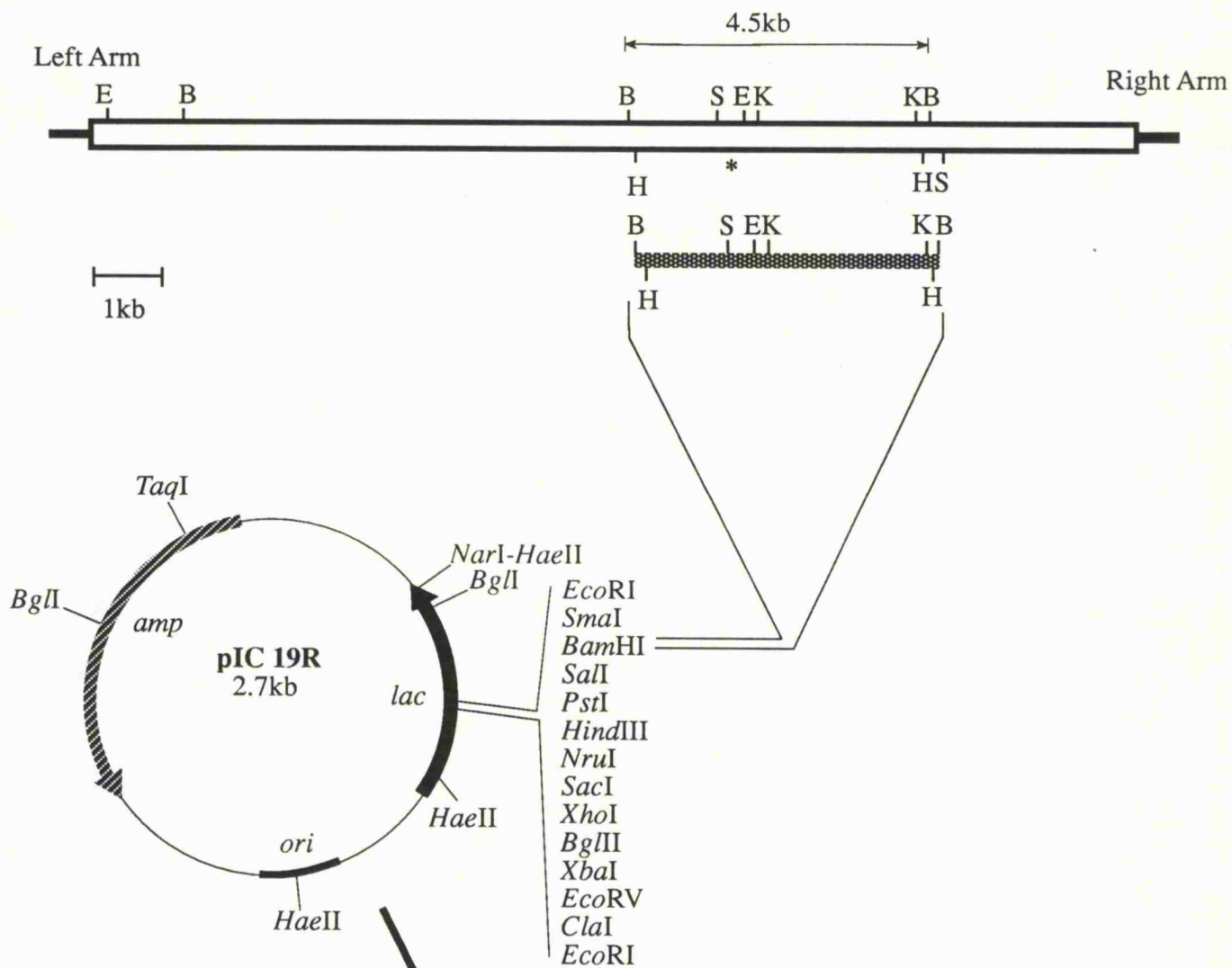
The λ EMBL3 ALA2 clone was digested with *Bam*HI (Section 2.5.1) and the DNA separated using a 1.0% (w/v) low gelling temperature agarose gel for recovery of the 4.5kb fragment (Figure 3.4, Lane 1) by the GeneClean method described in Section 2.5.4.1. The plasmid vector pIC 19R (Section 2.2.4) was linearised by digestion with *Bam*HI. The 4.5kb fragment and the 2.7kb linearised vector were ligated as described in Section 2.5.5. Following transformation and recovery the new plasmid, depicted as pALAS (Figure 3.6B), was used for further routine analysis of the clone.

Figure 3.6. Restriction map of the λ EMBL3 cloned insert.

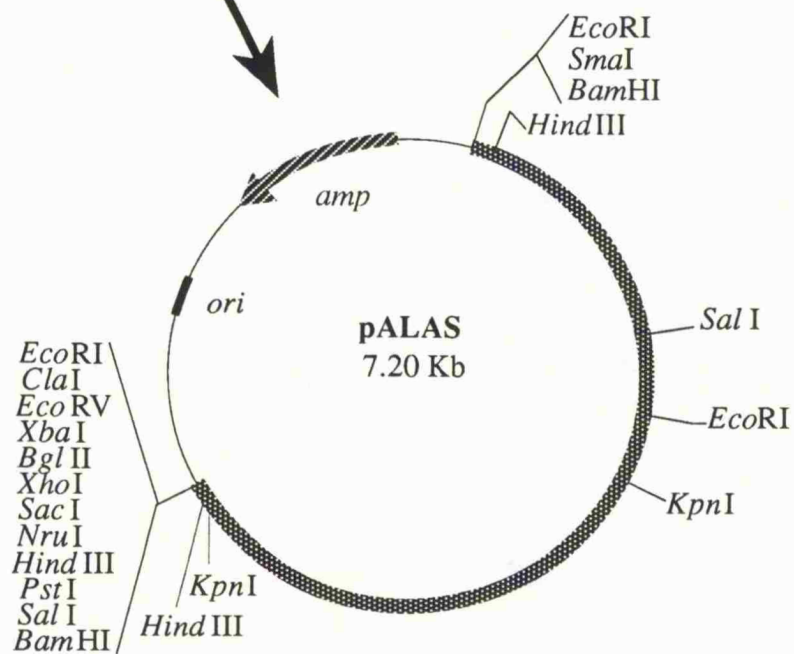
Panel A represents the restriction map of the isolated λ EMBL3 genomic clone ALA2, deduced from the data presented in Section 3.7, and containing the putative *Aspergillus nidulans* δ -aminolaevulinate synthase gene. The position at which the 23mer oligonucleotide probe hybridised to the clone is indicated by the asterisk (*). This was shown to be enclosed within a 4.5kb *Bam*HI fragment which was subsequently isolated and ligated into the *Bam*HI digested plasmid vector pIC19R to produce the plasmid pALAS as shown in panel B.

The restriction sites are as follows: B - *Bam*HI; E - *Eco*RI; H - *Hind*III; K - *Kpn*I
S - *Sal*I. Enzyme sites in the polylinker region are listed (Marsh *et al.*, 1984).

A.



B.



3.9. *Aspergillus nidulans* contains a single δ -aminolaevulinate synthase gene.

To establish the copy number of the putative δ -ALAS gene present within the *Aspergillus nidulans* genome and that the cloned fragment is not rearrangement of the genomic map, Southern blot analysis was carried out on genomic DNA which was prepared from the *A. nidulans* strain R153 as described in Section 2.4.3. The 0.4kb δ -ALAS probe was prepared from the λ EMBL3 clone ALA2 which was digested with the restriction enzymes *Eco*RI and *Sal*I (Section 2.5.1) and the DNA separated using a 1.0% (w/v) low gelling temperature agarose gel to recover the fragment (Figure 3.5 A, Lane 6) as described in Section 2.5.4.

The genomic DNA was subjected to restriction digests using the enzymes *Bam*HI, *Hind*III, *Sal*I or *Sma*I, the DNA separated by gel electrophoresis and transferred to Hybond-N filter. Southern blot analysis was carried out to determine whether *A. nidulans* contained one or more copies of the δ -ALAS gene. Hybridisation of the 0.4kb *Eco*RI/*Sal*I fragment to the genomic *A. nidulans* DNA revealed single hybridisation bands identical to that depicted in Figure 3.4 B (Figure 3.7). These hybridisation signals were stable having washed the membrane under moderate stringency conditions in 3x SSC, 0.1% (w/v) SDS at 65°C. These results indicate that *A. nidulans* contains a single copy of the δ -ALAS gene within the haploid genome.

Figure 3.7. Southern blot analysis of *Aspergillus nidulans* genomic DNA.

A. nidulans genomic DNA was digested with the restriction enzymes *Bam*HI, *Hind*III, *Sal*I or *Sma*I. The DNA separated by gel electrophoresis and Southern blot hybridisation of the 0.4kb *Eco*RI/*Sal*I fragment to the genomic *A. nidulans* DNA revealed single hybridisation bands after washing the membrane under moderate stringency conditions in 3x SSC, 0.1% (w/v) SDS at 65°C.



3.10. Summary.

The putative δ -aminolaevulinate synthase gene of *A. nidulans* has been successfully isolated from a λ EMBL3 genomic library. Three positive clones were found, one of which, ALA2 was used for further characterisation by restriction analysis. A restriction map of the cloned insert was constructed and estimated to be 16kb in length. The putative δ -ALAS gene was shown to be located within a 4.5kb *Bam*HI fragment. Southern blot analysis of the genomic DNA indicated that the putative δ -ALAS gene is present as a single copy within the haploid genome and that the cloned fragment had not been rearranged.

This fragment was subcloned into a the plasmid vector pIC 19R producing the plasmid pALAS which was used to provide templates for DNA sequencing analysis.

CHAPTER 4.

SEQUENCING AND ANALYSIS OF THE *ASPERGILLUS NIDULANS* δ -AMINOLAEVULINATE SYNTHASE GENE.

4.1. Introduction.

The previous chapter described the isolation of the *Aspergillus nidulans* putative δ -ALAS gene and its subsequent subcloning into the plasmid vector pIC 19R to produce pALAS. At this stage in the project it was not known if the full open reading frame (ORF) for the δ -ALAS gene was present within the subclone. This chapter describes the use of the unique enzyme restriction sites within the presumptive δ -ALAS gene in pALAS to construct M13 recombinant phages to provide templates for preliminary sequence analysis. Having obtained a detailed restriction map from the sequence data, further subclones were constructed in M13 recombinant phages to complete the sequence analysis.

4.2. Construction of subclones to provide templates for sequence analysis.

The plasmid pALAS (Figure 3.6B) containing the putative δ -ALAS gene was used to generate smaller DNA fragments which were ligated into the multiple cloning site (MCS) of the replicative form of the phages M13 mp18 or mp19 to generate single stranded DNA templates for sequencing (Figure 4.1). The M13mp vectors are viable phages in which DNA sequences of up to 2.5kb can be cloned into the MCS which is located in a non-essential region of the phage genome. The MCS is placed in frame at the 5' end of the *E. coli lacZ* gene encoding the N-terminal (alpha (α)) peptide of β -galactosidase. These M13mp derivatives form blue plaques on a lawn of cells that contain the *lacZ* C-terminal (omega (ω)) peptide of β -galactosidase, when grown on plates supplemented with Xgal and IPTG. However if the *lacZ* gene has been disrupted by cloning a sequence into the MCS, the resultant phage forms white plaques due to the inability to produce the α -peptide. Double stranded phage DNA can be isolated from phage infected cells of *E. coli* for further manipulation. Single stranded phage DNA can be isolated from the viral particles released into the medium by infected cells and provides a template for sequencing. Detailed sequence analysis was carried out by the dideoxy method (Sanger *et al.*, 1977) using the universal primer.

4.2.1 ALA-1 clones.

The plasmid pALAS was digested with the restriction enzymes *Bam*HI and *Sal*II to generate a 1.4kb δ -ALAS fragment. The nucleic acids were separated by electrophoresis in a 1.0% (w/v) agarose gel and the 1.4kb fragment was recovered by the GeneClean method (Section 2.5.4.1). Separate digestions of the phage M13 mp18 and mp19 were carried out using the above enzymes. The proteins were removed by phenol extraction and the DNA precipitated with

ethanol and sodium acetate. Two ligation reactions were set up both containing the 1.4kb δ -ALAS fragment, one with M13 mp18 and the other M13 mp19. This strategy enables provision of single stranded DNA templates to allow sequencing in both orientations using the universal primer.

The ligation mixtures were diluted 10 fold with sterile distilled water. One half of the ligation reaction mixture was used to transfect the *E. coli* strain NM522 and plated onto BBL agar plates supplemented with Xgal and IPTG (Section 2.9.1). White plaques were selected and analysed for the presence of an insert by comparing the electrophoretic mobility of DNA purified from phage in which the insert is present to that of DNA from phage lacking an insert (Section 2.9.2). Cells containing the required recombinant vector DNA were replated to purify the virus such that well isolated plaques were formed (Section 2.9.3). Several independently isolated recombinant phage were used to inoculate separate *E. coli* cultures to prepare single stranded template DNA for sequence analysis (Section 2.9.4). The phage vector M13 mp19 containing the 1.4kb *Bam*HI/*Sal*I fragment was designated ALA-1(+) and enabled sequence analysis to be carried out in the 5' to 3' direction using universal primer (Figure 4.2). Sequence in the opposite orientation was obtained using the vector, designated ALA-1(-), which comprised the M13 mp18 vector containing the 1.4kb *Bam*HI/*Sal*I fragment (Figure 4.2).

This strategy for the preparation of single stranded DNA templates using M13mp phage vectors was followed when preparing all the other templates.

4.2.2. ALA-2 clones.

The plasmid pALAS was digested with the restriction enzymes *SalI* and *EcoRI* and the 0.4kb δ -ALAS fragment recovered. The phage vectors were digested with the enzymes *SalI* and *EcoRI* and the DNA purified prior to religation with the 0.4kb fragment and transfection into the strain NM522 as described. The two plasmids containing the δ -ALAS fragment in opposite orientation, designated ALA-2(+) and ALA-2(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.2).

4.2.3. ALA-3 clones.

The plasmid pALAS was digested with the enzymes *EcoRI* and *KpnI* and the 0.3kb δ -ALAS fragment recovered. The phage vectors were digested with the enzymes *EcoRI* and *KpnI* and the DNA purified prior to religation with the 0.3kb δ -ALAS fragment and transfection into the strain NM522 as described. The two plasmids containing the δ -ALAS fragment in opposite orientation, designated ALA-3(+) and ALA-3(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.2).

4.2.4. ALA-4 clones.

The plasmid pALAS was digested with the enzyme *KpnI* and the 2.3kb δ -ALAS fragment recovered. The phage vector M13 mp18 was digested with the enzyme *KpnI* and the DNA purified prior to religation with the 2.3kb δ -ALAS fragment and transfection into the strain NM522 as described. Following the confirmation of the presence of the insert, further analysis were carried out to

locate vectors carrying the 2.4kb δ -ALAS fragment in opposite orientation prior to the isolation of single plaques. The difference in the orientation of the insert can be distinguished since only the "+" strand of phage DNA is incorporated into the phage particle. Thus recombinant phage containing identical sequences in opposite orientation will contain single stranded DNA that is able to form a double strand hybrid along the stretch of insert DNA. This partial hybrid molecule can be detected by its altered electrophoretic mobility when compared to the non-hybridised single stranded species (Section 2.9.2). At this stage, it is not known which of the two recombinant phage contain the insert DNA in the 5'-3' direction and in the 3'-5' direction, with respect to the MCS, therefore the two plasmids are arbitrarily designated as ALA-4(+) and ALA-4(-). This description was confirmed, or if necessary, altered in the appropriate manner once the initial sequence data was obtained. The plasmids were prepared as single stranded DNA for sequence analysis using universal primer (Figure 4.2).

Figure 4.1. The M13mp phage vector.

The M13mp cloning vector (Messing *et al.*, 1977) contains a portion of the *Escherichia coli lac* operon which bears a multiple cloning site at the start of the *lacZ* gene producing the α -peptide of β -galactosidase. When plated in a host strain producing the ω -peptide, the phage forms blue plaques, but when the phage contains an fragment inserted within the MCS white plaques are formed. The MCS is composed of a DNA sequence which contains contiguous recognition sites for a number of different restriction endonucleases and the orientation of the MCS determines the vector type, that is mp18 or mp19.

The vector also contains a stretch of DNA which is complementary to the universal primer used for sequencing reactions and lies adjacent to the MCS.

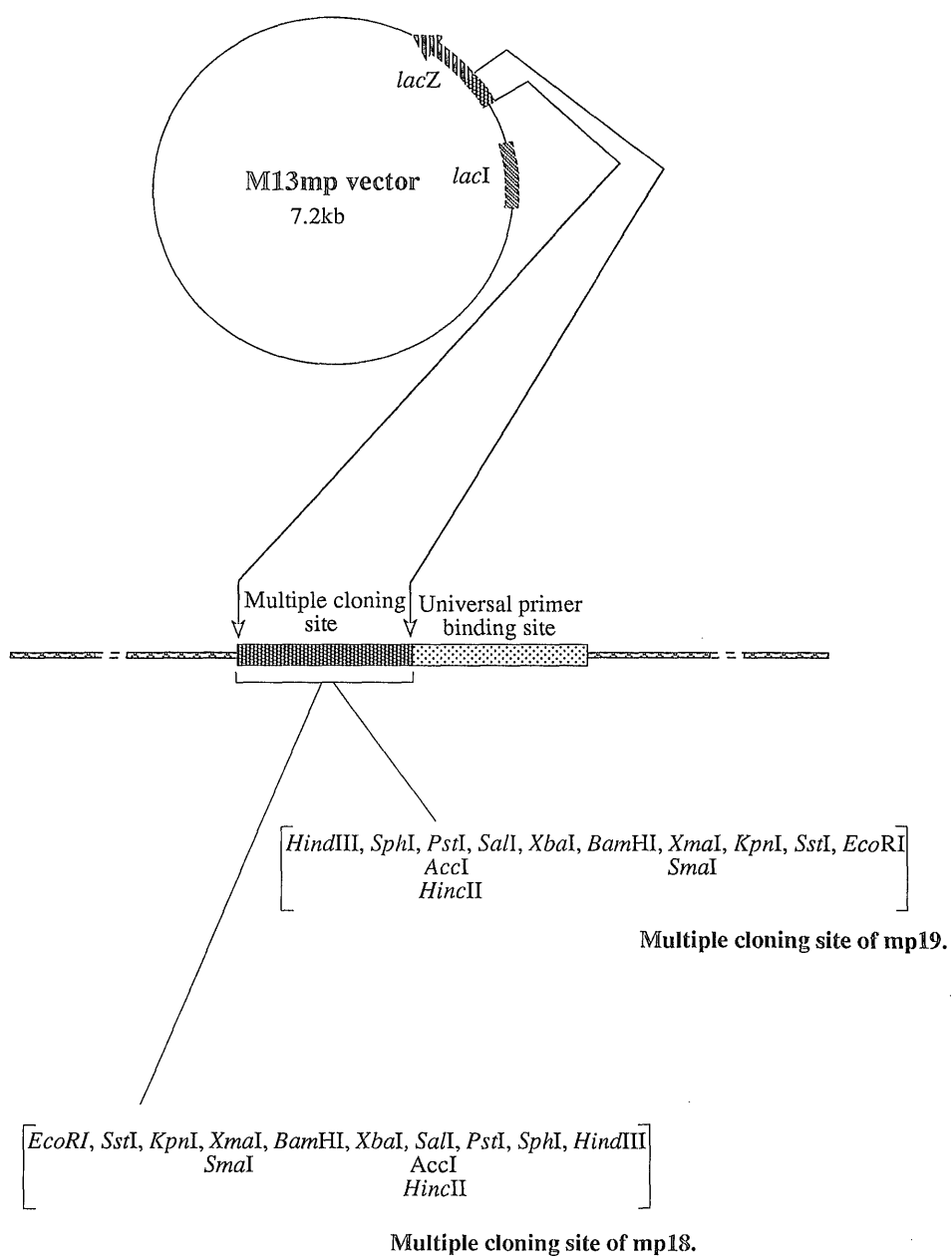


Figure 4.2. Construction of subclones for sequence analysis.

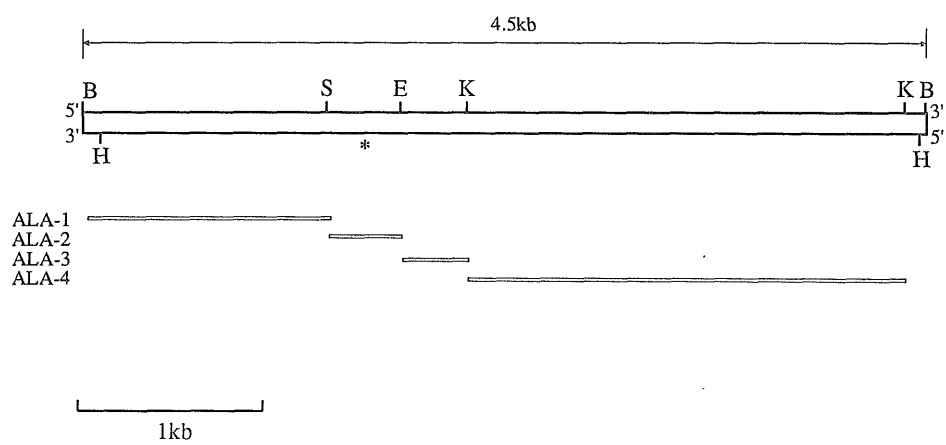
The restriction map of the putative δ -ALAS gene subcloned into pIC 19R is shown. The bold lines depicts the fragments used to generate subclones into the replicative forms of M13mp18 and mp19. Eight subclones were constructed, whereby each fragment were prepared in opposite orientation with respects to the universal primer binding site, allowing sequencing in the 5'-3' direction (+ clones) and in the 3'-5' direction (- clone).

ALAS-1(+) 1.3kb *Bam*HI/*Sal*I fragment subcloned into M13 mp19,
ALAS-1(-) 1.3kb *Bam*HI/*Sal*I fragment subcloned into M13 mp18,
ALAS-2(+) 0.4kb *Eco*RI/*Sal*I fragment subcloned into M13 mp18,
ALAS-2(-) 0.4kb *Eco*RI/*Sal*I fragment subcloned into M13 mp19,
ALAS-3(+) 0.35kb *Eco*RI/*Kpn*I fragment subcloned into M13 mp19,
ALAS-3(-) 0.35kb *Eco*RI/*Kpn*I fragment subcloned into M13 mp18,
ALAS-4(+) 2.1kb *Kpn*I fragment subcloned into M13 mp18,
ALAS-4(-) 2.1kb *Kpn*I fragment subcloned into M13 mp18.

The restriction sites are as follows: B - *Bam*HI; E - *Eco*RI; H - *Hind*III; K - *Kpn*I
S - *Sal*I; .

The asterisk (*) indicates the position at which the 23mer oligonucleotide probe hybridised to the clone.

The ALAS-4 plasmids were arbitrarily designated (+) and (-), this being confirmed or altered in the appropriate manner once the initial sequence data was obtained.



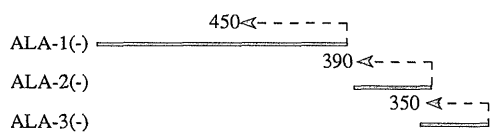
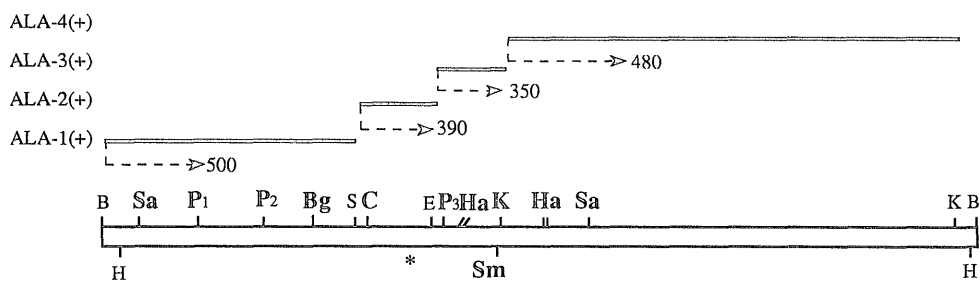
4.3. Sequencing of the cloned templates.

The templates were sequenced following the dideoxynucleotide chain termination sequencing method (Section 2.9) developed by Sanger *et al.*, 1977 using modified phage T3 DNA polymerase (Sequenase, USB Corp). The length of DNA sequence data generated by each template is indicated in Figure 4.3. The information revealed the presence of further enzyme restriction sites which allowed further subcloning of smaller fragments for overlapping sequence analysis on both DNA strands of the *A. nidulans* gene for δ -ALAS.

Figure 4.3. Sequencing of the putative δ -ALAS gene.

M13 templates were sequenced as indicated by the broken arrow (---->) and the length of sequence information obtained for each templates is shown in base pairs (bp). The sequence data revealed further enzyme restriction sites which are indicated by bold typescript. Three *Pst*I restriction sites revealed in the sequence are distinguished from each other as *Pst*I1, *Pst*I2 and *Pst*I3.

The restriction enzymes are as indicated in Figure 4.2 with the following additions: Bg - *Bgl*III; C - *Cla*I; Ha - *Hae*III; P - *Pst*I; Sa - *Sau*3A; Sm - *Sma*I



1kb

4.4. Construction of intermediate δ -ALAS DNA plasmid vectors.

The plasmid pALAS (Figure 3.6B) containing the putative δ -ALAS gene was used to generate further fragments which were subcloned into the plasmid vector pBS M13+, for easier manipulation prior to subcloning into the replicative form of the phage M13 mp18 and mp19 to generate single stranded DNA templates for sequencing.

4.4.1. Construction of plasmid pALAS-a.

The presence of three *Pst*I restriction sites, designated *Pst*II1, *Pst*II2 and *Pst*II3, within the fragment containing the putative δ -ALAS gene in pALAS made it necessary to produce further subclones in a plasmid vector prior to subcloning into the replicative form of the phage M13 mp18 and mp19. Partial digestion of the plasmid pALAS was achieved by incubating the DNA for 5 minutes with 1 unit of the restriction enzyme *Pst*I, under standard conditions.

The nucleic acids were separated on a 1.0% (w/v) agarose gel and a 3.5kb fragment was recovered. The fragment contains the whole of the plasmid vector and 0.8kb δ -ALAS sequence, the remainder having been removed as smaller *Pst*I fragments. The 3.5kb fragment has *Pst*I complementary ends and was religated into circular form before transformation into the *E. coli* strain NM522 (Section 2.3.2). The plasmid was recovered (Section 2.4) and is depicted as pALAS-a (Figure 4.4).

4.4.2. Construction of plasmid pALAS-b.

A separate partial digestion reaction of the plasmid pALAS was carried out using the enzyme *Pst*I as described above. The nucleic acids were again

separated on a 1.0% (w/v) agarose gel and a 1.4kb δ -ALAS fragment recovered and ligated into the pBSM13+ vector. Following transformation the DNA was recovered and the plasmid depicted as pALAS-b (Figure 4.5).

4.4.3. Construction of plasmid pALAS-c.

The plasmid pALAS was digested to completion with the enzyme *Pst*I (Section 2.5). The nucleic acids were separated on a 1.0% (w/v) agarose gel and the 0.9kb δ -ALAS fragment was recovered and ligated into the *Pst*I digested pBSM13+ plasmid vector. Following transformation the DNA was recovered and the plasmid depicted as pALAS-c (Figure 4.6).

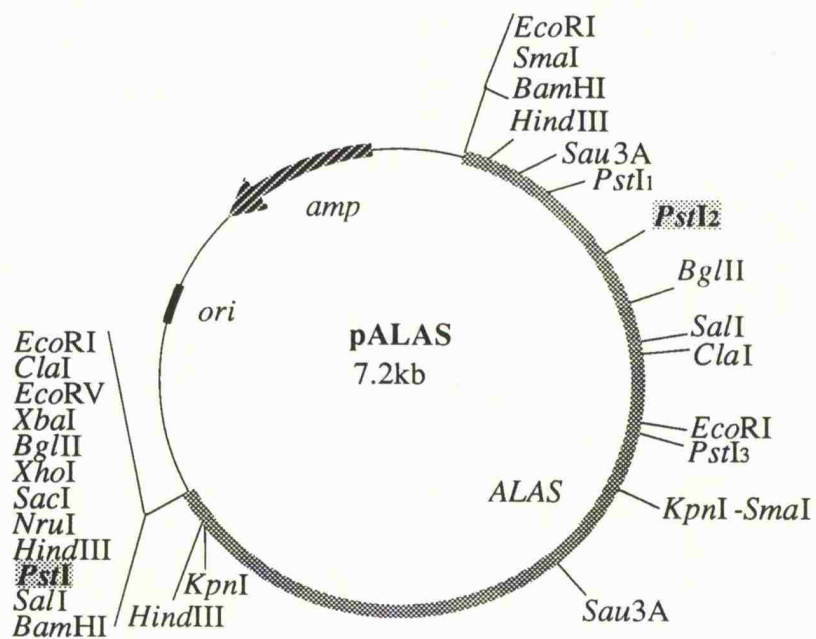
4.4.4. Construction of plasmid pALAS-d.

The plasmid pALAS was digested to completion using the enzymes *Eco*RI and *Sau*3A. The nucleic acids were separated on a 1.0% (w/v) agarose gel and the 0.8kb δ -ALAS fragment was recovered and ligated into the *Bam*HI and *Eco*RI digested plasmid vector pBSM13+. Religation was successful since *Sau*3A digested fragment ends are compatible with *Bam*HI digested fragment ends, however this ligation event resulted in the loss of the *Bam*HI restriction site. Following transformation the DNA was recovered and the plasmid depicted as pALAS-d (Figure 4.7).

Figure 4.4. Construction of the vector pALAS-a.

The plasmid pALAS containing a 4.5kb DNA fragment encompassing the putative δ -ALAS gene was partially digested with the restriction enzyme *Pst*I and the resulting 3.5kb fragment recovered. The targeted restriction sites are highlighted by bold typescript.

The 3.5kb fragment, consisting a 0.8kb δ -ALAS fragment attached to the pIC P16R vector and was religated to produce the plasmid pALAS-a.



Partial *Pst*I digest and recovery of the 3.5kb fragment.

Ligation and transformation.

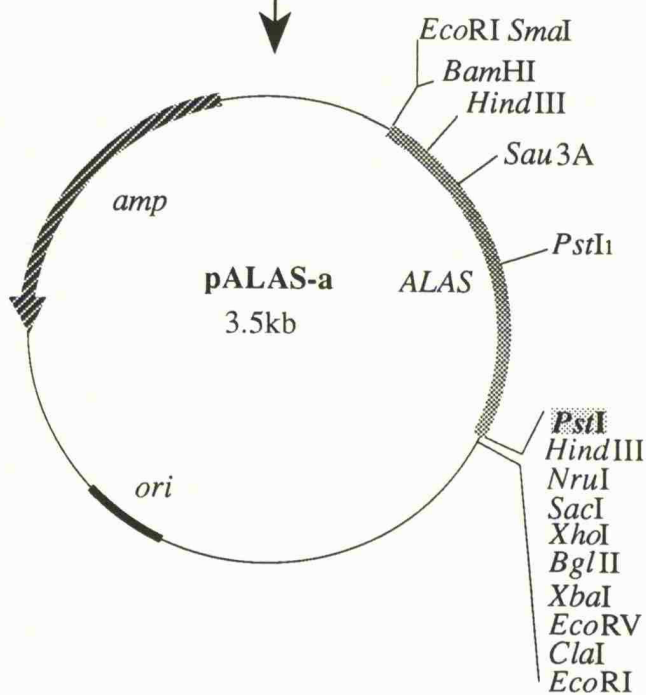


Figure 4.5. Construction of the vector pALAS-b.

The plasmid pALAS containing a 4.5kb DNA fragment encompassing the putative δ -ALAS gene was partially digested with the restriction enzyme *Pst*I and the resulting 1.4kb δ -ALAS fragment recovered. The targeted restriction sites are highlighted by bold typescript.

The plasmid vector pBS M13+ was digested with the above enzyme and ligated with the 1.4kb δ -ALAS fragment to produce the plasmid pALAS-b.

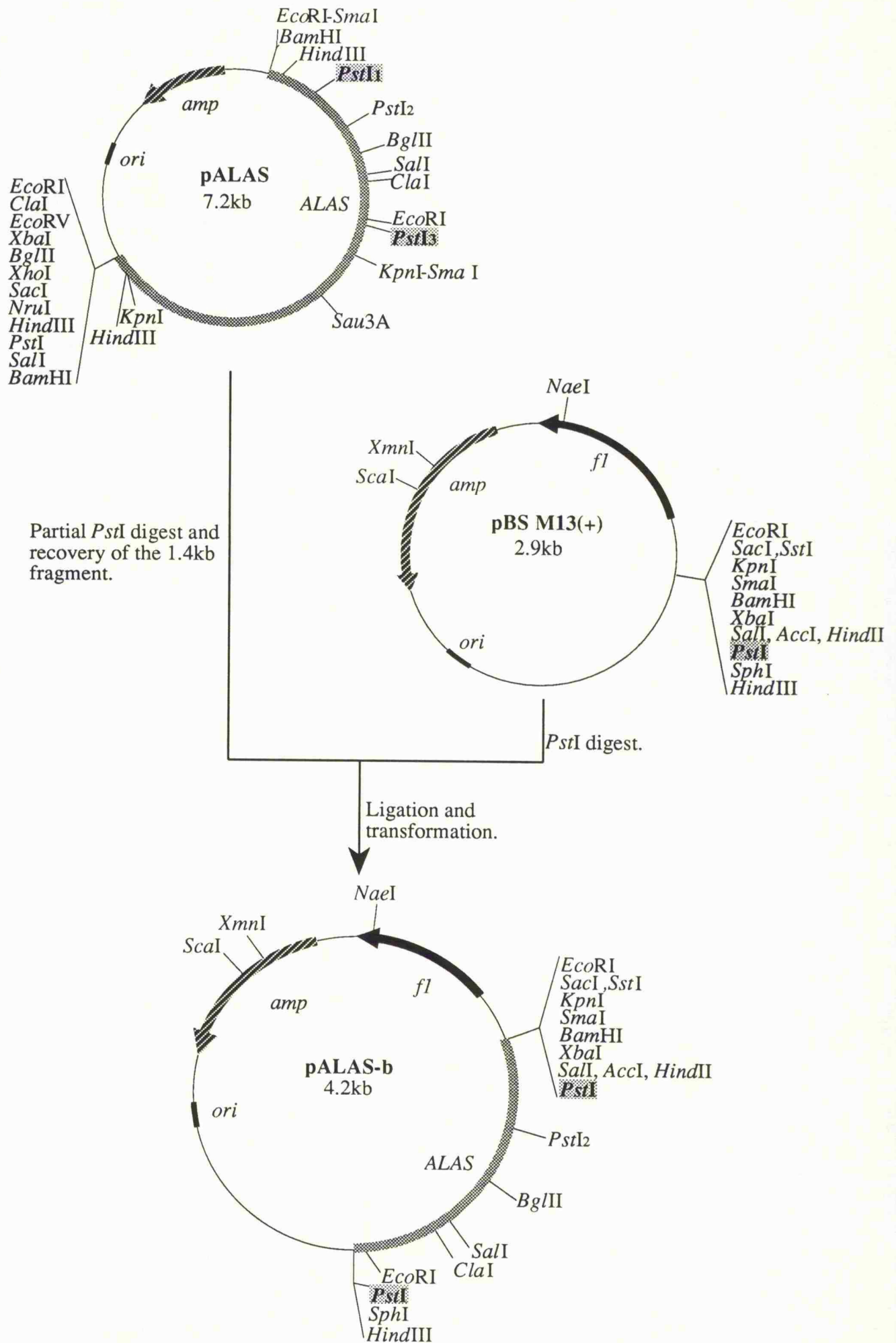


Figure 4.6. Construction of the vector pALAS-c.

The plasmid pALAS containing a 4.5kb DNA fragment encompassing the putative δ -ALAS gene was digested with the restriction enzyme *Pst*I to completion and the resulting 0.9kb δ -ALAS fragment recovered. The targeted restriction sites are highlighted by bold typescript.

The plasmid vector pBS M13+ was digested with the above enzyme and ligated with the 0.9kb δ -ALAS fragment to produce the plasmid pALAS-c.

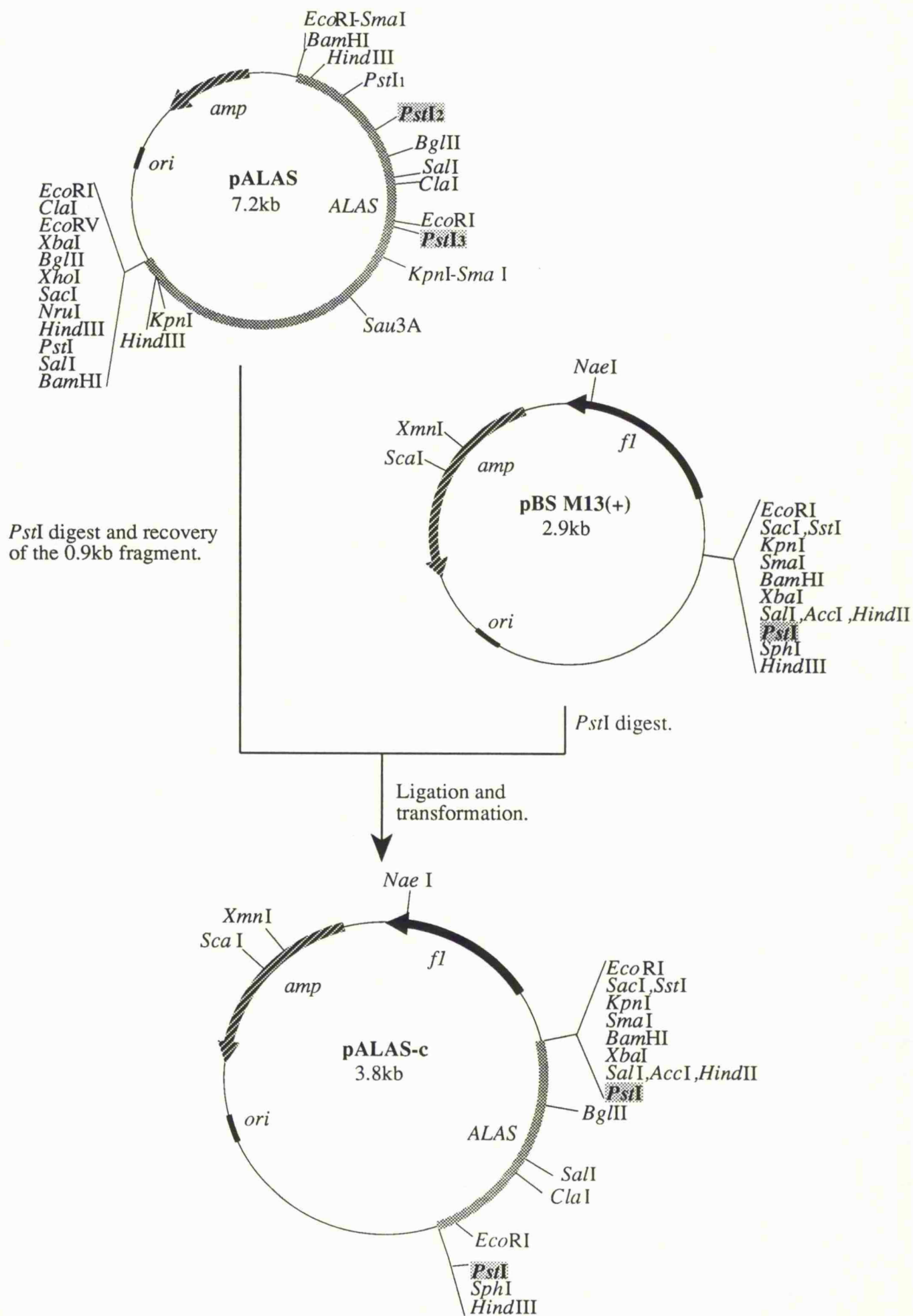
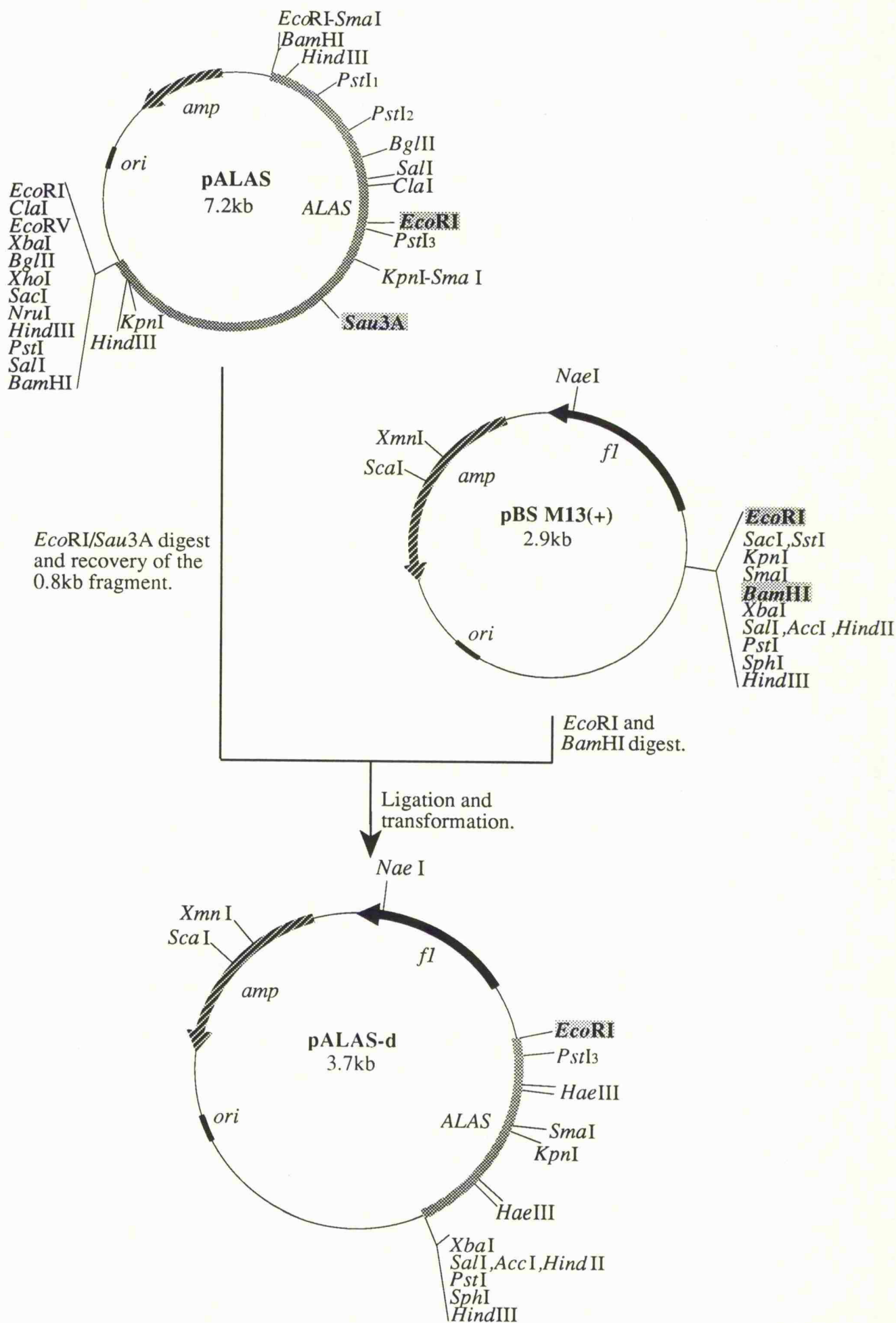


Figure 4.7. Construction of the vector pALAS-d.

The plasmid pALAS containing a 4.5kb DNA fragment encompassing the putative δ -ALAS gene was digested with the restriction enzymes *Eco*RI and *Sau*3A to completion and the resulting 0.8kb δ -ALAS fragment recovered. The targeted restriction sites are highlighted by bold typescript.

The plasmid vector pBS M13+, digested with the restriction enzymes *Bam*HI and *Eco*RI was ligated with the 0.8kb *Eco*RI and *Sau*3A δ -ALAS fragment, with the loss of both the *Bam*HI and *Sau*3A restriction sites, to produce the plasmid pALAS-d.

The restriction enzymes *Bam*HI and *Sau*3A produce complementary single stranded DNA ends .



4.5. Construction of a further set of subclones for sequencing.

4.5.1. ALA-5 clones.

Restriction enzyme sites *Bam*HI and the *Pst*I within the putative δ -ALAS gene of the plasmid pALAS-a and in the multiple cloning sites of M13 mp18 and mp19 were used to construct ALA-5 clones as described above (Figure 4.8). The plasmid pALAS-a was digested with the enzyme *Pst*I and the DNA separated in a 1.0% (w/v) agarose gel to allow recovery of the 3.1kb fragment, this fragment being composed of the plasmid vector and a 0.4kb δ -ALAS fragment. Following recovery, the DNA was further digested with *Bam*HI, the DNA separated and the 0.4kb δ -ALAS fragment recovered. The phage vectors were digested with the enzymes *Bam*HI and *Pst*I and the DNA purified prior to religation with the 0.4kb δ -ALAS fragment and transfection into the strain NM522. The two plasmids containing the δ -ALAS fragment in opposite orientation, designated ALA-5(+) and ALA-5(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

4.5.2. ALA-6 clones.

The plasmid pALAS-a was digested with the enzyme *Eco*RI and the 0.8kb δ -ALAS fragment recovered. The *Eco*RI enzyme restriction sites, within the polylinker of the plasmid pALAS-a, are adjacent to the *Bam*HI and *Pst*I restriction sites present within the cloned ALAS fragment. The phage vector M13 mp18 was digested with the enzyme *Eco*RI and the DNA purified prior to religation with the 0.8kb δ -ALAS fragment and transfection into the strain NM522. Following the analysis for the presence of the insert, further analysis was carried out to locate vectors carrying the 0.8kb δ -ALAS fragment in the

opposite orientation (Section 2.9.2). The two plasmids containing the δ -ALAS fragment in opposite orientation, arbitrarily designated ALA-5(+) and ALA-5(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

4.5.3. ALA-7 clones.

The plasmid pALAS-a was digested with the enzymes *Hind*III and *Sau*3A and the 0.6kb δ -ALAS fragment recovered. The *Hind*III enzyme restriction site, within the polylinker of the plasmid pALAS-a, is adjacent to the *Pst*I restriction site present within the cloned δ -ALAS fragment. The phage vectors M13 mp18 and mp19 were digested with the enzymes *Hind*III and *Bam*HI and the DNA purified prior to religation with the 0.6kb δ -ALAS fragment and transfection into the strain NM522. Religation was successful since *Sau*3A digested fragments are compatible with *Bam*HI digested fragments. The possible regeneration of the *Bam*HI site following ligation was not investigated. The two plasmids containing the δ -ALAS fragment in opposite orientation, designated ALA-7(+) and ALA-7(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

4.5.4. ALA-8 clones.

The plasmid pALAS-b was digested with the enzymes *Bam*HI and *Bgl*II and the 0.65kb δ -ALAS fragment recovered. The *Bam*HI enzyme restriction site, within the polylinker of the plasmid pALAS-b, is adjacent to the *Pst*I restriction site present within the cloned δ -ALAS fragment. The phage vector M13 mp18 was digested with the enzymes *Bam*HI and the DNA purified prior to religation with the 0.65kb δ -ALAS fragment. Religation of the *Bgl*II digested end to

*Bam*HI digested end of the vector was successful since they both generate compatible DNA ends thereby ensuring a complete ligation with the loss of both restriction sites. Following transfection into the strain NM522 and confirmation of the presence of the insert, further analysis was done to locate vectors carrying the 0.65kb δ -ALAS fragment in the opposite orientations. The two plasmids containing the δ -ALAS fragment in opposite orientation, arbitrarily designated ALA-8(+) and ALA-8(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

4.5.5. ALA-9 clones.

The plasmid pALAS-c was digested with the enzymes *Sal*I and the 0.5kb δ -ALAS fragment recovered. A *Sal*I enzyme restriction site, within the polylinker of the plasmid pALAS-c, is adjacent to the *Pst*I restriction site present within the cloned δ -ALAS fragment. The phage vector M13 mp18 was digested with the enzyme *Sal*I and the DNA purified prior to religation with the 0.5kb δ -ALAS fragment and transfection into the strain NM522. Following the analysis for the presence of the insert, further analysis were carried out to locate vectors carrying the 0.5kb δ -ALAS fragment in the opposite orientation (Section 2.9.2). The two plasmids containing the δ -ALAS fragment in opposite orientation, arbitrarily designated ALA-9(+) and ALA-9(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

4.5.6. ALA-10 clones.

The plasmid pALAS-c was digested with the enzymes *Cla*I and *Bgl*II and the 0.3kb δ -ALAS fragment recovered. The phage vectors M13 mp18 and mp19

were digested with the enzymes *Sma*I and *Bam*HI and the DNA purified prior to religation with the 0.3kb δ -ALAS fragment. Religation of the *Bgl*III digested end to *Bam*HI digested end of the vector was successful since they generate compatible DNA ends as described above. However with *Cla*I being incompatible to *Sma*I digested DNA ends a fill-in reaction was carried out (Section 2.5.5) to create a blunt ended fragment. Following transfection into the strain NM522 the two plasmids containing the δ -ALAS fragment in opposite orientation, designated ALA-10(+) and ALA-10(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

4.5.7. ALA-11 clones.

The plasmid pALAS-c was digested with the enzyme *Sal*I and the 3.3kb fragment recovered, this fragment being composed of the plasmid vector and 0.45kb δ -ALAS fragment. Following recovery, the DNA was further digested with *Pst*I, the DNA separated and the 0.45kb δ -ALAS fragment recovered. The phage vector M13 mp18 and mp19 were digested with the enzymes *Pst*I and *Sal*I and the DNA purified prior to religation with the 0.45kb δ -ALAS fragment and transfection into the strain NM522. The two plasmids containing the δ -ALAS fragment in opposite orientation, designated ALA-11(+) and ALA-11(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

4.5.8. ALA-12 clones.

The plasmid pALAS-d was digested with the enzyme *Hae*III and the 0.4kb δ -ALAS fragment recovered. The phage vector M13 mp18 was digested with the enzyme *Sma*I and the DNA purified prior to religation with the 0.4kb

δ -ALAS fragment and transfection into the strain NM522. Ligation was successful since both restriction enzymes produced blunt ended fragments. Following the analysis for the presence of the insert, further analysis was carried out to locate vectors carrying the 0.4kb δ -ALAS fragment in the opposite orientations (Section 2.9.2). The two plasmids containing the δ -ALAS fragment in opposite orientation, arbitrarily designated ALA-12(+) and ALA-12(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

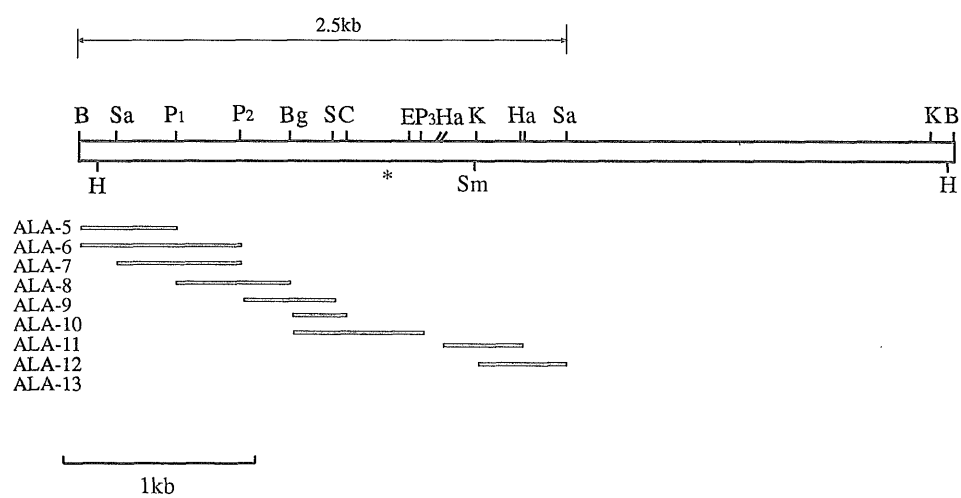
4.5.9. ALA-13 clones.

The plasmid pALAS-d was digested with the enzymes *KpnI* and *SalI* and the 0.45kb δ -ALAS fragment recovered. The *SalI* enzyme restriction site, within the polylinker of the plasmid pALAS-d, is adjacent to the *Sau3A/BamHI* ligation site; the *Sau3A* restriction site being present within the cloned δ -ALAS fragment, and removed upon formation of the plasmid pALAS-d. The phage vectors M13 mp18 and mp19 were digested with the enzymes *KpnI* and *SalI* and the DNA purified prior to religation with the 0.45kb δ -ALAS fragment and transfection into the strain NM522. The two plasmids containing the δ -ALAS fragment in opposite orientation, designated ALA-13(+) and ALA-13(-), were prepared as single stranded DNA and used for sequence analysis of both strands using universal primer (Figure 4.8).

Figure 4.8. Construction of further subclones for sequence analysis.

The restriction map of the putative δ -ALAS gene subcloned into pIC 19R is shown along with the fragments used to generate subclones into the replicative forms of M13mp18 and mp19. Twenty subclones were generated, and each fragment was prepared in opposite orientation to allow sequencing in the 5'-3' direction (+ clones) and in the 3'-5' direction (- clone). The restriction sites are as depicted in Figure 4.3.

ALAS-5(+)	420bp <i>Bam</i> HI/ <i>Pst</i> I fragment subcloned into M13 mp19,
ALAS-5(-)	420bp <i>Bam</i> HI/ <i>Pst</i> I fragment subcloned into M13 mp18,
ALAS-6(+)	838bp <i>Bam</i> HI/ <i>Pst</i> I fragment subcloned into M13 mp19,
ALAS-6(-)	838bp <i>Bam</i> HI/ <i>Pst</i> I fragment subcloned into M13 mp18,
ALAS-7(+)	640bp <i>Sau</i> 3A/ <i>Pst</i> I fragment subcloned into M13 mp19,
ALAS-7(-)	640bp <i>Sau</i> 3A/ <i>Pst</i> I fragment subcloned into M13 mp18,
ALAS-8(+)	670bp <i>Pst</i> I/ <i>Bgl</i> II fragment subcloned into M13 mp18,
ALAS-8(-)	670bp <i>Pst</i> I/ <i>Bgl</i> II fragment subcloned into M13 mp19,
ALAS-9(+)	470bp <i>Pst</i> I/ <i>Sal</i> I fragment subcloned into M13 mp18,
ALAS-9(-)	470bp <i>Pst</i> I/ <i>Sal</i> I fragment subcloned into M13 mp19,
ALAS-10(+)	280bp <i>Bgl</i> II/ <i>Cla</i> I fragment subcloned into M13 mp18,
ALAS-10(-)	280bp <i>Bgl</i> II/ <i>Cla</i> I fragment subcloned into M13 mp19,
ALAS-11(+)	460bp <i>Sal</i> I/ <i>Pst</i> I fragment subcloned into M13 mp19,
ALAS-11(-)	460bp <i>Sal</i> I/ <i>Pst</i> I fragment subcloned into M13 mp18,
ALAS-12(+)	400bp <i>Hae</i> III fragment subcloned into M13 mp18,
ALAS-12(-)	400bp <i>Hae</i> III fragment subcloned into M13 mp18,
ALAS-13(+)	810bp <i>Kpn</i> I/ <i>Sau</i> 3A fragment subcloned into M13 mp19,
ALAS-13(-)	810bp <i>Kpn</i> I/ <i>Sau</i> 3A fragment subcloned into M13 mp18.



4.6. Further sequencing of the cloned templates.

The templates were prepared such that 2.5kb of the putative δ -ALAS was sequenced in both directions with maximum possible sequence overlaps in accordance with the dideoxynucleotide chain termination sequencing method as described above. The lengths of sequence information generated by each template are indicated in Figure 4.9. Gels were read visually and the sequence data was transferred to a VAX 8600 computer and reformatted using the University of Wisconsin Genetics Computer (UWGCG) suite of programs (Dereux *et al.*, 1984). The complete sequence of the δ -ALAS gene designated *hemA* is presented below in Figure 4.10.

Figure 4.9. Sequencing of the putative δ -ALAS gene.

The M13 templates were sequenced as indicated by the broken arrow (---->) and the length of sequence information obtained for each templates is shown in base pairs (bp). The figure gives a detailed restriction map of a 2.5kb *Bam*HI fragment from the original clone which was used for sequencing analysis. The dark grey region indicates the open reading frame (ORF) of the putative δ -ALAS gene with the potential intron being depicted by the light grey box. The white rectangles depicts non-coding region of the clone. The position at which the 23mer oligonucleotide probe hybridised is also indicated (*). The bold arrow indicates the direction of transcription of the putative δ -ALAS gene.

The restriction sites are as depicted in Figure 4.3.

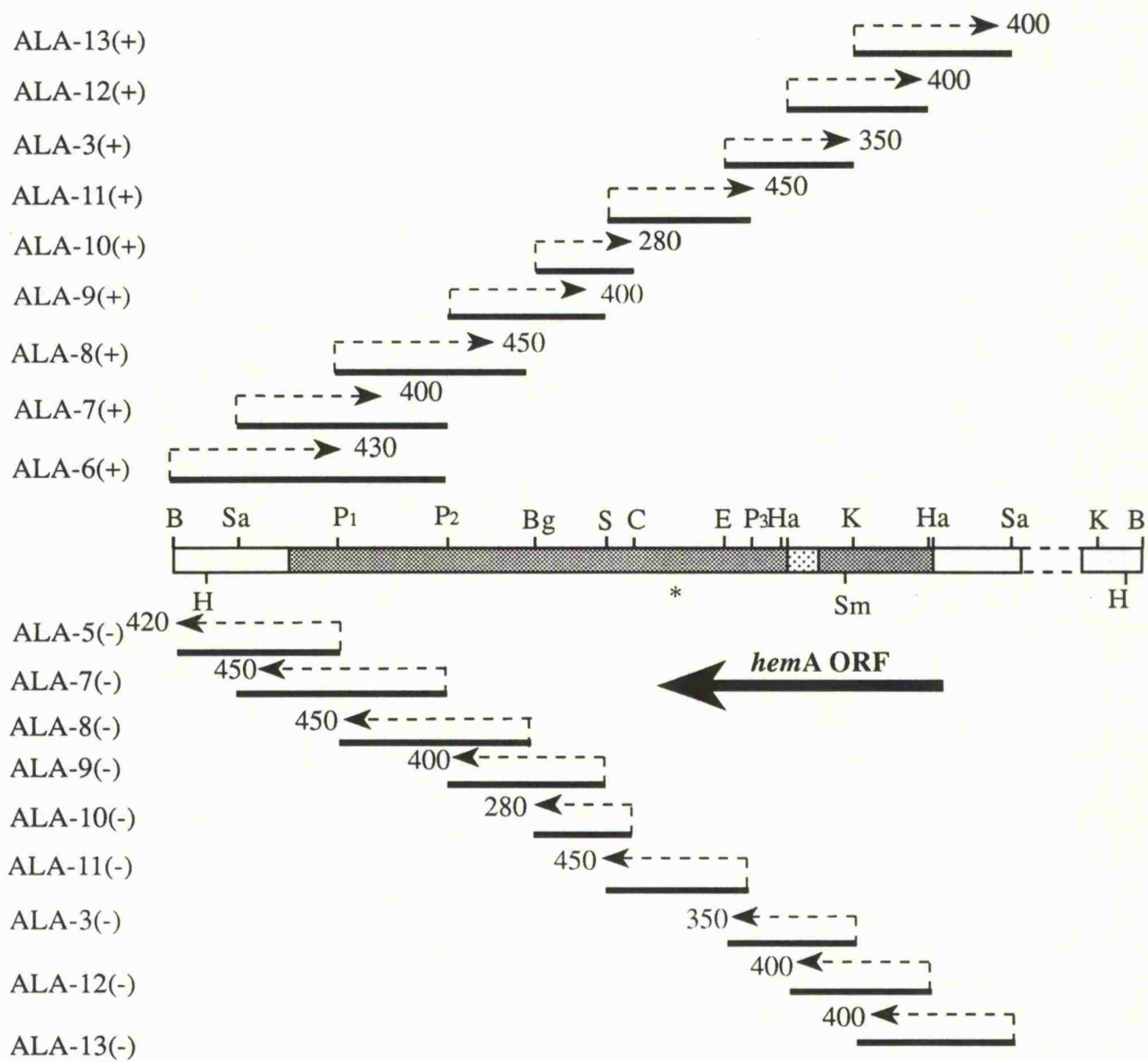


Figure 4.10. Nucleotide sequence of the *hemA* gene of *A. nidulans* encoding δ -ALAS.

Nucleotides are numbered on the left of the figure, commencing from the first ATG codon (A = +1). Other potential translation start codons downstream of position +1 are indicated by the underlined amino acid residue (M). Amino acids are numbered on the right. The conserved block of amino acids **VWSNDYL** in the δ -ALAS polypeptide is boxed, and is located from residues 196 to 203. The intron is shown in lower case letters with the consensus splicing signals underlined. An AT rich region upstream of the first ATG is overscored.

-206 GGATCTGGTAAGAGGCTCCTACCCACAGTCTTAGTCACGGGCTCTGAATAACCTGCCGAGATTTCACGAGTTGCGAATCCGCTGCCGCTCGTATT
-106 TTAGTGACACATCCCTCGTGGTTCTGTCGATTCAGTACTTTGTCACATATTCATCAATTGCTTGTCACCTTCCTCATATCGAATCAACCGATATTC
V
-6 TGCTCAATGAGGCCCTTCCAGCAGTCTCGGGCCATGTGTCGGTTTCACAGCGCAGCTACCGAACCCTTCGTTGCTTGCGTACCGCTACCGCTC
M E A L L Q Q S R A M C P F L K R S S P N T L R S L A T A T R P 32
94 CCAGCACAGCCCTGGCGGTGTTACTATGACCAATTTGCAAGAAATCGCTCGCCGCTGCGCGCTCATGAGCAAGCGCTCGCTGTCAGAGTGCCTGCAT
S T S P G G G T M T N L Q R I A R R C P V M S K A L A V Q S A R M 65
194 GACTGGCACCAAGCGATTACTTCTCTGCGCTGGTGTACCTGGCGCTGGAGCTGGTACCCCAAGGCCACCGGGGTTGCGCGGAAAGAGGGCGCTG
T G T K R F T S S A A G V P G A G A G T P K P T R G S P G K R A L 98
294 CACTCGACTGGAGAAACGGGCGCAATATGAGCACTGAGTTTCACAAAGGAGCCGACAGAGtaccgttttctgagagcgatagactagacaaattgtcg
H S T G G N G A N M S T E F H K G A Q Q I
394 aatataactgaactgggatgtttcaagttccacccctggcctttccaaagccacccgttctacgctcggggctcagcaaccgtttccgggtctactctcgtg
H P G L S N A T R S H V G A S A T V S G P T P R A 144
494 CCCCCTGGCTGGCCATTCGACTACGACGCTTCTACAAATGCAAGACTGCAGAAAGCACCAGGACAAGTCGTATCTTAAACAATCAACCG
P V A A P F D Y D A F Y N A E L Q K K H Q D K S Y R Y F N N I N R 177
594 TCTCGCTCAGGAATCCCGCGCTCATACGGCTTCGAAGGATGAGAAAGTGAACCGTCTGGTCTCGAAGGACTACTCGGCATGGGACGTAATCCGAA
L A Q E F P R A H T A S K D E K V T V W C S N D Y L G M G R N P E 210
694 GTTCTCGGACCATGATGAAGACCTCGATGACGAGAGCGCGCGGAGGAACCTGAACATTTCCGGGACAAATCAACATCCGCTCGCTGGAGA
V L A T M H K T L D T Y G A G A G G T R N I S G H N Q H A V S L E N 244
794 ATACTCTGGCAAACTCCATGGTAAAGAGCTGCGCTGGTTTTCAGCTCGCTTTGTAGCCAAAGAGCTACTTTGGCTACTCTCGGCAAGATGCC
T L A K L H G K E A A L V F S S C F V A N D A T L A T L G S K M P 277
894 TGACTCGCTATTTTGTGCGACTCGCTCAACGCGATGATGATCCAGGGTATCCGCAATTCGCGCGAAGAAATGGTCTTCAAGCACAACGATTG
D C V I L S D S I L N H A S M I Q G I R H S G R K K M V F K H N D L 310
994 GTCGACCTCGAGCAGAGCTCGCTCTCTCTGCTGCTGCTTCCAAAGATCATTTGCTTTCGAATCGGTACAGATATGTTGCTCTTCTGCTCGGATG
V D L E T K L A S L P L H V P K I I A F E S V Y S M C G S I A P I E 344
1094 AGCGGATTTGTGATTTGGCGGCAAGTACGGAGCCATTACTTTCTAGACAGGTTACGCGCTGGGAATGTACGGACCGCACGGCTGAGTTGCCGA
A I C D L A D K Y G A I T F L D E V H A V G M Y G P H G A G V A E 377
1194 GCATCTGACTACGAGATCTATGCTTCGAGGACACCGCAACCCCTCAGCACCAAGGCACTGTATGACCGGATCAACATCATCCGGTACTCTT
H L D Y E I Y A S Q D T A N P L S T K G T V M D R I N I I T G T L 410
1294 GGTAAAGGCTACGGATGCTCGTGGTTACATTTGCTGGCTTCGCGCTTTAGTTGACACCATCGCTCCCTGCGCCCTGGGTTTATTTTACCACTTCCC
G K A Y G C V V G G Y I A G S A A L V D T I R S L A P G F I F T T S L 444
1394 TTCCTCCAGCAACCATGGCGGTGCGGATATCGCAATCGGTATCAGGCACGCCACGACGAGGACCGTATCTGACGAGTTGCACACTCGGCTGTAA
P P A T M A G A D T A I R Y Q A R H Q Q D R I L Q Q L H T R A V K 477
1494 GCAATCATTTAAGGATTTAGATATCCCGCTCATCCCTAACCCCTCTCACATCGTCCCTCTCTCTGCGGTGATGCGGAGCTCGGCAAGCAGGCTCGGAC
Q S F K D L D I P V I F N P S H I V P L L V G D A E L A K Q A S D 510
1594 AAGCTCCTTGAAGAGCACGGCTACCTTCAAGCAATTAACCTACCCCACTGCGCGGTGAAGAGCGGCTCGGATTAACCCCACTGCTGTCACA
K L L E E H G I Y V Q A I N Y P T V P R G E E R L R I T P T P G H T 544
1694 CGCAAGAGCTTCTGACCACTTTGTCGAAGCAGTCAACACTGCTTGAACGACCTCGGCATCAAGCGCGCAAGCGACTGGAAGGCAATGGCGGATTCGT
Q E L R D H L V E A V N T V W N D L G I K R A S D W K A M G G F V 577
1794 CGGTGTTGGCTGGAAGCTGCGAGCTCGAGATCAACCCATCTGGAACGACCGGAGTGAATATGCGACCTGATGAAACCTCGAGGCTGCAAGTTGA
G V G V E A A E L A E N Q P I W T D A Q L N M R P D E T L E A A V E 610
1894 CGCGAGTTTCAGGCGCGGTTCTCGGCTGAAGAGCGGGGTGCAAGGCGAAGCCCGTGGCTCAATCGCGGCCAACCTTATCGGTGCTCAATCCAG
R E F Q A A V P G M K A G G A K A K P V G S I A A N P I G A S I P V 644
1994 TCGCGGCTGCTGCTTAAGTGCCTGCTGCTGCTTTTATTTGTTGGGTTGTTGTTTATATATACGTATATATAGCCCTCCCGCTCCGCTCATTAGTTAT
A A A A
2094 TCGGTGATCTGCTTTGTGCAACAATTTTTCGATGTGATGCTTTCAGTATGACCGGCCGCTCATTTACATTAGGCTTAATACAAAGTTGTCATAC
2194 ACTCGAAGCTTCTCTATTTGTCAGCACGTAGCGTCCATACATGATTCACACAACTTAAGTTGATTTTCAAGATTGACTACCAATTAATGATCTCAGGCCA
2294 GTCGGATCC 2302

4.7. General features of the *A. nidulans* *hemA* gene encoding δ -aminolaevulinate synthase.

The δ -ALAS gene (*hemA*) isolated from *A. nidulans* encodes a large protein (60kDa) which is composed of 648 amino acid residues. The deduced amino acid sequence includes the conserved amino acid sequence 'VWCSNDYL' upon which the degenerate 23mer oligonucleotide probe was designed. The open reading frame (ORF) is interrupted by an intron with the consensus sequences indicative of intron splicing (Ballance, 1986) at the 5'-GTANGTY-3' junction, the lariat 5'-T/ARCTRAC-3' and the 5'-YAG-3 junction. The size of the intron is 64bp which is consistent with those found in other genes in filamentous fungi where the average intron lengths are in the range of 60-70bp.

The translation start site, ATG at position +1 is preceded by a T residue at -3. This observation is in contrast to the findings of Gurr *et al.*, 1987 where it is shown that in filamentous fungi this position is most often occupied by an A (83%) or a purine (90%). Other potential translation start sites are located downstream of this first ATG at positions +31, +121, +160 and +193. The ATG start codon, at position +31, as well as possessing a G at position -3, has the sequence context GCCATG, which matches the consensus sequence for translation initiation in higher eukaryotes, CCA/GCCATG(G) (Kozak, 1984). However, at this stage the actual translation start site cannot be determined.

The enzyme δ -ALAS is synthesised within the cytosol as a precursor protein with the N-terminal mitochondrial targeting sequence being proteolytically cleaved upon entry into the mitochondrion (May *et al.*, 1986). The resulting protein is enzymatically active and catalyses the formation of δ -aminolaevulinic acid from glycine and succinyl CoA. The catalytically active core of the protein, lying within the C-terminal domain is predicted to consist

of approximately 440 amino acids (Cox et al., 1991). The C-terminal putative catalytic domain of the *A. nidulans hemA* gene when compared with that of the yeast *Saccharomyces cerevisiae* shows an amino acid sequence identity of 64% (Figure 4.11). This indicates of a high degree of conservation within the catalytic domain of δ -ALAS proteins.

In yeast, the first 35 amino acids comprising the N-terminal mitochondrial targeting peptide is rich in serine, threonine and the basic amino acids arginine, histidine and lysine. Similarly in *A. nidulans* 15 of the first 35 residues consist of these amino acids. The residues following N-terminal mitochondrial targeting sequences, residues 36 to 106 are is rich in uncharged amino acids which may allow hydrophobic interactions with the inner mitochondrial membrane.

The percentage identities of the *A. nidulans hemA* gene with known δ -ALAS sequences (Table 4.1) shows the greatest homology with the *HemA* gene of the bacterium *Bradyrhizobium japonicum* (McClung et al., 1987) at 63% at the DNA level. The amino acid sequence of the *A. nidulans hemA* gene was aligned with known δ -ALAS amino acid sequences from bacteria, chicken, human, mouse and yeast (Figure 4.12). The alignment shows a high degree of homology throughout the entire length of the sequence, with the conserved amino acid sequence 'VWCSNDYL' being a common feature among all of the sequences.

Figure 4.11. Alignment of two δ -ALAS polypeptide sequences.

Comparison of the deduced complete amino acid sequences of *A. nidulans* *hemA* (A upper) and *S. cerevisiae* *HEM1* (Urban-Grimal *et al.*, 1986) (Y lower), numbered on the right. Alignments were made using the Bestfit programme of the UWGCG group (Devereux *et al.*, 1984). Gaps in the *S. cerevisiae* sequence are represented by a period and perfect matches are shown by a vertical line while the colon (:) represented similar amino acid residues.

[illegible]

Figure 4.12 Alignment of five δ -ALAS polypeptide sequences.

Alignments were made using the Bestfit programme of the UWGCG group (Devereux *et al.*, 1984) to show a comparison of amino acid sequences of prokaryotic and eukaryotic δ -ALAS polypeptide sequence. The conserved block of amino acids, **VWCSNDYL**, is boxed. The sequences used for this comparison were obtained from the following organisms: Yeast, *Saccharomyces cerevisiae* (Urban-Grimal *et al.*, 1986); Bacteria, *Bradyrhizobium japonicum* (McClung *et al.*, 1987); Human, human housekeeping (Bawden *et al.*, 1987); Mouse, mouse erythroid (Schoenhaut and Curtis, 1986); Chicken, chicken housekeeping (Borthwick *et al.*, 1985).

Gaps in the sequences are represented by a dash (-),

Matching amino acids are shown by an asterisk (*) ,

Related amino acid residues are indicated by periods (.).

Multiple sequence alignment

```
Aspergillus MEA---LLQQRAMCF---FLKRSSP-NTL-----RSLATAT-----RPSTSPGGGTMNLR-----
yeast M-----QRSIFAR-----FGNSSAAVSTL-----NRLSTTA-----APHAING-----
Bacteria M-----
human MDS---VVGRCPPLSRVQAFLLQKAGKS---LLFYAQCNCPKMMEVGAKQPSRIVHCSSTLPQDQETPPASEKDQTAKAKVQQTLMDPSPRVQMAHSPRLDSV
Mouse MVAAMLLRSCPLVSGQFTLGLRVAKTYQPLFSIGRCPLATQGPCTCSQIHLKATKA-----
chicken MEA---VVRRCPLARVSAFLQKAGFS---LLFYAQCNCPKMMEAFPAARGLATSAARGQVVEETPAAQPEAKKAKEVAQNTDGSQPPAGH-----
*

Aspergillus -----IARRCPVMSKALAVQSAARMGTGKRTFTSSAAGVPGAGAGTPKPTRGSPGKRALHSTGNGANMSTEFHKAQQTIIHPGLSNATRSHV GASAT
yeast -----YATATGAGAAATATASSTH-----AAAA
Bacteria -----
human WFLAATSQGTASKCPFLAAQMTREAAVSSAKPVLSPRRDQVEMNAVKK---EGAETSAGPSVSVKTDGQDPSGLL-KRVQDIMGQKQRPVRSVHLLHNDL
Mouse -----GGELQDRKSKIVORAAPEVEDVKTFTKD-----LLSTMDSSTRSHSP-PSFQE--PEQTGAVPHLIQNNM
chicken --PPAAAVQSSATKCPFLAAQMNHSSNVFCASLELQEDVKEMQVDRKGKEFAKIPTNSSVVRNTEABGEEQSGLL-KKFKDIMLKQRPESVSHLLQDNL

Aspergillus VSGPTTPAPVAAPFDYDAFYNAELQKHQDKSYRYFNINRLAQEFRA---HTASKDEKVTWCSNDYLGMRNPEVLATMHKTLDTYGAGAGGTRNI
yeast AAAAAHSTQESSGPDYECILDSLEQKKRLDKSYRYFNINRLAKEPPLA---HQREADKVTWCSNDYLGMRNPEVLATMHKTLDTYGAGAGGTRNI
Bacteria -----DYSPFNSSALDLHTERRRYRVFADLERMAGRPFA---IHNSPKGRDVTWCSNDYLGMRNPEVLATMHKTLDTYGAGAGGTRNI
human PKSV-----STFYHDFRFEKALDEKNDHTYRVFKTVNRRAHIFPMADDYSDSLITTKQVSVCSNDYLGMRNPEVLATMHKTLDTYGAGAGGTRNI
Mouse T-GS-----QAFGYDQFFRDKIMEKKQDHTYRVFKTVNRWANAYPPAQHSEASMASKDVSVCSNDYLGMRNPEVLATMHKTLDTYGAGAGGTRNI
chicken PKSV-----STFYQDQFFRDKIMEKKQDHTYRVFKTVNRKAQIFPMADDYSDSLITTKQVSVCSNDYLGMRNPEVLATMHKTLDTYGAGAGGTRNI
* * * * *

Aspergillus SGHNQHAVSENLTLAKLHGKEAALVFSSCFVANDATLATLGSKMPDCVILSDLNHSMIQQIRSHGRKKMVFPHNDLVLETKLASLPLHVPKI IAPES
yeast AGHNPTLNLAEALATLHGKEAALVFSSCFVANDAVLILGQKMKDLVIFSDLNHSMIQQIRSHGRKKMVFPHNDLVLETKLASLPLHVPKI IAPES
Bacteria AGTHHPLVLEAELADLHGKEAALVFSSCFVANDSTLFTLVKMMPGCEIYSDGNHSMIQQIRSHGRKKMVFPHNDLVLETKLASLPLHVPKI IAPES
human SGTSKFHVLELELADLHGKEAALVFSSCFVANDSTLFTLVKMMPGCEIYSDGNHSMIQQIRSHGRKKMVFPHNDLVLETKLASLPLHVPKI IAPES
Mouse SGTSKFHVLELELADLHGKEAALVFSSCFVANDSTLFTLVKMMPGCEIYSDGNHSMIQQIRSHGRKKMVFPHNDLVLETKLASLPLHVPKI IAPES
chicken SGTSKFHVLELELADLHGKEAALVFSSCFVANDSTLFTLVKMMPGCEIYSDGNHSMIQQIRSHGRKKMVFPHNDLVLETKLASLPLHVPKI IAPES
* * * * *

Aspergillus VYSMCGSIAPIEAICDLADKYGAITFLDEVHAGVMPGHGAGVAEHLDEYIYASQDTANP-LSTKG---TVMDRINIITGLKAYGCVGGYIAGSAALV
yeast VYSMGVSADIEKICDLADKYGALITFLDEVHAGVMPGHGAGVAEHLDEYIYASQDTANP-LSTKG---TVMDRINIITGLKAYGCVGGYIAGSAALV
Bacteria LYSMGVDVAPLAKICDLAEKYNMTYVDEVHAGVMPGRGGGIAER-----DGMHRIIDLEGTLAKAFGLGGYIANGRII
human VHSVDGAVLPLEELCDVAHEFGAITFVDEVHAGVMPGRGGGIGDR-----DGMHRIIDLEGTLAKAFGLGGYIANGRII
Mouse VHSMDGAVCPLEELCDVAHEFGAITFVDEVHAGVMPGRGGGIGDR-----DGMHRIIDLEGTLAKAFGLGGYIANGRII
chicken VHSMDGAVCPLEELCDVAHEFGAITFVDEVHAGVMPGRGGGIGDR-----DGMHRIIDLEGTLAKAFGLGGYIANGRII
* * * * *

Aspergillus DTIRSLAPGFIFTTSLPPATMAGADTAIRVQARHQD--RILQQLHTRAVKQSFKDLDIPVFNPNHSHIVPLLVGDALAKQASDKLLEHGIYVQAINYP
yeast DWFRSAPGFIFTTSLPPSVMAGATAIRVQARHIDL--RTSQQHTMVPKAFHGLGIPVFNPNHSHIVPLLVGDALAKQASDKLLEHGIYVQAINYP
Bacteria DAVRSYAPGFIFTTSLPPATMAGADTAIRVQARHIDL--REHODRAARVKAITMAGLEPVMSSTHIVPLLVGDALAKQASDKLLEHGIYVQAINYP
human DTVRSYAGGFIFTTSLPPMLLAGAESVRLKSGEGRVRRQHORNVKLMRQMLMDAGLPVVPCHSHIIPRVADAANKTEVCDELMRSHNIVQAINYP
Mouse DMVRSYAGGFIFTTSLPPMLLAGAESVRLKSGEGRVRRQHORNVKLMRQMLMDAGLPVVPCHSHIIPRVADAANKTEVCDELMRSHNIVQAINYP
chicken DTVRSYAGGFIFTTSLPPMLLAGAESVRLKSGEGRVRRQHORNVKLMRQMLMDAGLPVVPCHSHIIPRVADAANKTEVCDELMRSHNIVQAINYP
* * * * *

Aspergillus TVPRGEELRLITPTPGHTQELRDHLVEAVNTVMNDLGIKRASDWKMGGFVGVGVEAAELENQPIWTDALQNNRDETELEAAVEREFQAAVPMKAGGAK
yeast TVARGTERLRITPTPGHTNDLSDILINAVDVFVNEQLPRVRDWEQGGLLGVG-ESGFVEESNLWTSSQLSL-TNDDLENVR-----LK
Bacteria TVAKGSELRITPTSPYHDDGLDQLAEALLQVWRDLGLP-----
human TVPRGEELRLIAPTPTPHHTPOMMYFLENLLVTVKQVGLLELKPSSAECNFCRRPLHFEVMSER-----EKSY-----
Mouse TVPRGEELRLIAPTPTPHHTPOMMYFLENLLVTVKQVGLLELKPSSAECNFCRRPLHFEVMSER-----EKSY-----
chicken TVPRGEELRLIAPTPTPHHTPOMMYFLENLLVTVKQVGLLELKPSSAECNFCRRPLHFEVMSER-----EKSY-----
* * * * *

Aspergillus AKFVGSIANPPIGASIPVAAA--A
yeast -----DPIVQLEVSSGIIQ
Bacteria QKSLAA-----E
human --PSGLS-KLV-----SAKA
Mouse --FGNMGPOVY-----TTYA
chicken --FSGMS-KLL-----SVSA
```

Table 4.1. δ -ALAS sequence comparisons.

The *A. nidulans hema* gene and the derived polypeptide sequence was compared to other sequences within the EMBL database using the FASTA and TFASTA programs in the University of Wisconsin Genetics Computer package. The sequences used for this comparison were obtained from the following organisms: Yeast, *Saccharomyces cerevisiae* (Urban-Grimal *et al.*, 1986); Bacteria, *Bradyrhizobium japonicum* (McClung *et al.*, 1987); Human, the housekeeping gene (Bawden *et al.*, 1987); Mouse, mouse erythroid gene (Schoenhaut and Curtis, 1986); Chicken, the housekeeping gene (Borthwick *et al.*, 1985).

Table 4.1. Identity of the *A. nidulans hema* gene and δ -ALAS protein with other organisms.

Species	Nucleotide sequence (%)	Derived amino acid sequence (%)	C-Terminal Domain (%)
Yeast	60	58	64
Bacterium (<i>Bradyrhizobium japonicum</i>)	63	43	53
Mouse	60	54	54
Chicken	58	40	53
Human	58	41	52

4.8. Summary.

The *Aspergillus nidulans* *hemA* gene, encoding δ -aminolaevulinate synthase, has been cloned and sequenced using subclones prepared from the restriction sites within the original λ EMBL3 ALA2 clone. The first ATG is designated +1 (A), however the precise translation start site cannot be determined due to the presence of further start codons downstream of position +1. The presence of intron splicing consensus sequences gives strong indication for the occurrence of a 64bp intron located between 355 and 419nt in the gene; between amino acids 119 and 120 of the protein.

The gene shows a 60% nucleotide similarity with the *HEM1* gene of the yeast *Saccharomyces cerevisiae*. Comparisons of the amino acid sequences revealed a 58% identity along the entire length of the amino acid sequence, however this identity increases to 64% over the predicted C-terminal catalytic domain indicating a highly conserved protein.

CHAPTER 5.

ANALYSIS OF THE TRANSCRIPTION OF THE *ASPERGILLUS* *NIDULANS hemA* GENE.

5.1. Introduction.

The previous chapter has described the sequencing of the *Aspergillus nidulans hemA* gene encoding δ -aminolaevulinate synthase (δ -ALAS). Computer analysis of the sequence data revealed an open reading frame of 2010bp which is interrupted by a single intron of 64bp in length, and therefore the size of the *hemA* mRNA transcript is expected to be about 2.0kb. A study of DNA transcription will establish the number of mRNA's, their sizes and abundance. In addition it is useful to map the transcription sites and using the same experimental methods to provide direct evidence for the occurrence of an intron, which is suggested by the presence splicing consensus sequences within the coding sequence.

5.2. Identification and estimation of the size of the *hemA* mRNA.

The *hemA* mRNA transcript was investigated by northern blot hybridisation to total cellular RNA with a DNA probe containing part of the *hemA* coding sequence. *A. nidulans* strain R153 was grown in glucose minimal medium in a 37°C shaking incubator for 18 hours, harvested by vacuum filtration and the total cellular RNA isolated (Section 2.4.4). A 10µg sample of this RNA was maintained in its denatured form by glyoxalation, which removes secondary structure (McMasters and Carmichael, 1977), prior to separation by electrophoresis on a 1x MOPS agarose gel. RNA size markers (Gibco BRL) were also glyoxalated prior to separation on the same gel with the total cellular RNA and used later for size estimation. The nucleic acids were transferred to a Hybond-N membrane filter which was then exposed to UV irradiation to bind the nucleic acids to the membrane (Section 2.6.2). Following UV irradiation glyoxal residues present within the nucleic acids were removed to allow DNA/RNA hybridisation, by baking the filter at 80°C for 2 hours in a vacuum oven.

The DNA used as a probe to identify the *hemA* mRNA was the 0.4kb *EcoRI/SalI* fragment which contains the conserved sequence to which the original oligonucleotide was designed to isolate the gene (Section 3.7). The plasmid pALAS (Figure 3.7) was digested with the enzymes *EcoRI* and *SalI* in a single reaction, and the DNA separated by electrophoresis on a 1.0% (w/v) low gelling temperature agarose gel prior to recovery of the 0.4kb δ -ALAS fragment by the GeneClean method (Section 2.5.4.1). Approximately 200ng of the purified fragment was radioactively labelled with [α -³²P]dCTP by random oligonucleotide priming (Section 2.6.1.2) and used as a probe for hybridisation analysis. The RNA, immobilised on Hybond-N membrane, was hybridised to the radiolabelled DNA probe for 18 hours at 65°C in 5x SSPE, 5x Denhardt's,

0.2% (w/v) SDS and 50 μ g.ml⁻¹ sonicated salmon sperm DNA (Section 2.6.2). Following hybridisation, the filter was washed using high stringency conditions of 65°C in a 0.1% (w/v) SDS, 1x SSPE solution.

A single hybridisation signal was observed indicating that the *hemA* gene encodes a single mRNA transcript the size of which was estimated by its comparison to the size of the RNA size markers (Figure 5.1). The size of the single *hemA* mRNA transcript is approximately 2.3kb which is consistent with the expected size deduced from the sequence analysis data. The intensity of the hybridisation signal with respect to the quantity of nucleic acid used in the analysis indicates that the *hemA* mRNA is relatively high in abundance.

Figure 5.1. Identification of the *hemA* message by northern blot analysis.

Northern blot analysis was carried out on 10µg *A. nidulans* total cellular RNA. The RNA was glyoxalated and separated by electrophoresis on a 1% (w/v) MOPS agarose gel, transferred to a Hybond-N filter and hybridised to a 0.4kb [α -P³²P]dCTP labelled *Eco*RI/*Sal*I fragment which contained the conserved region from which the original oligonucleotide probe was designed. The hybridisation was carried out for 18hours at 65°C in 5x SSPE, 5x Denhardt's, 0.2% (w/v) SDS and 50µg.ml⁻¹ sonicated salmon sperm DNA. Following hybridisation high stringency washes were carried out at 65°C in a 0.1% (w/v) SDS 1x SSPE solution.

The of the size markers are indicated and based upon standard RNA samples supplied by Gibco BRL.

kb

9.5 -

7.5 -

4.4 -

2.4 -

1.4 -



5.3. Preliminary mapping of the 5' and 3' termini of the *hemA* mRNA by northern blot analysis.

The 5' and 3' termini of the *hemA* mRNA were first located by northern blot analysis using DNA probes prepared from the cloned *hemA* gene. To localise the 5' terminus, northern blot hybridisations were carried out using a series of three overlapping DNA probes spanning the translation start site (Figure 5.2). These probes were obtained by cleaving the plasmid, pALAS-d (Figure 4.7), in three separate endonuclease reactions: Probe A, a 199bp *Xba*I/*Hae*III fragment; Probe B, a 455bp *Xba*I/*Kpn*I fragment and Probe C, a 401bp *Hae*III fragment (Figure 5.2 A, B and C respectively). The *Xba*I enzyme restriction site, within the polylinker of the plasmid pALAS-d is adjacent to the *Sau*3A/*Bam*HI ligation site; the *Sau*3A restriction site being present within the cloned δ -ALAS fragment, pALAS, was removed upon formation of the plasmid pALAS-d. The reaction products were separated by agarose gel electrophoresis and the corresponding fragments recovered (Section 2.5.4.1).

To localise the 3' terminus, a further three overlapping DNA probes derived from the plasmid pALAS-a (Figure 4.4) were used for northern hybridisation (Figure 5.2). These probes were obtained by cleaving the plasmid, pALAS-a. Probes D and E were obtained by a two step strategy since restriction of pALAS-a with *Pst*I and *Bam*HI yields two 413bp fragments which cannot be separated by agarose gel electrophoresis. The plasmid was first cleaved with the restriction enzyme *Pst*I, to release Probe D, a 413bp *Pst*I₁/*Pst*I₂ (Figure 5.2 D) and a 3.1kb fragment; the fragments were separated by agarose gel electrophoresis and both were recovered. The 3.1kb fragment was then subjected to a further restriction endonuclease reaction with *Bam*HI to release Probe E, a 413bp *Pst*I/*Bam*HI fragment (Figure 5.2 E). The plasmid pALAS-a, was again cleaved in a separate reaction to release Probe F, a 198bp

Sau3A/BamHI fragment (Figure 5.2 F). The reaction products were separated by agarose gel electrophoresis and the required fragments recovered (Section 2.5.4.1).

Approximately 200ng of each of the purified fragments were radioactively labelled with [α - 32 P]dCTP by random oligonucleotide priming (Section 2.6.1.2) and used as a probes for hybridisation analysis. Total cellular RNA immobilised on Hybond-N filter was hybridised to the radiolabelled DNA probes (Section 2.6.2) followed by high stringency washes with 0.1% (w/v) SDS/1x SSPE solution at 65°C. Separate hybridisation reaction were carried out with each DNA probe.

Northern blot hybridisation analysis using DNA probes derived from the 5' end of the *hemA* gene resulted in strong hybridisation signals with the probes prepared from fragments B and C indicating a high degree of sequence homology between the *hemA* gene DNA probe and the mRNA species (Figure 5.3 lanes B and C). However, the probe which was derived from fragment A produced a weak hybridisation signal indicating that there is limited sequence identity between DNA probe and the mRNA resulting in the probe being largely released during the high stringency wash (Figure 5.3 lane A). These results indicate that the 5' end of the mRNA spans the sequences common to probes B and C but probably extends only to a limited extent into the sequences of probe A. Thus the transcript start site of the *hemA* gene lies within the region represented by fragment A, that is the 199bp sequence from the *Sau3A* site (-205nt)[†] to the *HaeIII* site (+6nt)[†], and most likely closer to the *HaeIII* restriction site.

[†] The first translation codon is defined as +1 as shown in Figure 4.10

Northern analysis of the 3' terminus gave similar results, in that strong hybridisation signals were observed with the probes prepared from fragments D and E and a relatively weak signal with the probe derived from fragment F (Figure 5.3 lanes D, E and F). The same rationale for the interpretation of the position of the 5' terminus applies for the evaluation of the 3' terminus. Thus the transcript stop site of the *hemA* gene lies within the region represented by fragment F; that is, the 198bp sequence from the *Sau3A* site (+2099) to the *Bam*HI site (+2297); most likely closer to the *Sau3A* restriction site.

Figure 5.2. The DNA fragments used for the analysis of *A. nidulans hemA* mRNA.

The restriction map of the putative δ -ALAS gene subcloned into pIC 19R is shown together with the fragments used to generate probes for northern blot and S1 nuclease analysis. The positions of the restriction enzyme sites are given with reference to the translation start site (+1) of the derived open reading frame

- A. 199bp fragment; *Sau*3A (-205nt) to *Hae*III (+6nt),
- B. 455bp fragment; *Sau*3A (-205nt) to *Kpn*I (+250nt),
- C. 401bp fragment; *Hae*III (+27nt) to *Hae*III (+428nt),
- D. 413bp fragment; *Pst*I (+1884) to *Pst*I (+1471),
- E. 413bp fragment; *Bam*HI (+2297) to *Pst*I (+1884),
- F. 198bp fragment; *Bam*HI (+2297) to *Sau*3A (+2099),
- G. 276bp fragment; *Sma*I (+266) to *Pst*I (+542).

The restriction enzyme sites are as depicted in Figure 4.2.

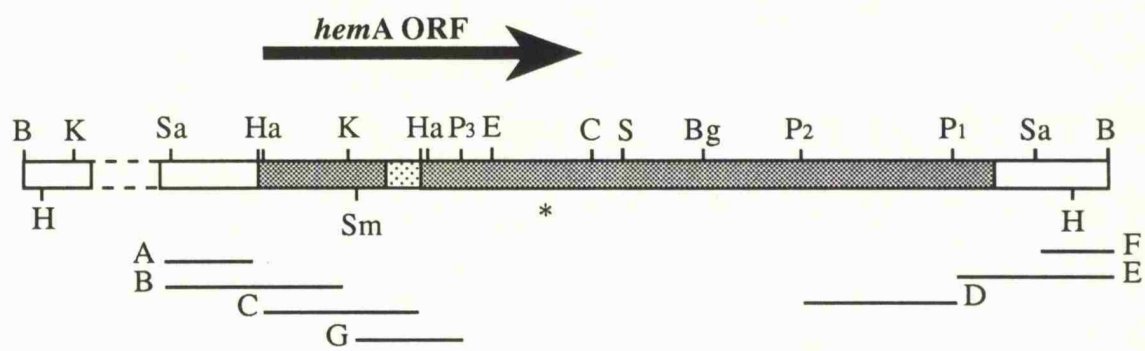
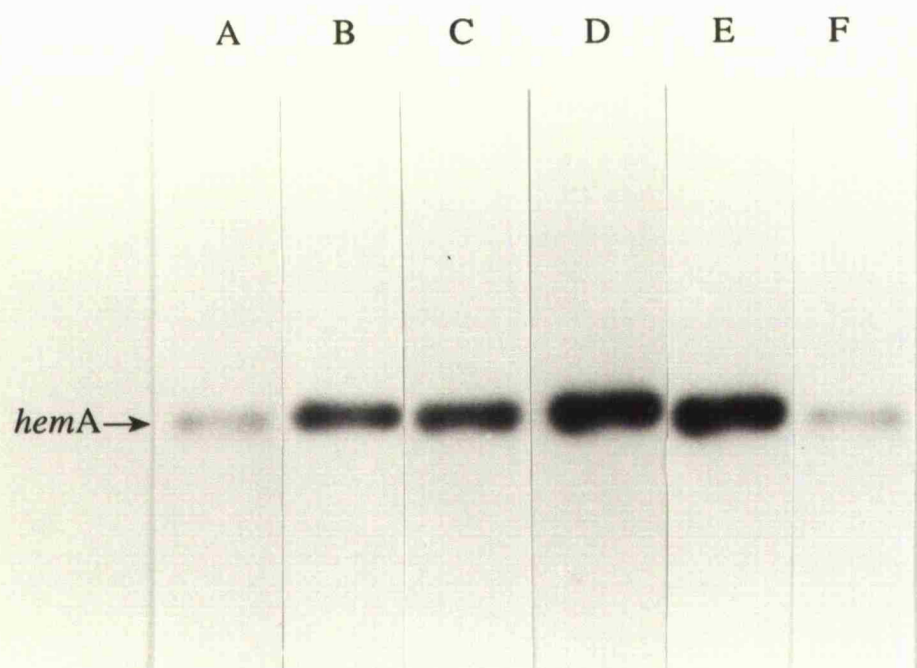


Figure 5.3. Preliminary mapping of the 5' and 3' termini of the *hemA* mRNA by northern blot analysis.

Northern blot analysis was carried out on 5µg *A. nidulans* total cellular RNA with the probes as listed in Figure 5.2 . Total cellular RNA was separated by electrophoresis on a 1% (w/v) MOPS agarose gel, transferred to a Hybond-N filter and hybridised to a single [α -³²P]dCTP labelled fragment. The hybridisation was carried out for 18hours at 65°C in 5x SSPE, 5x Denhardt's, 0.2% (w/v) SDS and 50µg.ml⁻¹ sonicated salmon sperm DNA. Following hybridisation high stringency washes were carried out at 65°C in a 0.1% (w/v) SDS/1x SSPE.

The probes A-F are as listed in Figure 5.2.



5.4. Mapping of the 5' terminus by primer extension analysis.

Primer extension analysis was used in an attempt to accurately map the 5' terminus of the *hemA* mRNA. The DNA primer, prepared from an oligonucleotide complementary to the *hemA* mRNA is end labelled, hybridised to the RNA and extended using reverse transcriptase and dNTPs to form a complementary DNA strand. The length of the resulting DNA strand maps the position of the 5' end of the mRNA (Section 2.7).

Since there are five ATG codons and thus a number of potential translation start sites in the 5' region of the *hemA* ORF, the first primer was chosen to lie 3' of the fifth ATG codon so that the DNA strand synthesised from the *hemA* mRNA would span all potential transcription start sites (Figure 5.5).

A 19mer oligonucleotide primer complementary to the *hemA* mRNA 5'-GCGGGCACTCTGGACAGCG-3' was synthesised based upon the nucleotide sequence of the *hemA* gene at +174 to +192nt (Figure 4.11). The 19mer oligonucleotide was isolated and the DNA 5' end-labelled to a high specific activity using T4 polynucleotide kinase and [γ -³²P]dATP (Section 2.7). The end-labelled fragment was annealed in separate reactions to 50 μ g and 25 μ g of total cellular RNA which had been isolated from young mycelium of *A. nidulans* strain R153 grown in glucose minimal medium. The extension reaction was carried out with reverse transcriptase and dNTPs. The reaction products, consisting of the RNA template and the newly synthesised end-labelled DNA, were recovered by ethanol precipitation and separated by electrophoresis on a 6% acrylamide gel alongside the a set of sequencing reactions prepared from the same 19mer oligonucleotide primer.

A major signal and several minor signals were observed as the products of primer extension analysis using the 19mer oligonucleotide (Figure 5.4, Lanes 1 and 2). The major signal corresponds to a transcription start site within the coding region at position +104nt, with respect to the potential translation start site +1[†] (Figure 4.10). The minor signals corresponds to an apparent transcription start site within the coding region at position +144nt.

If the translation start site were at position +104 the first ATG codon 3' to this is at +121 suggesting a RNA leader sequence of 17nt (Figure 5.5 Panel A). Such an occurrence is rare in filamentous fungi (Gurr *et al.*, 1987). Furthermore, preliminary mapping of the 5' terminus (Section 5.3) has indicated that the position of the transcription start site is within a 199bp region, from a *Sau3A* site (-205nt) to a *HaeIII* site (+6nt) and it appears a strong possibility that the signals observed are products of premature termination of the extension reaction. Similarly the weak signals, corresponding to transcription start site at positions +144nt, are probably also a result of premature termination. The possibility of the formation of secondary structures in *hemA* transcript which terminates the extension reactions on the mRNA templates was explored by examination of the nucleotide sequences in this region.

Position +104nt could be at the base of a stable stem loop structure which would then account for the apparent transcript start site observed above (Figure 5.6 Panel A). Similarly position +144nt could be at the 3' end of the loop structure structure which would account for the minor signal. This observation infers a possible cause for premature termination of the primer extension reaction and hence questions the validity of the of the transcription start sites mapped using 19mer oligonucleotide primer 1 (+174 to +192nt).

[†] The first translation codon is defined as +1 as shown in Figure 4.10

Due to the ambiguous results obtained using primer 1 a second oligonucleotide primer was prepared 37bp upstream of nucleotide position +104nt and used for further primer extension analysis. The second 20mer oligonucleotide primer complementary to the *hemA* mRNA 5'-GGGTGTTTCGGTGAGCTGCGC-3' was prepared from the nucleotide sequence *hemA* gene +47 to +66nt (Figure 4.11). The extension reaction using this 20mer oligonucleotide (primer 2) was carried as described above. The products were recovered by ethanol precipitation and separated by electrophoresis on a 6% acrylamide gel alongside a set of sequencing reactions prepared from the same primer.

A major signal is observed which corresponds with respect to the translation start site +1, to a transcription start site at -92nt (Figure 5.7). This result provides a 92bp leader which lies within the range found in other sequenced fungal genes (Figure 5.5 B) (Gurr *et al.*, 1987). Additionally, this start site is consistent with data produced by preliminary mapping of the 5' terminus.

Minor sites are also observed at positions -8nt and at +4nt with primer extension analysis. A small potential secondary structure can be constructed whereby one of the minor signal, which corresponds to position -8nt, appears to form the base of the stem structure with the other minor signal, corresponding to position +4nt, forming the 3' end of the loop structure (Figure 5.6 B). The occurrence of further potential secondary structures within this region would indicate that these minor signals could be the result of premature termination of the extension reaction.

From the results obtained using the 19mer oligonucleotide primer, it would appear that there is the possibility of secondary structures within the 5' region of the *hemA* transcript which impairs the activity of the primer extension reaction. Primer extension analysis using the 20mer oligonucleotide primer

produced a transcription start site at -92nt, with the appearance of minor signals corresponding to positions -8 to +4nt. Due to the ambiguity of the results obtained by primer extension analysis it would be highly speculative at this stage to infer a specific transcriptional initiation site.

Figure 5.4. Primer extension analysis of the 5' terminus of the *A. nidulans* *hemA* mRNA using the 19mer oligonucleotide probe.

A radioactively end-labelled 19mer oligonucleotide primer was hybridised to 25µg (lane 1) and 50µg (lane 2) of total cellular RNA from the *A. nidulans* strain R153. An extension reaction was then carried out using reverse transcriptase. The extended products were separated on a 6% polyacrylamide/urea denaturing gel along with the sequence pattern of the 455bp *Sau3A/KpnI* fragment using the 19mer oligonucleotide primer. The major transcript initiation site is indicated by the arrow along with the sequence data at the deduced 5' terminus.

1 G A T C 2

C
A
C
C
A
G
C
C
C
T
G
G
C
G
T

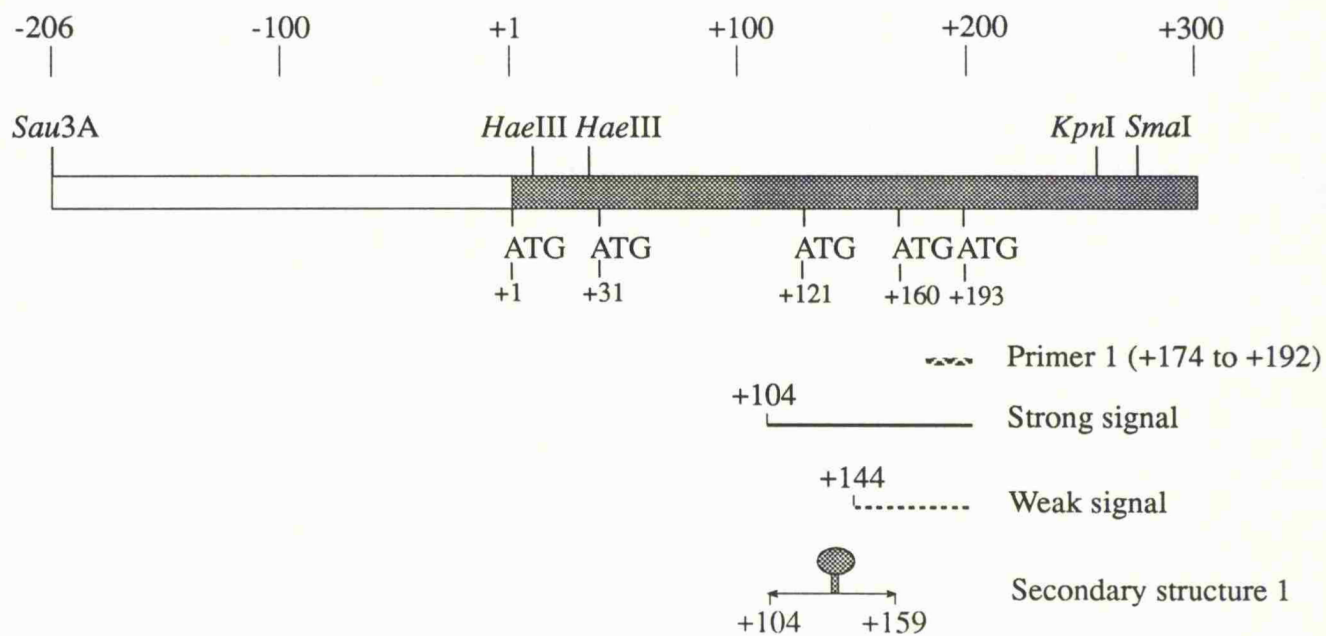


Figure 5.5. Illustration of the primer extension results.

Panel A shows the first 500bp of the 5' region (-206 to +300) of the *hem A* gene depicted by the long rectangle with the predicted open reading frame depicted by the shaded region. Primer extension analysis using the 19mer oligonucleotide, +174 to +192 (Primer 1) produced a strong signal corresponding to position +104nt and weak signals corresponding to positions +132 to +144. A stable secondary structure can be constructed over the entire region, +104 to +159, within which both the major and minor the signal are generated.

Panel B shows the 5' region of the *hem A* gene and the predicted open reading frame. Primer extension analysis using the 20mer oligonucleotide, +47 to +66 (Primer 2) produced a strong signal corresponding to position -92nt and weak signals corresponding to positions -8 to +4. A small secondary structure can be constructed over the region -9 to +8 within which minor the signals are generated.

A.



B.

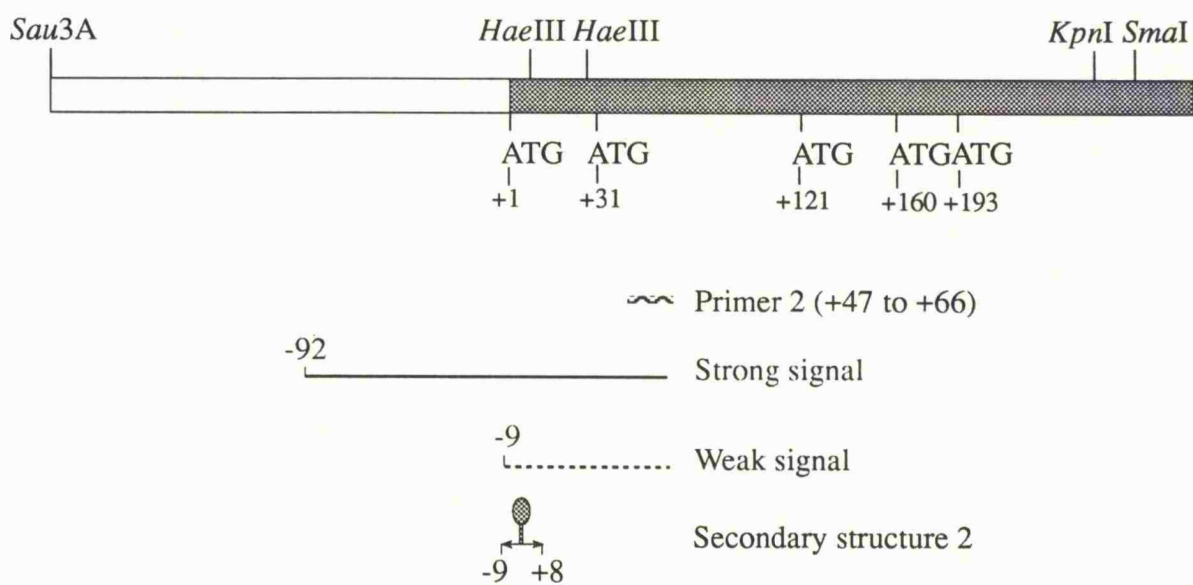


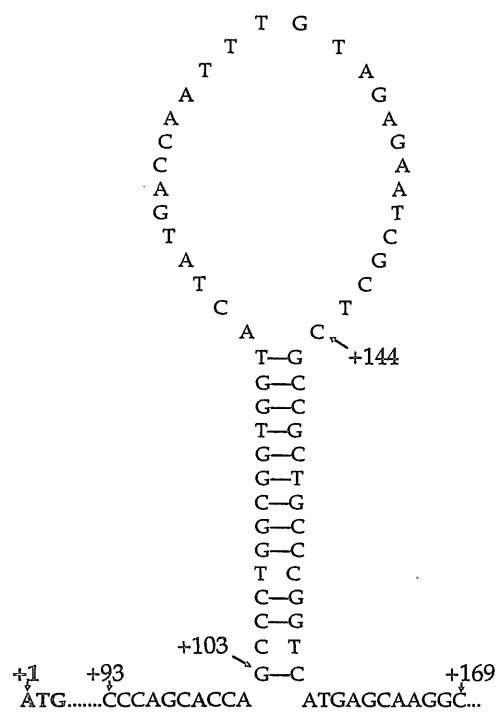
Figure 5.6. Possible secondary structures in the 5' region of the *A. nidulans* *hemA* mRNA.

Panel A Primer extension analysis of the 5' terminus using the 19mer oligonucleotide probe revealed the presence of a possible stem loop structure (secondary structure 1) from 104nt to 158nt. A major primer extension signal was observed at 104nt and at 144nt, the latter is predicted to be the 5' start of the stem and former predicted as the 3' end of the loop. As a result of this stem loop premature termination of the extension reaction occurred.

Panel B A 20mer oligonucleotide probe was used for further primer extension analysis and the results revealed the possibility of a smaller stem loop (secondary structure 2) from -8nt to +7nt. A major primer extension signal was observed at -8nt and at +4nt, the latter is predicted to be the 5' start of the stem and former predicted as the 3' end of the loop.

The position of each nucleotide is referred to the first translation start site **ATG**, at position +1.

A.



B.

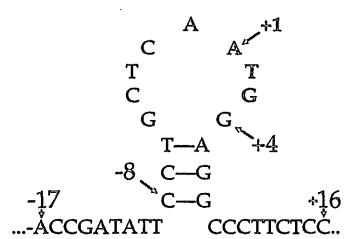
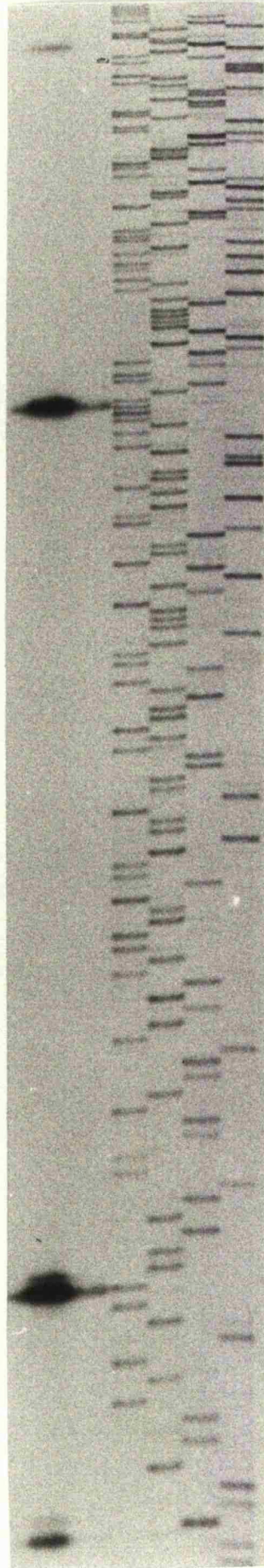


Figure 5.7. Primer extension analysis of the 5' terminus of the *A. nidulans* *hemA* mRNA using the 20mer oligonucleotide probe.

A radioactively end-labelled 20mer oligonucleotide primer was hybridised to 50µg (lane 1) and 25µg (lane 2) of total cellular RNA from the *A. nidulans* strain R153. An extension reaction was then carried out using reverse transcriptase. The extended products were separated on a 6% polyacrylamide/urea denaturing gel along with the sequence pattern of the 455bp *Sau3A/KpnI* fragment using the 20mer oligonucleotide primer. The major transcript initiation site is indicated by the arrow along with the sequence data at the deduced 5' terminus.

1 2 G A T C

C
C
A
T
C
C
C
C
C
C
T
C
G
C
T
G
G



5.5. Mapping the *hemA* mRNA by S1 nuclease analysis.

Protection against S1 nuclease digestion was used to map the 5' and 3' termini of the *hemA* mRNA using two radioactive DNA probes, one spanning the 5' terminus and another spanning the 3' terminus of the mRNA (Section 2.8). These probes were hybridised to total cellular RNA in separate experiments. The resulting RNA/DNA hybrids were subjected to S1 nuclease, a single stranded exonuclease which degrades the single stranded non-hybridised portion of the probe and the mRNA. The resultant radiolabelled DNA fragment reflects the size of RNA that is homologous to the DNA probe with reference to a known restriction enzyme site in the coding region. S1 nuclease analysis was also used to map the location and size of the intron by the use of a DNA probe which spans the predicted intron splice site junction.

The M13 mp plasmids constructed in the DNA sequence analysis, were used to generate templates for the synthesis of the uniformly radioactively labelled single stranded DNA probes which are complementary to the mRNA of interest. A general strategy was followed whereby the radioactively labelled DNA probe was synthesised from the single stranded DNA of M13 bacteriophage using the Klenow fragment of *Escherichia coli* DNA polymerase, [α -³²P]dCTP, dNTPs and the universal primer to initiate the synthesis (Section 2.8). Having produced a double stranded DNA the nucleic acid was digested with an appropriate restriction enzyme to release the desired fragment which was then separated by gel electrophoresis and the fragment recovered by electroelution (Section 2.5.4.2). The double stranded fragment, which consists of both the non-radioactive template strand and the newly synthesised radioactive DNA strand, was denatured and the radioactively labelled DNA strand hybridised to total cellular RNA isolated from young mycelium of the *A. nidulans* strain R153 grown in glucose minimal medium. The S1 nuclease

reaction was carried out for 30 minutes and the products separated by electrophoresis on a 6% acrylamide denaturing gel.

5.5.1. Determination of the 5' terminus of the *hemA* mRNA.

The 455bp *Sau3A/KpnI* fragment (B) at the 5' end of the sequenced fragment was used to map the 5' transcription terminus (Figure 5.2 B). The probe was complementary to the mRNA, extending from the *KpnI* site (+250nt) in the first exon to the *Sau3A* site (-205nt) 5' to the translation start (Figure 5.2 B). This probe was prepared from the M13 recombinant phage ALA-13(-) (Section 4.5.9) which was used as a single stranded template for the synthesis of the radioactively labelled complementary DNA strand. The double stranded fragment was released by digestion with the restriction enzymes *SalI*, adjacent to the *Sau3A/BamHI* ligation site, and *EcoRI*, adjacent to the *KpnI* restriction site, since the original *Sau3A/KpnI* fragment was cloned within the polylinker to construct phage ALA-13(-). The recovered fragment was resuspended in TE buffer (Section 2.8) and the incorporation of radioactivity determined by Cherenkov counting.

The radioactive probe solution was divided into five aliquots. Three aliquots of the probe were separately hybridised to 50µg of *A. nidulans* R153 total cellular RNA as described in Section 2.8.2. The most suitable hybridisation temperature was 55°C. A fourth aliquot was hybridised to 50µg of yeast tRNA and the fifth aliquot stored for use later as a positive control for confirmation of the size of the fragment and labelling efficiency. Following hybridisation, S1 nuclease was added and the samples immediately transferred to 37°C (Section 2.8.3). Digestion of the experimental samples was carried out using 300, 600 and 900 units of S1 nuclease for 30 minutes. The fourth aliquot, hybridised to yeast tRNA was digested using 900 units of S1 nuclease for 30 minutes. The nucleic

acids were recovered by precipitation with ethanol and resuspended in TE buffer. The samples were denatured, by heating in a 105°C PEG bath for 3 minutes and transferring immediately to ice, and then separated by electrophoresis in a 6% acrylamide gel alongside the positive control and a set of sequencing reactions as size markers.

A single highly abundant S1 nuclease protected fragment was observed at 309bp in length (Figure 5.8, Lane 3, 4 and 5). This fragment length, with reference to the *KpnI* site at +250nt indicates a major transcription initiation site 55nt upstream of the first translation start site (+1) in the nucleotide sequence. A minor S1 nuclease protected fragment of 346bp was also observed corresponding to a minor transcription initiation site at 92nt, 5' of the predicted translation start site. The positive control shows a single fragment at 455bp which corresponds very well to the actual length of the fragment used, and indicates that the probe was efficiently radiolabelled (Figure 5.8, Lane 1). The tRNA negative control only shows the original 455bp fragment, and no other protected fragments were observed (Figure 5.8, Lane 2).

The presence of the original 455bp fragment in Lane 2, the negative control, where hybridisation was carried out with unrelated yeast tRNA, shows that the fragment has in part escaped S1 nuclease digestion. This may represent a failure to fully denature the original double stranded probe, or more likely, reformation of double stranded DNA by rapid hybridisation between the labelled fragment and its unlabelled complement. The presence of strong 455bp bands in the experimental samples can also be accounted for in a similar manner (Figure 5.8, Lanes 3, 4 and 5).

5.5.2. Determination of the 3' end of the *hemA* mRNA.

The 413bp *Bam*HI/*Pst*I fragment (E) from the 3' end of the coding region was used to map the 3' transcription terminus (Figure 5.2 E). The probe was complementary to the mRNA, extending from the *Pst*I site (+1884nt), 142bp 5' of the deduced *hemA* translation stop codon, to the *Bam*HI site (+2297), 393bp 3' of this site. The probe was prepared from the M13 recombinant phage ALA-5(-) (Section 4.5.1). The radioactively labelled complementary DNA strand was synthesised and the double stranded fragment released by digestion with the restriction enzymes *Pst*I and *Bam*HI. The recovered fragment was resuspended in TE buffer (Section 2.8) and the incorporation of radioactivity determined by Cherenkov counting. Hybridisation and S1 nuclease reaction were carried out as described above.

Two highly abundant S1 nuclease protected fragments, 305bp and 309bp in length, were observed (Figure 5.9). This indicates the presence two transcription termini, at 174nt and 178nt down stream of the predicted translation termination site, TAA at +2010nt. The positive control shows a single fragment at 420bp which corresponds very well to the actual length of the fragment used and indicates an efficiently radiolabelled DNA probe (Figure 5.9, Lane 1). The presence of this 420bp fragment in the experimental lanes are due to DNA/DNA hybrids as explained above (Figure 5.9, Lanes 2-5).

5.5.3. Determination of the intron splice sites within the *hemA* mRNA.

S1 nuclease analysis was used to determine the intron boundaries within the *hemA* mRNA using a 276bp *Sma*I/*Pst*I3 fragment (Figure 5.2 fragment G). The *Sma*I restriction site (+266nt) is located within the first exon, 89bp 5' of the intron splice consensus sequence, 5'-GTACGTT-3' (+355 to +362), and the

restriction site *Pst*II3 (+542nt) is located 124bp 3' of the splice junction consensus sequence, 5'-CAG-3' (+417 to +419), within the second exon.

The plasmid pALAS-d was digested with the restriction enzymes *Sma*I and *Pst*II and a 276bp δ -ALAS fragment recovered and ligated into the *Sma*I/*Pst*II digested phage M13 mp18 (Section 4.5). The recombinant phage DNA was prepared as a single stranded template for synthesis of the radioactively labelled complementary strand. The double stranded fragment was released by digestion with the restriction enzymes *Eco*RI, adjacent to the *Sma*I restriction site, since the original *Sma*I/*Pst*II fragment was cloned within the polylinker to construct the phage, and *Pst*II which resulted in the size of the fragment being increased from 276 to 290bp. The recovered fragment was resuspended in TE buffer (Section 2.8) and the incorporation of radioactivity determined by Cherenkov counting. Hybridisation and S1 nuclease reaction were carried out as described above.

Two clusters, each of five highly abundant S1 nuclease protected fragments were observed. One cluster corresponds to the region +88 to +93bp while the second cluster appeared at +122bp and +127bp from the translation start site at position +1 (Figure 5.10). The fragments were centred upon at +89bp and +124bp in length. The probe contained 89bp of the coding sequence from exon 1 and 124bp from exon 2 which would hybridise to the mRNA and protect against S1 nuclease digestion. This result provides confirmation for the presence of the predicted intron.

The presence of the clusters of protected fragments is due to incomplete protection by the mRNA resulting in variable S1 nuclease activity on the DNA/mRNA hybrids at the intron boundaries. The positive control shows a single fragment at 290bp which corresponds very well to the actual length of

the fragment used (Figure 5.10, Lane 1). The presence of this 290bp fragment in the experimental lanes are due to DNA/DNA hybrids as explained above (Figure 5.10, Lanes 2-5).

S1 nuclease analysis has provided evidence confirming the presence of an intron which was predicted by analysis of the sequence data. The S1 nuclease analysis was also successful in mapping both the 5' and 3' termini. The 5' terminus is mapped 55nt upstream of the first translation start site, at position +1 of the nucleotide sequence, with a minor site at 92nt upstream of the predicted translation start site. Two major 3' termini are observed at 174nt and 178nt down stream of the predicted translation termination site, the codon TAA at position 2010nt (Figure 5.11).

Figure 5.8. S1 nuclease analysis of the 5' terminus of the *A. nidulans* *hemA* mRNA.

A 455bp *Sau3A/KpnI* fragment was uniformly labelled was used to map the 5' terminus. The radioactively labelled DNA probe (Figure 5.2 E) complementary to the 5' end of the *hemA* mRNA and likely to extend 5' to the start position was hybridised to 50µg of total cellular RNA from the *A. nidulans* strain R153. The probe was also hybridised to 50µg of yeast tRNA and used as a negative control. The DNA/RNA hybrids were digested with 300 units (lane 3), 600 units (lane 4) and 900 units (lane 5) of S1 nuclease. The protected fragments were separated on a 6% polyacrylamide/urea denaturing gel. The major protected fragments are indicated by the arrow along with the sequence data at the deduced 5' terminus.

The sequence reactions GATC were used as size markers for the S1 digested products and were derived from the 455bp *Sau3A/KpnI* fragment.

Lanes	
1	Positive control - Untreated radioactive probe (455bp)
2	Negative control - DNA probe hybridised to tRNA plus 900 units S1 nuclease digest
3	DNA/RNA hybrids plus 300 units S1 nuclease
4	DNA/RNA hybrids plus 600 units S1 nuclease
5	DNA/RNA hybrids plus 900 units S1 nuclease
GATC	Sequence reactions

T
A
T
T
C
T
C
A
A
T
T

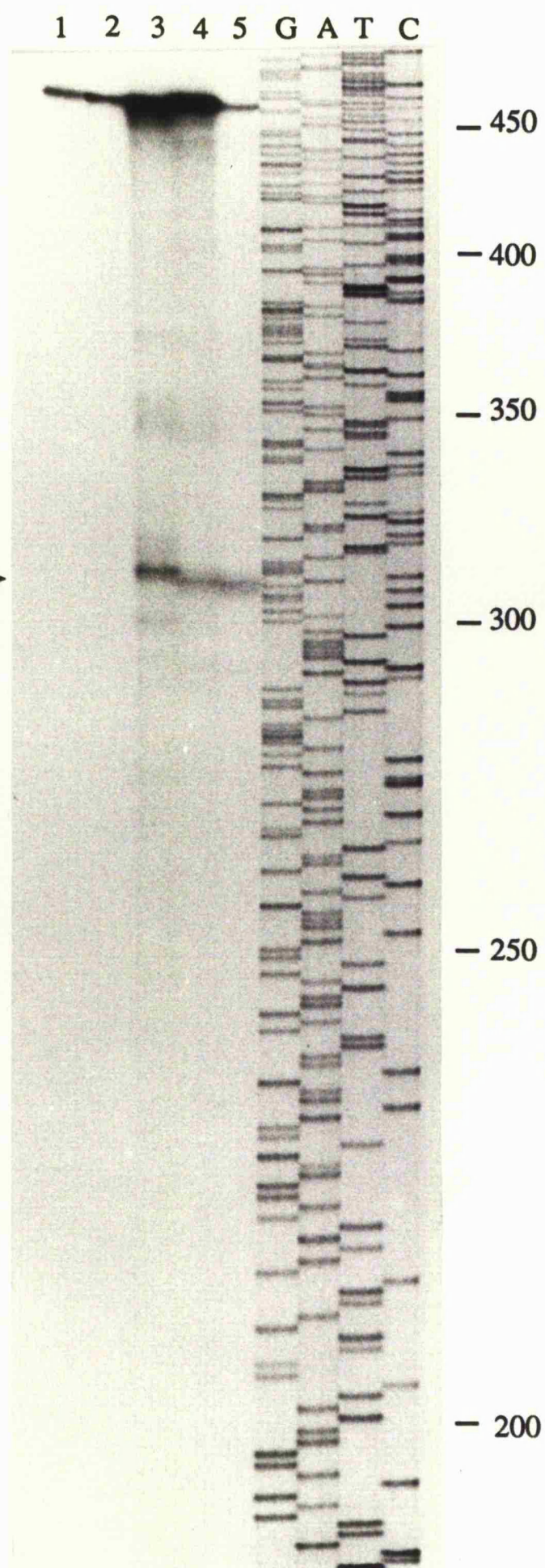


Figure 5.9. S1 nuclease analysis of the 3' terminus of the *A. nidulans* *hemA* mRNA.

A 413bp *Pst*I/*Bam*HI fragment was uniformly labelled and used to map the 5' terminus. The radioactively labelled DNA probe (Figure 5.2 B) complementary to the 5' end of the *hemA* mRNA and likely to extend 5' to the start position was hybridised to 50µg of total cellular RNA from the *A. nidulans* strain R153. The probe was also hybridised to 50µg of yeast tRNA and used as a negative control. The DNA/RNA hybrids were digested with 300 units (lane 3), 600 units (lane 4) and 900 units (lane 5) of S1 nuclease. The protected fragments were separated on a 6% polyacrylamide/urea denaturing gel. The major protected fragments are indicated by the arrow along with the sequence data at the deduced 5' terminus.

The sequence reactions GATC were used as size markers for the S1 digested products and were derived from the 455bp *Sau*3A/*Kpn*I fragment.

Lanes	
1	Positive control - Untreated radioactive probe (413bp)
2	Negative control - DNA probe hybridised to tRNA plus 900 units S1 nuclease digest
3	DNA/RNA hybrids plus 300 units S1 nuclease
4	DNA/RNA hybrids plus 600 units S1 nuclease
5	DNA/RNA hybrids plus 900 units S1 nuclease
GATC	Sequence reactions

A
T
A
C
A
A
A
G
T
T

1 2 3 4 5 G A T C

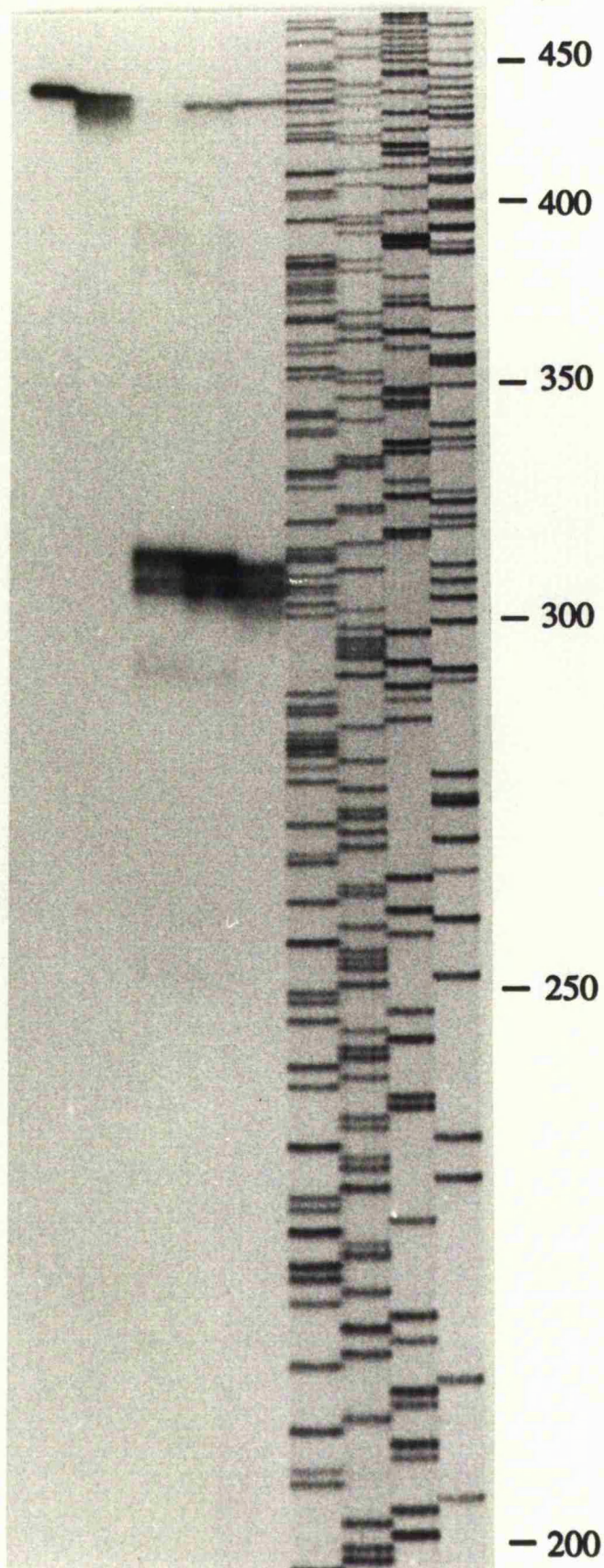


Figure 5.10. S1 nuclease analysis of the intron boundaries of the *A. nidulans* *hemA* mRNA.

A 276bp *SmaI/PstI* fragment was uniformly labelled was used to map the 5' terminus. The radioactively labelled DNA probe (Figure 5.2 G) complementary to the 5' end of the *hemA* mRNA and likely to extend 5' to the start position was hybridised to 50µg of total cellular RNA from the *A. nidulans* strain R153. The probe was also hybridised to 50µg of yeast tRNA and used as a negative control. The DNA/RNA hybrids were digested with 300 units (lane 3), 600 units (lane 4) and 900 units (lane 5) of S1 nuclease. The protected fragments were separated on a 6% polyacrylamide/urea denaturing gel. The major protected fragments are indicated by the arrow along with the sequence data at the deduced 5' terminus.

The sequence reactions GATC were used as size markers for the S1 digested products and were derived from the 455bp *Sau3A/KpnI* fragment.

Lanes	
1	Positive control - Untreated radioactive probe (290bp)
2	Negative control - DNA probe hybridised to tRNA plus 900 units S1 nuclease digest
3	DNA/RNA hybrids plus 300 units S1 nuclease
4	DNA/RNA hybrids plus 600 units S1 nuclease
5	DNA/RNA hybrids plus 900 units S1 nuclease
GATC	Sequence reactions

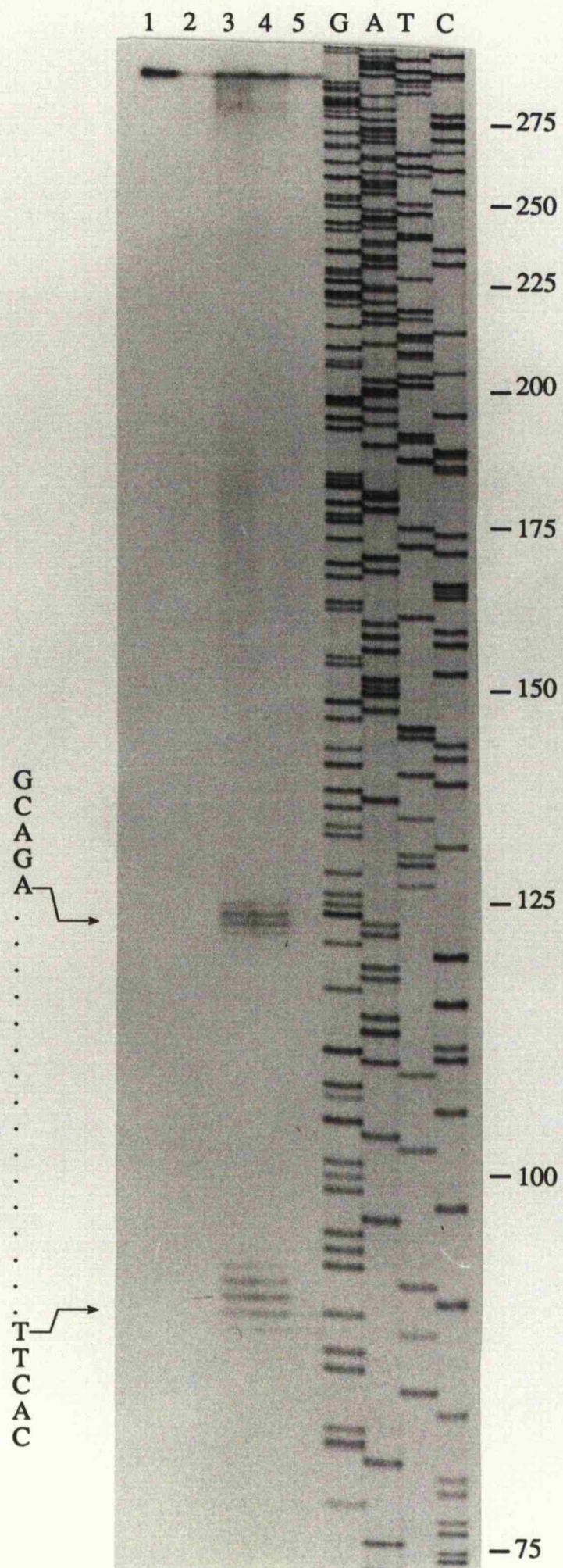


Figure 5.11. Nucleotide sequence of the *hemA* gene of *A. nidulans*.

Nucleotides are numbered on the left, commencing from the first ATG (+1). Amino acids are numbered on the right. The conserved block of amino acids **VWSNDYL** is boxed, from amino acids 196 to 203. The intron is shown in lower case letters with the consensus splicing signals underlined. An AT rich region upstream of the first ATG is overscored. The transcriptional start sites are indicated at -92 (†) and -55 (↓) as deduced by primer extension and S1 nuclease analysis respectively. Transcription termination sites are indicated at positions 2181nt and 2185nt (∇), 3' of the coding sequence.

-206 GGATCTGGTAAGAGGCTCCACCCACAGTCTTAGTCACGGGCTCTGAATAACCTGCGGAGATTTCACAGTTGCGAAATCCCGTCGCCCTCGTATT
-106 TTAGTGACACCATCCCCCTGGCTGGTCTGTCGGATTCTAGTACTTTGTCCACTATTTCAATTGCTTGTCCACTTCCATATCGAATCAACCGATATTCC
-6 TGCTCAATGGAGGCCCTTCTCCAGCAGTCTCGGCCATGTGCTGTTCTCAAGCGCAGCTACCGAACAACCTTCTGTTGCTGGCTACCGCTACCGCTC
M E A L L Q Q S R A M C P F L K R S S P N T L R S L A T A T R P 32

94 CCAGCACCAGCCCTGGCGGTGGTACTATGACCAATTGCAAGAGAATCGCTCGCCGCTGCGCCGCTCATGAGCAAGCGCTCGCTGTCAGAGTCCCGCAT
S T S P G G G T M T N L Q R I A R R C P V M S K A L A V Q S A R M 65

194 GACTGGCACCAGCGATTACTTCTCTCGGGCTGGTGTACTGGCGCTGGAGCTGGTACCCCAAGCCACCCCGGGTTGCGCGGAAAGAGGGCGCTG
T G T K R F T S S A A G V P G A G A G T P K P T R G S P G K R A L 98

294 CACTCGACTGGAGAAACGGCGCAATATGAGCACTGAGTTTCAAGGGAGCCCGCAGAGTACGTTTTCTGAGAGCGATAGACTAGACAATGtgg
H S T G G N G A N M S T E F H K G A Q Q I

394 aatataactgaatgggatgtttgaTTTCACTCGGCTTTCCCAAGCCACCGCTTCTACGTCGGGCGCTCAGCAACCGTTTCCGGTCTTACTCTCTGTG
H P G L S N A T R S H V G A S A T V S G P T P R A 144

494 CCCCGTGGCTCGGCCATTGCTACGACGCTTCTACAACTGAGAACTGAGAAAGTACCGTCTGGTCTCGAAGCACTACCTGCGCATGGGAGCTAACTCGAA
P V A A P F D Y D A F Y N A E L Q K K H Q D K S Y R Y F N N I N R 177

594 TCTGCTCAGGAATTCCTCCGCTCATGCGCTTCGAAGGATGAGAAAGTACCGTCTGGTCTCGAAGCACTACCTGCGCATGGGAGCTAACTCGAA
L A Q E F P R A H T A S K D E K V T V W C S N D Y L G M G R N P E 210

694 GTTCTCGGACCATGATAGACCTCGATGCTAGCAGGAGCGGGCGGGGAGGAAGCTGTAACATTTCGGGCAACATCAACATGCCCTCTCGTGGAG
V L A T M H K T L D T Y G A G A G G T R N I S G H N Q H A V S L E N 244

794 ATACTCTGGCAAACTCCATGTAAGAACTCGGCTGGTTTTCAGCTCGTCTTTAGTGAACAGCAGCTACTTTGGCTACTCTCGGCAAGAGTCC
T L A K L H G K E A A L V F S S C F V A N D A T L A T L G S K M P 277

894 TGACTCGTCTATTGTCGAGCTCGCTCAACACGATCGATGATCCAGGTATCCGCCATTCTGGCCGGAAGAAATGGTCTTCAAGCAACAGGATTG
D C V I L S D S L N H A S M I Q G I R H S G R K K M V F K H N D L 310

994 GTCGACCTGAGAGCAAGCTGCACTCTCTCTCTGCAATGTTTCAAGATCATTCGCTTCGAATCGGTATCAGTATGTTGGCTCTATTGCTCGGATCG
V D L E T K L A S L P L H V P K I I A F E S V Y S M C G S I A P I E 344

1094 AGCGGATTGTTGATTTGGGAGCAAGTACGAGGCTTACTTTCTTAGACGAGGTTACGCGCTGGGAATGTACGAGCCGACCGTCTGAGTTCGGA
A I C D L A D K Y G A I T F L D E V H A V G M Y G P H G A G V A E 377

1194 GCATCTTGAAGAGCAGGATCTATGCTCGGAGACACCGCAACCTCTCAGCACCAAGGACCTGTCATGGACCGGATCAACATCATTTACCGGTACTCTT
H L D Y E I Y A S Q D T A N P L S T K G T V M D R I N I I T G T L 410

1294 GGTAAGCGTACGAGTGGCTGGTGGTTCATTTGCTGGCTCTGCGCTTTAGTTGACACCATCCGCTCCCTCGCCCTGGGTTTATTTTACCACTTCCG
G K A Y G C V G G Y I A G S A A L V D T I R S L A P G F I F T T S L 444

1394 TTCTCCAGCAACCATGGCGGTGCGGATCTGCAATCCGATTCAGGACCGCCACGACGAGACCGTATCTCGACGATTCACACCTCGGCTGTAA
P F A T M A G A D T A I R Y Q A R H Q Q D R I L Q Q L H T R A V K 477

1494 GCAATCATTCAGGATTTAGATATCCCCGTCATCCCAACCTCTCAGATCGTCCCTCTCTCTGCTGGTGTATCCGAGCTCGCAAGCAGGCTCGGAC
Q S F K D L D I P V I P N P S H I V P L L V G D A E L A K Q A S D 510

1594 AAGCTCTTGAAGAGCAGGATCTACGTTCAAGCAATTAACCTACCCACCGTGGCAAGCGGTGAAGAGCGCTCCGGATTACCCCAACCTCTGTCACA
K L L E E H G I Y V Q A I N Y P T V P R G E E R L R I T P T P G H T 544

1694 CGCAAGAGCTTCTGACCACTTGTGCAAGCAGTCAACACTGTCTGGAACGACCTCGGATCAAGCGCGCAAGCGACTGGAAGGCAATGGCGGATCTGT
Q E L R D H L V E A V N T V W N D L G I K R A S D W K A M G G F V 577

1794 CGGTGTTGGCTGGAAGCTGCGAGCTGGAAGTCAACCATCTGGACGAGCCGCTTGAATATGCGACCTGATGAACCTCGAGGCTGCAATGAA
G V G V E A A E L E N Q P I W T D A Q L N M R P D E T L E A A V E 610

1894 CGCGAGTTTCAGGCGCGGCTTCTGGCATGAAAGCGGGGTGCAAGGCGAAGCCGCTCGGCTCAATCGCGGCAACCTTACGCTGCTCAATCCAG
R E F Q A A V P G M K A G A K A K P V G S I A A N P I G A S I P V 644

1994 TCGCGGCTGCTGCTTAAGTCGCTTGTGGTCTTTTATTGTTGGTGTGTTTATATATACGTATATATAGCCCTCCCGTCCGCTCATTTAGTTAT
A A A A

2094 TCGTGATCATGCTTTGTCGAACAATTTTTCGATGTGATGATGCTTCAAGTATGACCGGCCCTCATTTTACATTTAGGTCTAATCAAGATTGTCCATAC
2194 ACTCGAAGCTTCTCTATTTGATGACGATGCTGCTCATATGATTCACACAACTTAAGTTGATTTTCAAGATTGACTACCTTAATGATATCTCAGGCCA
2294 GTCGGATCC 2302

5.6. Summary.

A. nidulans hemA mRNA was shown to be in ample abundance since it was easily detectable by northern blot hybridisation analysis. A single species of *hemA* mRNA was observed and found to be approximately 2.3kb in length. Northern blot analysis using DNA probes derived from the 5' and 3' ends of the ORF (identified by sequencing the 2.5kb *Bam*HI/*Sau*3A fragment), clearly demonstrated by northern analysis that the region encodes the entire *hemA* transcript. The sequence data also enabled the selection of suitable DNA fragments used as probes to determine the 5' and 3' transcript termini by protection against degradation in S1 nuclease analysis and for use in primer extension experiments.

Primer extension analysis of the 5' terminus was unsuccessful since there were multiple signals arising from the 19mer primer. Similar analysis using a second 20mer primer, also revealed multiple signals, as well as a transcriptional start site at -92nt with respect to the start of the ORF. One of the problems associated with primer extension analysis is the generation of heterologous extension products due to the tendency of reverse transcriptase to terminate at positions of high secondary structure in the template RNA. This could have been minimised by performing the reaction in high concentrations of dNTP's and using primers at 50-100nt of its 5' terminus. Other causes of multiple reverse transcripts can include the presence of multiple start sites which may lead to mRNA sequences differing in length within their 5' non-coding region. Additionally cross hybridisation may occur due to a multigene family sharing sufficient homology over the region of the primer hence yielding multiple reverse transcripts in the primer extension, each deriving from a different mRNA.

It is necessary for the primer extension reaction to be carried out in excess DNA over the complementary RNA. The potential for templates to be in excess of the primer may present a situation where the primers selectively binds to the template. In the event of the *hemA* gene possessing multiple 5' start sites, hence producing different sizes mRNA, during primer extension analysis the larger message will be selectively primed and extended in preference to the smaller template. However a large excess of primer can lead to non specific priming during the extension reaction, and although not performed it is useful to perform RNA titrations around the expected optimum concentrations. A control where the extension reaction is performed in the absence of RNA was carried out to establish the absence of DNA-DNA hybrids.

S1 nuclease analysis of the 5' terminus revealed a major transcriptional start site at -55nt with respect to the start of the ORF and a minor point at -92nt. Analysis of the 3' terminus revealed two major transcriptional stop sites, 171bp (+2181nt) and 175bp (+2185nt) 3' of the translation stop codon (Figure 5.11). The S1 nuclease analysis also confirmed the presence of a 64bp intron. S1 nuclease analysis transcript gives the size of the *hemA* mRNA at between 2169-2213nt. The northern blot hybridisation analysis resulted in a single band of 2.3kb, a difference of approximately 0.1kb.

During S1 nuclease analysis the use of double stranded DNA probes required the hybridisation to be carried out in the presence of 80% formamide when DNA renaturation is suppressed. Under high formamide concentrations DNA-RNA hybrids are more stable than DNA-DNA duplexes allowing for the possibility of selecting a hybridising temperature which allows for hybridisation of the probe to the RNA while preventing reannealing of the probe. The optimum temperature will depend on the

length of the hybrid, the G+C content and the primary sequence. However, in the presence of 80% formamide the annealing temperature must ideally be above the melting temperature (T_m) of DNA-DNA duplexes but below that of DNA-RNA hybrids. Preliminary experiments were carried out to establish the optimum hybridisation for the S1 analysis.

Problems can occur with S1 nuclease analysis due to renaturing of DNA resulting in a diffused background of smaller fragments. The presence of multiple bands may be due to heterogeneity at the 5' and 3' regions of the RNA or exonucleolytic cleavage of the DNA-RNA hybrid by the S1 nuclease enzyme. Additionally if the probe is not completely denatured, the DNA duplexes will be present and will be resistant to the action of the S1 nuclease enzyme and therefore may be detected as distinct bands. These artefacts are observed in the S1 analysis and may be present when high concentrations of DNA is used and should show up in the control experiments.

Sequences within the 5' untranscribed region of eukaryotic genes which are implicated in transcription initiation are found 5' to the *hemA* ORF. An AT-rich region, indicative of a 'core promoter' TATAAA motif (Nussinov *et al.*, 1987; Guarente, 1984) is located upstream of the transcript sites as deduced by S1 nuclease analysis and primer extension analysis (Figure 5.11). The putative CAAT consensus sequence however, appears not to be present in the 5' untranscribed region of the gene. Such sequences, when present in filamentous fungi do not appear to perform any significant functional activity (Gurr *et al.*, 1987). With reference to the 3' region there were no obvious polyadenylation signal (AATAA) consensus sequences found at the transcription termini region again a feature not uncommon among filamentous fungi.

CHAPTER 6.

DISRUPTION OF THE *ASPERGILLUS NIDULANS* *hemA* GENE.

6.1. Introduction.

The enzymatic pathway for the biosynthesis of haem has been discussed in Chapter 1 and shown to be identical in all organism studied, beginning from the production of δ -aminolaevulinic acid (δ -ALA). The control mechanisms regulating the pathway have been extensively investigated, and are well known in the facultative anaerobe *Saccharomyces cerevisiae* (Labbe-Bois and Labbe, 1990). However, the regulation of the pathway is not well characterised in obligate aerobic organisms and a study of haem biosynthesis and its regulation in *Aspergillus nidulans* can contribute to a better understanding of this pathway. In addition, a study of haem production in *A. nidulans* will provide an insight into the possible involvement of oxygen in the regulation of the haem biosynthetic pathway and the differences between a facultative anaerobe, *S. cerevisiae*, and an obligate aerobe, *A. nidulans*.

6.1.1. Isolation of haem biosynthetic mutants in *Aspergillus nidulans*.

Mutant strains of *A. nidulans* deficient in haem biosynthesis would provide useful tools in which the pathway and its regulation can be studied, as has been the case in *S. cerevisiae* (Labbe-Bois *et al.*, 1980; Urban-Grimal and Labbe-Bois, 1981; Camadro *et al.*, 1982). Since initial attempts to isolate haem deficient mutants by classical procedure was unsuccessful (Section 6.2), it was necessary to employ a gene disruption strategy to isolate *hemA* mutants. This involved

the transformation of a diploid strain of *A. nidulans* with a construct which included a disrupted *hemA* gene and selecting for δ -ALA auxotrophy amongst haploid segregants (Section 6.3).

6.1.2. Transformation of filamentous fungi.

The availability of an effective DNA mediated transformation system provides a useful tool for the analysis of many aspects of gene function. Following the development of transformation procedure in the yeast *Saccharomyces cerevisiae* (Hinnen *et al.*, 1978), and reviewed in Boguslawski, (1985), and the filamentous fungi, *Neurospora crassa*, (Case *et al.*, 1979), efforts were concentrated on the development of a transformation system for the filamentous fungi, *A. nidulans* (Turner and Ballance, 1985). A recent review of transformation in filamentous fungi is by Fincham (1989).

6.1.3. Targeted gene disruption.

Stable inheritance form of transforming DNA requires the selectable marker to be either maintained on a self replicating vector molecule, or to be integrated into a chromosome of the recipient cell. In the yeast, *S. cerevisiae*, transforming DNA on non-replicating plasmids commonly integrates into the chromosome by homologous recombination upon transformation. In *N. crassa* however, the frequency of homologous recombination is low (Dhawale and Marzluf, 1985). In *A. nidulans* homologous recombination occurs at a frequency that is intermediate of these two systems (Yelton *et al.*, 1984; May *et al.*, 1985).

In *A. nidulans* DNA incorporation into the chromosome is utilised to alter its genome. Homologous recombination can occur with a circular DNA molecules between transforming DNA and the homologous locus on the chromosome

(reviewed by May, 1992). Transformation with circular DNA results in a single recombination event causing a duplication of the target sequences with the entire plasmid vector molecule separating the duplicated sequences (Figure 6.1). Due to the tandem duplication of the sequences within the genome, this event is usually unstable, resulting in the loss of the integrated vector sequences and the regeneration of the original vector sequences.

In contrast to the single recombination event which occurs as a result of integration of a circular DNA, integration of a linear DNA sequence involves a double recombination event (Figure 6.2). Since the DNA molecule is interrupted by the insertion of a selective marker, a double recombination event results in the transplacement of the chromosomal sequence. This type of event does not result in tandem duplication of the sequences within the genome, as is the case with integration with a circular molecule, and therefore ensures the stability of the chromosomal structure.

In *A. nidulans* the efficiency of transformation, and the frequency of integrative recombination is shown to increase when a linear plasmid vector is used as the transforming molecule (Streatfield *et al.*, 1992). To enhance the possibility of integrative recombination of the transforming molecule to a targeted locus, the selective marker gene must be flanked by sequence derived from the target locus. It is desirable to include extensive 5' and 3' *Aspergillus* flanking sequences thereby increasing the probability of integration by homologous recombination.

Figure 6.1. Integration of a circular DNA molecule by homologous recombination.

Integration of a circular molecule into the chromosome by homologous recombination where the sequence, A' to B', integrates at the homologous chromosome sequences, A to B. The integration leads to a tandem duplication of the homologue sequences separated by the vector sequences which carries the selective marker.

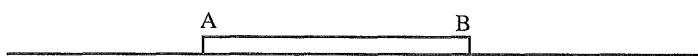
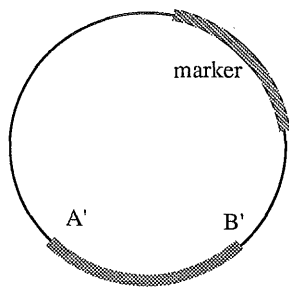
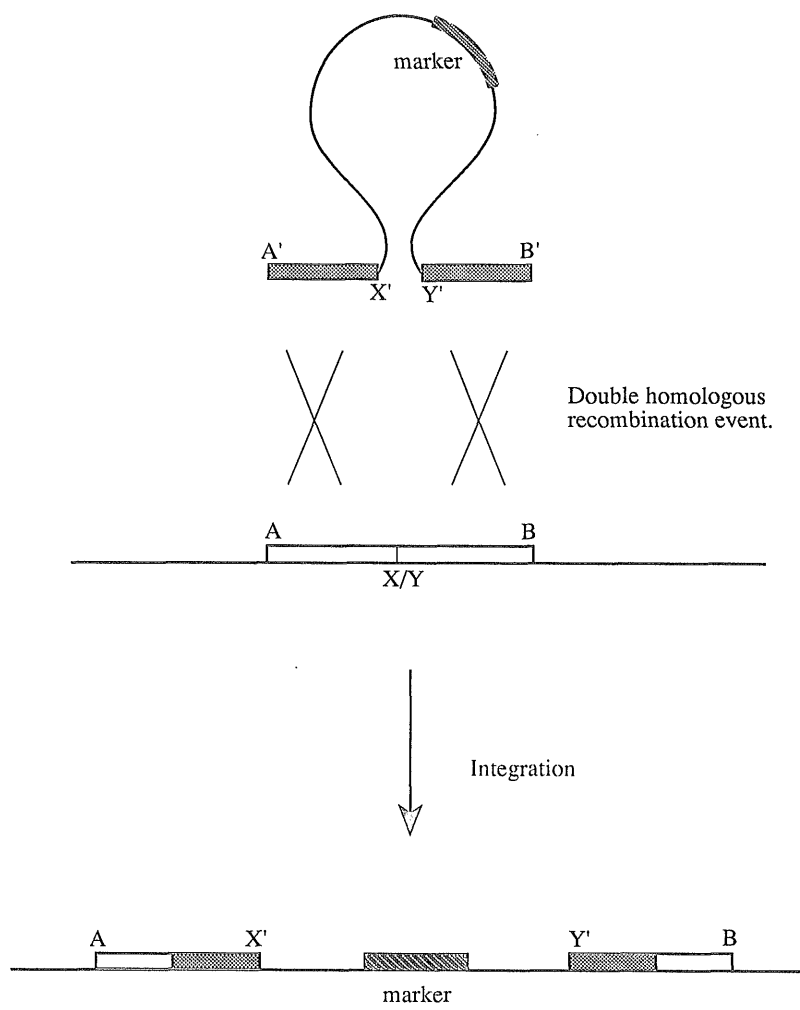


Figure 6.2. Integration of a linear DNA molecule by homologous recombination.

Integration of a linear molecule into the chromosome involved a double homologous recombination event. The linear molecule is interrupted by the insertion of a selective marker. The resulting sequence intervals, A' to X' and Y' to B', undergoes a double homologous recombination event with the equivalent chromosome sequences, A to X and Y to B. The result is the transplacement of the chromosomal sequences with those of the linear DNA molecule.



6.2. An attempt to isolate haem requiring mutants in *Aspergillus nidulans*.

Mutant strains of *S. cerevisiae* lacking an enzyme in the biosynthetic pathway require haem for growth, however if these mutants are deficient in one of the first four steps of the pathway L-methionine is also required (Gollub *et al.*, 1977) (Figure 6.3). Other derivatives of the haem pathway, such as ergosterol and polyoxyethylenesorbitan monooleate (Tween-80), can also be used as growth supplements in some *S. cerevisiae* mutants but L-methionine must again be supplied for those mutants deficient in the production of uroporphyrinogen.

Preliminary attempts to isolate haem requiring mutants of *A. nidulans* followed a well established method known as "filtration enrichment" (Woodward *et al.*, 1954; Bos *et al.*, 1992). The technique has been particularly effective in recovering mutants of *A. nidulans* deficient in utilisation of various carbon sources (Armitt *et al.*, 1976; Bos *et al.*, 1981; Uitzetter *et al.*, 1986). In principle, the method involves the incubation of conidiospores, previously exposed to mutagenic treatment, in minimal growth medium and at intervals removing growing mycelium by filtration through a sterile gauze. This process enriches for a population of the non-growing mutant spores which may be plated in the appropriately supplemented medium to recover the desired mutant strains.

An attempt to recover haem deficient mutants was carried out by plating the enriched spore suspension in minimal agar supplemented with haem and L-methionine. A total of 48 mutant strains were recovered. However when the growth requirements of these strains were further investigated, all of the mutants were found to specifically require L-methionine for growth and none responded to haem alone.

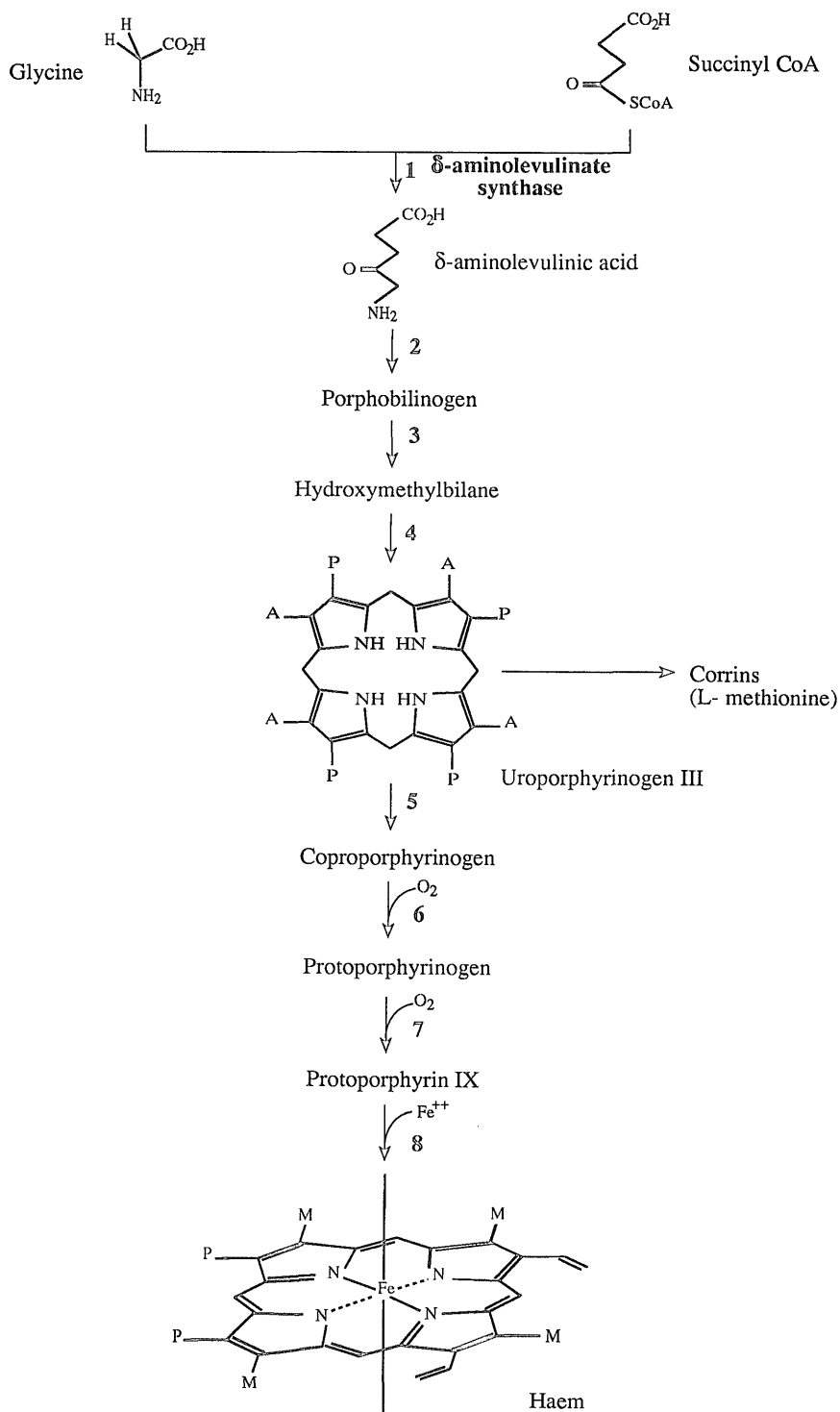
Further attempts were made to recover *A. nidulans* haem deficient mutants using the same method but plating the enriched spore suspension in minimal agar supplemented with L-methionine and various concentrations of haem. This resulted in the recovery of 136 mutant strains, but again all of these strains were found to be L-methionine requiring mutants. Thus although the filtration method proved efficient in recovering mutant strains none requiring haem for growth were isolated.

Since the filtration enrichment method for the recovery of mutants in *A. nidulans* follows a two step procedure, an enrichment step and a recovery step, the inability to isolate haem deficient may be due to flaws at either of these stages. During enrichment there is a considerable period of time, after exposure to UV irradiation, before possible haem deficient mutant strain are plated onto the recovery medium. This period, although essential for the enrichment of the spore suspension, may result in the loss of viability of potential haem deficient mutants. In recovery it is possible that the design of the medium to rescue the mutants may not be appropriate. Moreover, it is possible that *A. nidulans* is unable to accumulate haem from the medium and therefore this essential nutrient cannot be replaced exogenously. Taking these observations into account, lead me to conclude that it would be counter productive to persist with the filtration enrichment procedure to isolate *A. nidulans* haem deficient mutants.

Figure 6.3. The haem biosynthetic pathway.

A summary of the haem biosynthetic pathway showing the eight enzymic steps. One of the intermediate porphyrin compound, uroporphyrinogen, is used to form the porphyrin compound, including L-methionine.

1. δ -aminolaevulinate synthase;
2. porphobilinogen synthase;
3. porphobilinogen deaminase;
4. uroporphyrinogen III synthase;
5. uroporphyrinogen decarboxylase;
6. coproporphyrinogen III oxidase;
7. protoporphyrinogen oxidase;
8. ferrochelatase.



6.3. Isolation of *hemA* mutants in *Aspergillus nidulans*.

Since haem requiring mutants in *A. nidulans* were not isolated by the enrichment method and the *A. nidulans hemA* gene was already cloned, a different strategy was designed to recover a *hemA*⁻ mutant strain of *A. nidulans*. The principle is to disrupt one of the two alleles in a diploid strain of *A. nidulans* which would enable the recovery of the *hemA* mutation in the heterozygous state. It is reasonable to assume that the non-mutant allele would be dominant and thus the heterozygous diploid strain could be recovered on normal (non-supplemented) growth medium. The design of a recovery medium to isolate the mutant strains could then be formulated by segregating the alleles by haploidisation on appropriately supplemented media. In the case of *hemA*⁻ mutants, it is very likely that medium supplemented with δ -aminolaevulinic acid (δ -ALA) would support the growth of the strains.

The strategy for the disruption of fungal genes by targeted integrative transformation has been shown to be an effective tool for analysing many aspects of gene function. The procedure involves the use of a uracil requiring homozygous diploid strain *pyrG/pyrG* of *A. nidulans* which is transformed with a construct in which the cloned *A. nidulans* gene of interest is interrupted by the *pyr-4* gene of *Neurospora crassa* for orotidine decarboxylase (Newbury *et al.*, 1986). When integrated into the genome of *A. nidulans*, the *pyr-4* gene complements the *pyrG* enzyme lesion thereby allowing uracil independent growth of the transformed *A. nidulans* strain.

6.3.1. Transformation in *Aspergillus nidulans*.

Transformation in *Aspergillus nidulans* begins with the preparation of protoplasts from young growing mycelia or germlings using lytic enzyme and an osmotic stabiliser. The lytic enzyme used is the commercial enzyme Novozyme 234, with the osmotic stability of the protoplast being maintained by the presence of sodium or potassium ions (Ballance *et al.*, 1983; Yelton *et al.*, 1984). The uptake of DNA by the protoplasts is promoted by the addition of calcium ions and polyethylene glycol (PEG) to the transformation reaction.

Variations on the general method have been employed in an attempt to optimise the transformation frequencies. Protoplasts can be prepared where they are either filtered or floated to remove them from the undigested cells and cell walls (Ballance *et al.*, 1983; Tilburn *et al.*, 1983; Yelton *et al.*, 1984). Removal of the protoplasts has been shown to reduce the frequency of transformation (May, 1992). Other factors are shown to affect the transformation efficiency. Similar to *N. crassa*, during the digestion of the *A. nidulans* cell wall with the lytic enzyme, the particular batch of Novozyme 234 can alter the transformation rate (Atkins and Lambowitz, 1985).

The *A. nidulans* diploid strain, D10 (Streatfield *et al.*, 1992) was used as the recipient strain for the disrupted *hemA* gene. The D10 strain, containing genetic markers on five of the eight linkage groups (Figure 6.4), has green conidia, requires uracil for growth and yields abundant recessive colour marker segregants upon growth in the presence of the mitotic spindle inhibitor benomyl, which induces mitotic haploidisation (Hastie, 1970).

Southern blot analysis of *A. nidulans* genomic DNA has indicated that the *hemA* gene is present at a single locus (Section 3.9). The strategy to replace one of the

resident wild type alleles is outlined in Figure 6.5 in which the *pyr-4* gene of *N. crassa* (Oakley *et al.*, 1987) is introduced into the *hemA* coding sequence and the construct transformed into *A. nidulans*. The *A. nidulans pyrG* gene shows little DNA homology with the *pyr-4* gene of *N. crassa* and as a result, is not targeted to the *A. nidulans pyrG* locus (Turner and Ballance 1985). Transformants containing the *pyr-4* gene can therefore be selected by uracil independence and screened to identify a diploid strain in which one of the resident *hemA* genes has been replaced by the interrupted sequence.



Figure 6.4. Genetic map of the diploid strain D10.

The diploid strain contains genetic markers on five of the eight linkage groups. Phenotypically, the strain has green conidia, requires uracil for growth and yields visible recessive conidial colour marker, *yA* (yellow) or *wA3* (white), when haploidized by growth in the presence of the mitotic spindle inhibitor benomyl.

The relative lengths of the chromosomes are deduced from the linkage data (Clutterbuck, 1982).

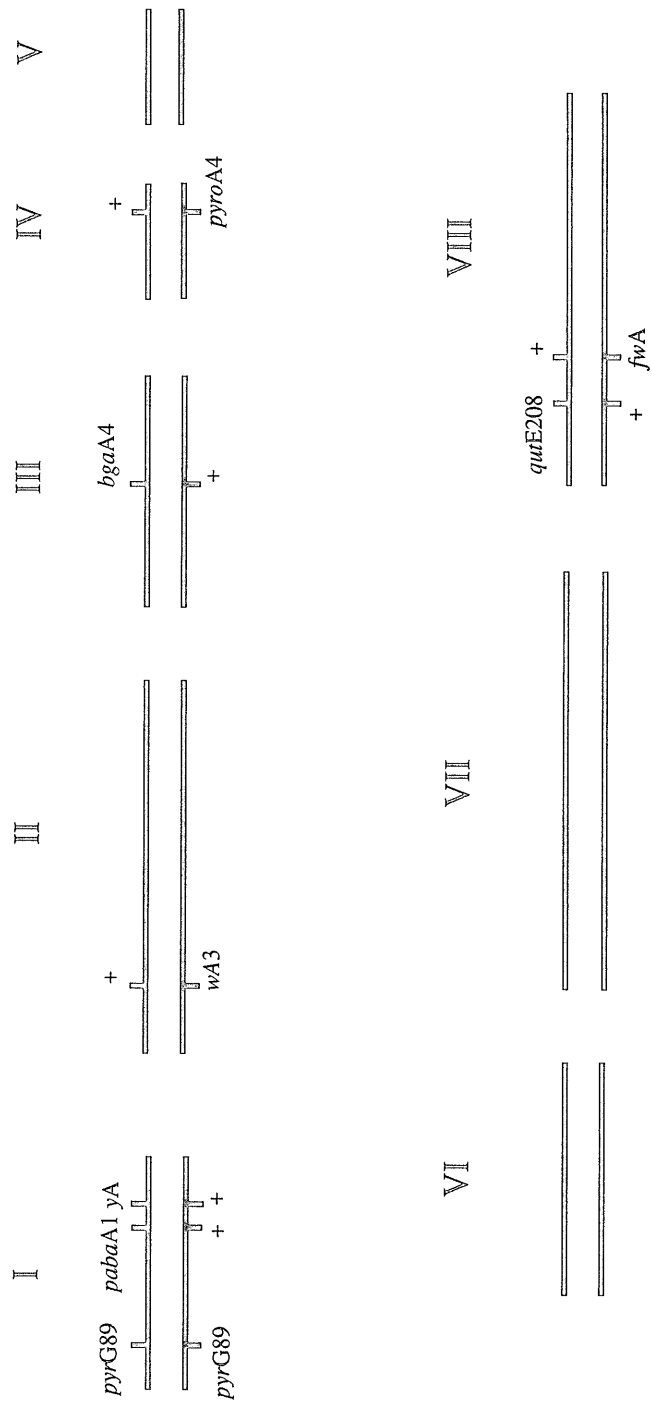
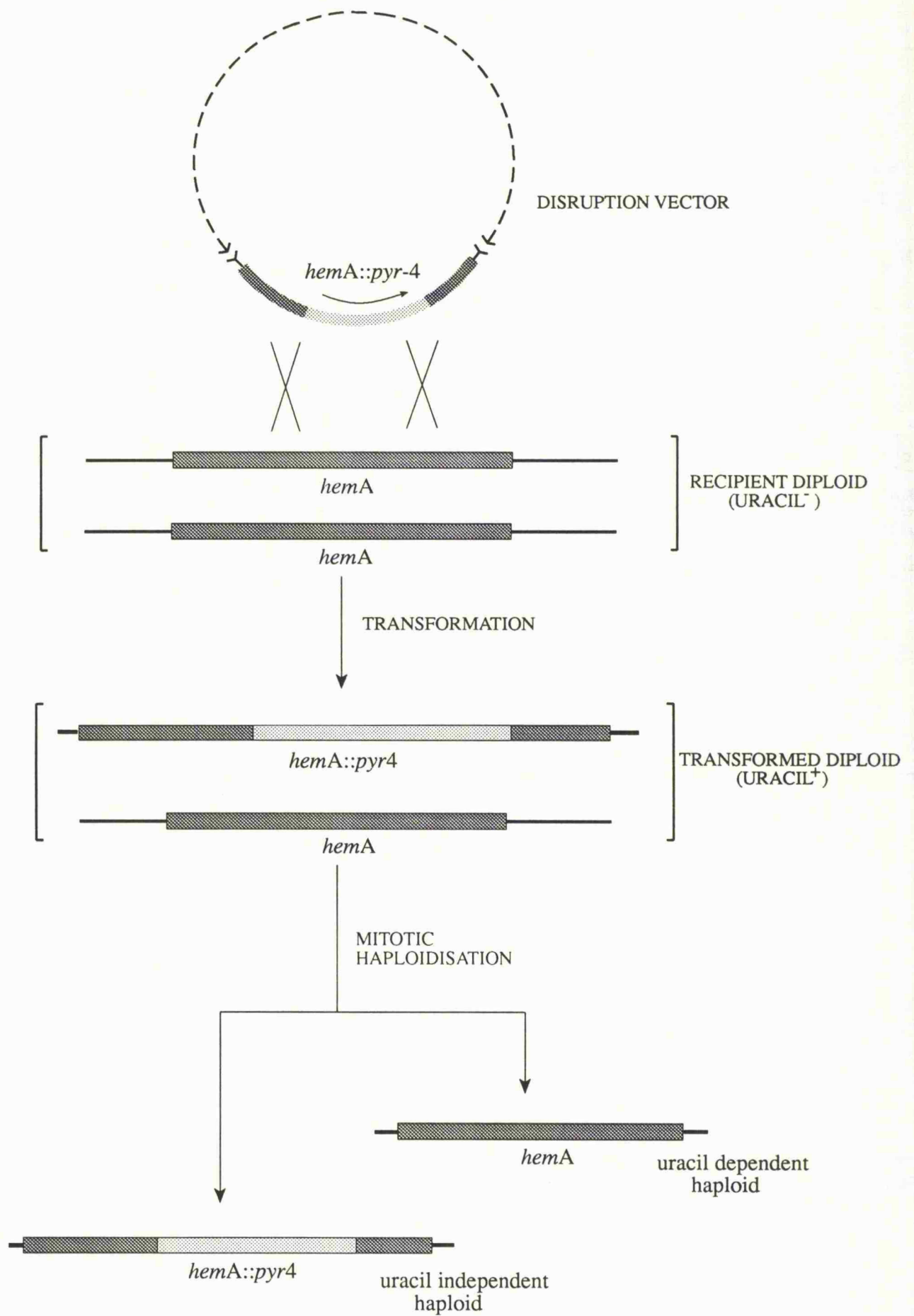


Figure 6.5. Strategy for the disruption of the *hemA* gene in a diploid strain of *A. nidulans*.

A disruption vector is constructed whereby the *A. nidulans hemA* gene is interrupted by the *pyr-4* gene of *N. crassa*. The disruption vector may be transformed into *A. nidulans* as the circular vector, the linear vector or as the released disruption fragment. When the vector is transformed into a homozygous *pyrG/pyrG* diploid strain a double homologous recombination event may occur between *hemA* sequences present on the vector and one of the native *hemA* loci. This would generate a uracil independent transformant with one native *hemA* locus and one disrupted *hemA* locus. Growing the strain in the presence of benomyl will induce haploidisation and segregation of the chromosome would yield both the uracil independent (*hemA::pyr-4*) and the uracil dependent (*hemA*⁺) haploid strains.

 *A. nidulans* sequences
 *N. crassa* sequences
 — — — — bacterial sequences



6.4. Disruption of the *Aspergillus nidulans* *hemA* gene .

Before embarking on the isolation of an *A. nidulans* *hemA* mutant by targeted gene disruption, a suitable vector must be designed and constructed prior to transformation into the diploid recipient strain D10 of *A. nidulans*. Furthermore, a strategy is required to enable the identification of those transformants in which one of the *hemA* alleles has been disrupted.

6.4.1. Design of the *hemA* disruption vector.

The design principles for a *hemA* disruption vector is to construct a plasmid containing the *A. nidulans* *hemA* gene in which a significant portion of the central coding region is replaced by the full coding sequence of the *pyr-4* gene of *N. crassa* for orotidine decarboxylase (Newbury *et al.*, 1986). The *hemA* gene is rendered inactive by the deletion of part of the coding sequences. To enhance the possibility of targeting the disruption vector to the *hemA* locus, the *pyr-4* gene, with its promoter sequences, must be flanked by *hemA* sequence. It is desirable to include extensive 5' and 3' *Aspergillus* flanking sequences thereby increasing the probability of integration by homologous recombination.

6.4.2. Construction of the *hemA* disruption vector.

In outline, the *hemA* disruption vector was constructed using the plasmid pALAS containing the *hemA* gene as the source of the *A. nidulans* sequence (Figure 3.6), with the *N. crassa* *pyr-4* gene originating from the plasmid pDJB1 (Ballance *et al.*, 1983). The disruption vector was prepared such that 700bp of the *hemA* coding sequence was removed by courtesy of the unique restriction enzyme sites, *Cla*I and *Sma*I present within the gene. The 700bp region was

replaced by a 3.5kb *PvuII*/*ClaI* fragment derived from pDJB1, containing the entire *pyr-4* coding sequence.

The plasmid vector pBS-M13(+) (Section 2.2) was used to deliver the construct since this allowed the introduction of unique flanking *XbaI* and *EcoRI* enzyme restriction sites, enabling linearisation of the vector or the release of the construct prior to transformation. The presence of three *BamHI* restriction sites, two flanking the construct and one within the *pyr-4* gene, will provide the necessary diagnostic restriction pattern when Southern blot analysis is carried out to identify a gene replacement at one of the two *hemA* loci.

In detail, the disruption vector was constructed by a series of three steps (Figures 6.6, 6.7, and 6.8). First, the plasmid pALAS (Figure 3.6) was digested with the restriction enzymes *BamHI* and *SmaI* to generate a 2.5kb δ -ALAS fragment containing the amino terminal codons of the *hemA* gene and 5' non-coding sequence. The nucleic acids were separated using 1.0% (w/v) agarose gel and the 2.5kb fragment recovered by the GeneClean method (Section 2.5.4.1). A separate digestion of the plasmid vector pBS-M13(+) was carried out using the above enzymes after which the proteins were removed by phenol extraction and the DNA precipitation with ethanol and sodium acetate (Section 2.5.2). Ligation reactions were set up containing the linear plasmid vector and the 2.5kb δ -ALAS fragment. The product of the ligation reaction was transformed into the *E. coli* strain NM522 (Section 2.3.2) and the plasmid recovered (Section 2.4) and depicted as pALAS-n (Figure 6.6). The plasmid was verified by restriction enzyme digestion and Southern blot analysis.

In the second step, plasmid pALAS-n (Figure 6.6) was digested with the restriction enzymes *EcoRI* and *SmaI* to linearise the 5.4kb plasmid. The nucleic acids were separated on 1.0% (w/v) agarose gel and the 5.4kb fragment recovered. A separate digestion of the plasmid pDJB1 was carried out using the enzymes *EcoRI* and *PvuII* to generate a 4.8kb fragment which contained the entire coding sequence of the *pyr-4* gene of *N. crassa*. The 4.8kb fragment was recovered and ligated with the linear 5.4kb plasmid, pALAS-n. Ligation between *SmaI* and *PvuII* digested fragments was successful since both enzymes generate blunt ended fragments with the subsequent loss of both restriction sites. The ligated fragments were transformed into the *E. coli* strain NM522 and the recovered plasmid, verified by restriction enzyme digestion and Southern blot analysis, depicted as pALAS-np (Figure 6.7).

The final step involved the digestion of plasmid pALAS-np (Figure 6.7) with the restriction enzymes *EcoRI* and *ClaI* to generate an 8.9kb fragment containing the non-coding 5' region of the *hemA* gene linked to the *pyr-4* gene as well as the plasmid vector. The nucleic acids were separated on a 1.0% (w/v) agarose gel and the 8.9kb fragment recovered. A separate digestion of the plasmid pALAS was carried out using the enzymes *EcoRI* and *ClaI* to generate a 1.4kb fragment which contained the carboxy terminal codons of the *hemA* gene. The 1.4kb fragment was recovered and ligated with the 8.9kb fragment. The plasmid was transformed into the *E. coli* strain NM522 and the recovered disruption plasmid, depicted as pALAS-pd (Figure 6.8).

To verify disruption vector restriction analysis by digesting the plasmid with the restriction enzymes *EcoRI* and *XbaI* was carried out. The nucleic acid was separated by electrophoresis on a 1.0% (w/v) agarose gel after which two bands were observed at 7.4, and 2.9kb. The 7.4kb band, this being the size of the

disruption fragment, was confirmed as such by Southern blot analysis using radioactively labelled *hemA* and *pyr-4* DNA sequences as probes. This construct lacks *hemA* codons including the essential amino acids from which the original DNA probe was based (Section 3.5) between the *Sma*I and *Cla*I sites in the *hemA* gene.

Figure 6.6. Construction of the vector pALAS-n.

The plasmid pALAS containing a 4.5kb DNA fragment encompassing the *hemA* gene was digested with the restriction enzyme *Sma*I and *Bam*HI and the 2.7kb fragment recovered. The plasmid vector pBS M13+ was digested with the same enzymes and ligated with the 2.7kb δ -ALAS fragment to produce the plasmid pALAS-n.

The relevant restriction sites are highlighted by bold typescript.

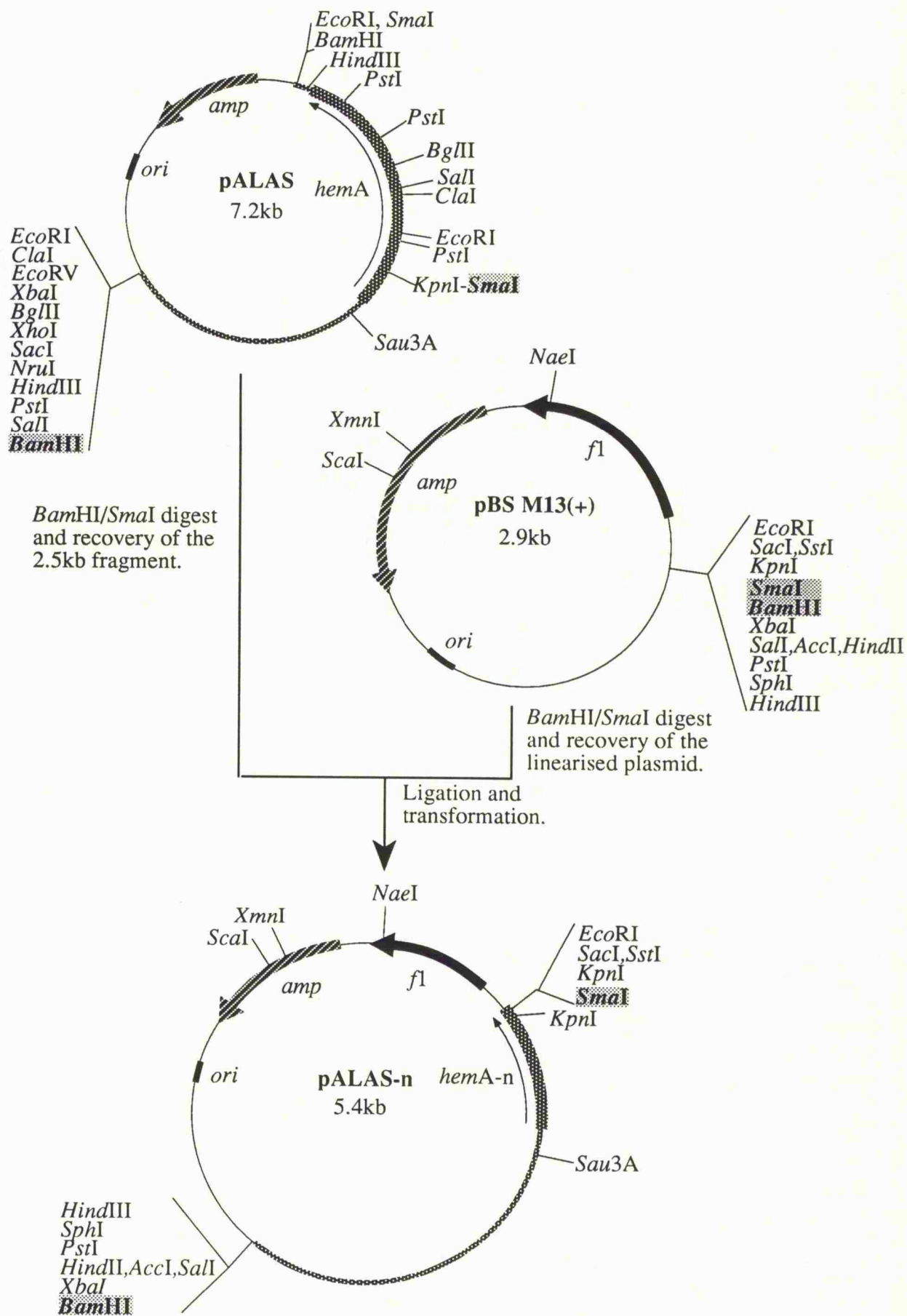


Figure 6.7. Construction of the vector pALAS-np.

The plasmid pALAS-n containing a 2.7kb DNA fragment with part of the *hemA* gene was digested with the restriction enzymes *EcoRI* and *SmaI* and the linearised plasmid purified. The plasmid vector pDJB1 was digested with the restriction enzymes *EcoRI* and *PvuII* and the 4.8kb fragment recovered and ligated with the linearised plasmid, pALAS-n, to produce the plasmid pALAS-np. Ligation between *SmaI* and *PvuII* digested fragments was based upon both enzymes generating blunt ended fragments with the subsequent loss of both restriction sites.

The relevant restriction sites are highlighted by bold typescript.

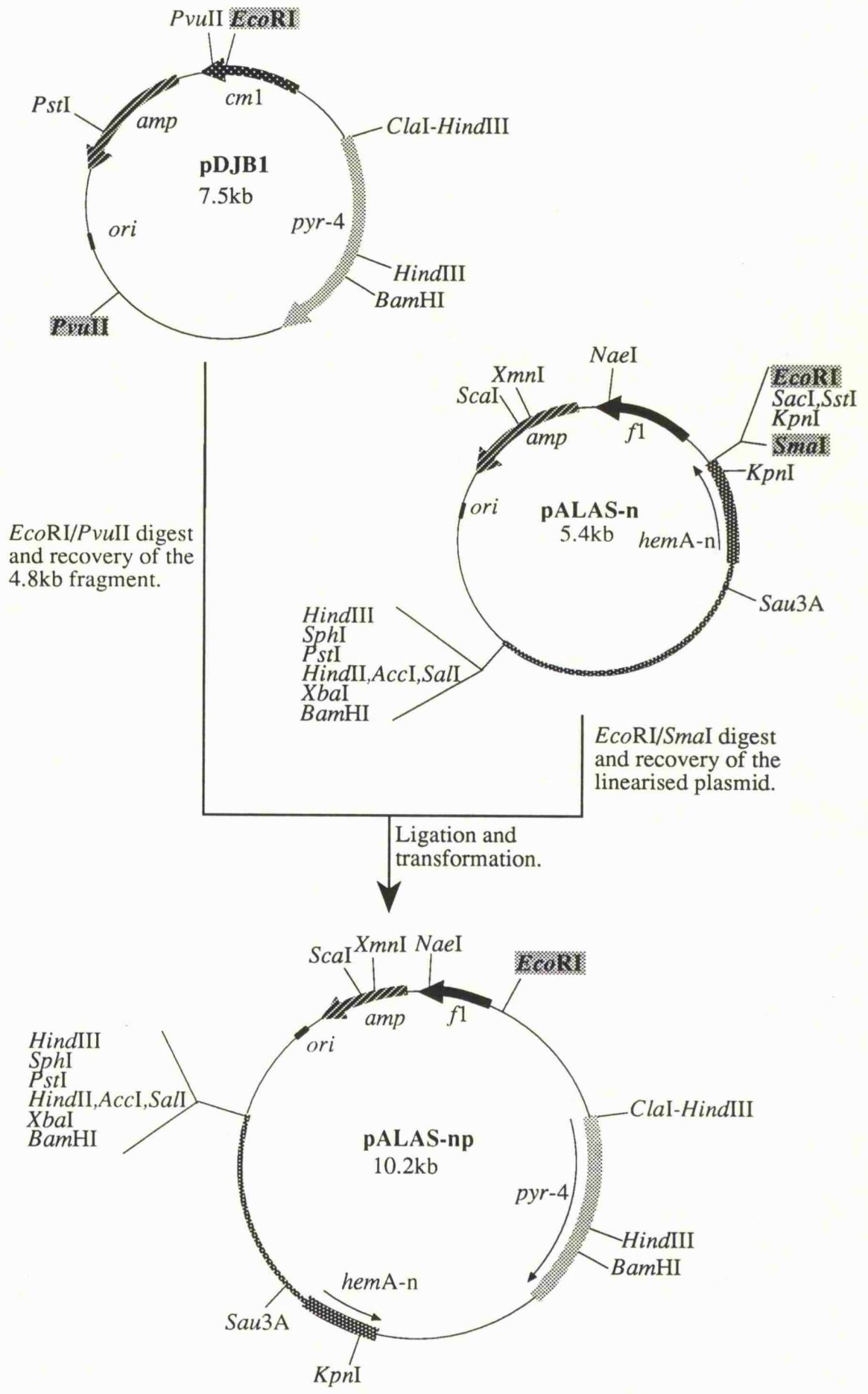
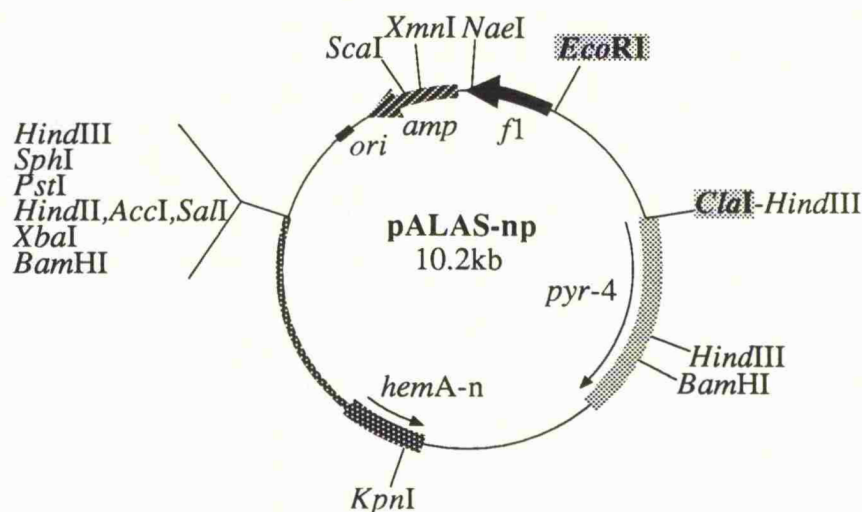


Figure 6.8. Construction of the disruption vector pALAS-pd.

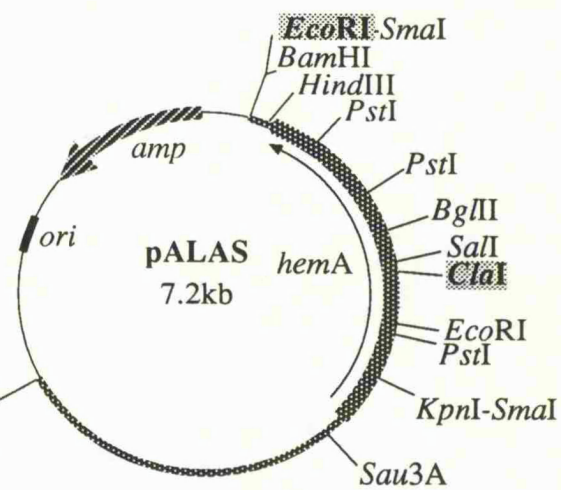
The plasmid pALAS-np was digested with the restriction enzymes *Eco*RI and *Cla*I and the 8.9kb fragment recovered. The plasmid vector pALAS was digested with the restriction enzymes *Eco*RI and *Cla*I and the 1.4kb fragment recovered and ligated with the 8.9kb fragment to produce the disruption plasmid pALAS-pd.

The relevant restriction sites are highlighted by bold typescript.



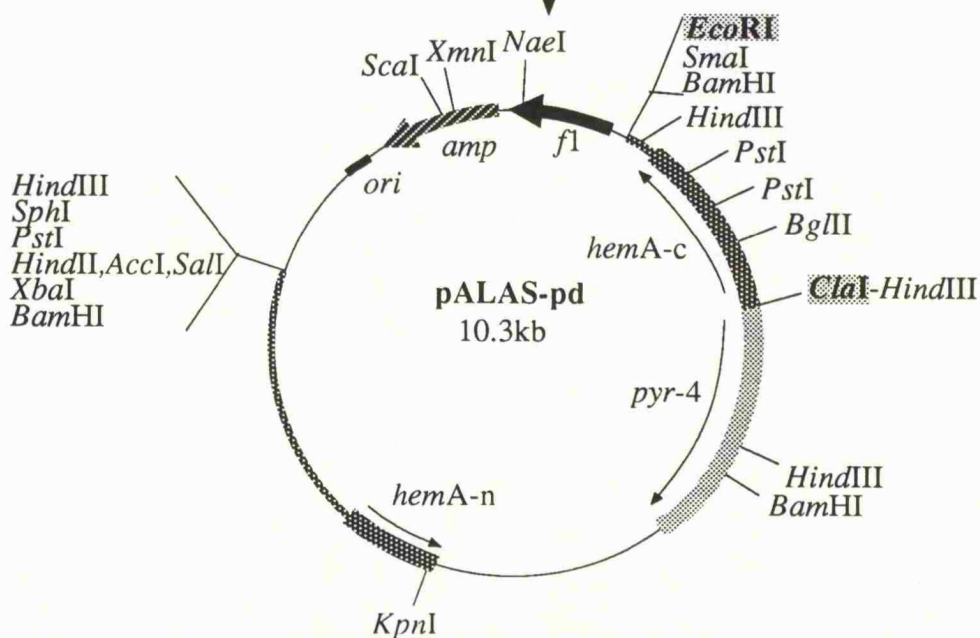
EcoRI/ClaI digest
and recovery of the
8.9kb fragment.

EcoRI
ClaI
EcoRV
XbaI
BglII
XhoI
SacI
NruI
HindIII
PstI
SalI
BamHI



EcoRI/ClaI digest
and recovery of the
1.4kb fragment.

Ligation and
transformation.



6.4.3 Identification of the targeted gene replacement.

Following transformation with the disruption vector, a suitable method of analysis is required to identify those transformants in which targeted gene replacement has occurred. A restriction map of the wild type *hemA* gene locus and a schematic representation of the expected restriction map following gene replacement is presented in Figure 6.9.

Southern blot analysis of genomic DNA from wild type *A. nidulans* (Section 3.7) confirms that upon digestion with the restriction enzyme *Bam*HI a single 4.5kb fragment is identified when hybridised with an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence. The same probe identifies two fragments of 4.4 and 3.5kb when the wild type genomic DNA is digested with *Sma*I (Figure 6.10).

Using identical conditions for Southern blot analysis, targeted gene replacement of one of the two *hemA* genes in the transformed diploid will generate a different series of hybridisation signals when the genomic DNA is digested with the restriction enzymes *Bam*HI and *Sma*I. Thus in addition to the 4.5kb *Bam*HI fragment, two further fragments of 4.7 and 2.7kb are predicted for the heterozygous diploid strain, and a 10.7kb *Sma*I fragment is predicted in addition to the 4.4 and 3.5kb fragments. Whilst the 4.7 and 4.5kb *Bam*HI fragments will not be readily separated by electrophoresis, each of the other predicted fragments should be clearly resolved in Southern blot analysis.

Figure 6.9. Schematic representation of the vector integration event.

A schematic representation of the molecular events during a classical targeted gene replacement. The table shows the molecular weights of the fragments predicted following Southern blot hybridisation using an [α - 32 P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment, consisting of the entire *hemA* sequence, with the *Bam*HI and the *Sma*I digests of the genomic DNA isolated from:- the recipient diploid (D10); the transformed heterozygous diploid (*hemA::pyr-4*); the two haploid segregants derived from the transformed diploid, the uracil dependent (*hemA*⁺) segregant and the uracil independent (*hemA::pyr-4*) segregant.

Strain	Predicted fragment sizes (kb)	
	<u><i>Bam</i>HI digest</u>	<u><i>Sma</i>I digest</u>
Recipient diploid (D10)	4.5	4.4, 3.5
Transformed heterozygous diploid (<i>hemA::pyr-4</i> ; <i>hemA</i> ⁺)	(4.7, 4.5) [§] , 2.7	10.7, 4.4, 3.5
Uracil dependent haploid segregant (<i>hemA</i> ⁺)	4.5	4.4, 3.5
Uracil independent haploid segregant (<i>hemA::pyr-4</i>)	4.7 2.7	10.7

[§] the 4.5kb and the 4.7kb bands are expected to be observed as a single hybridisation signal.

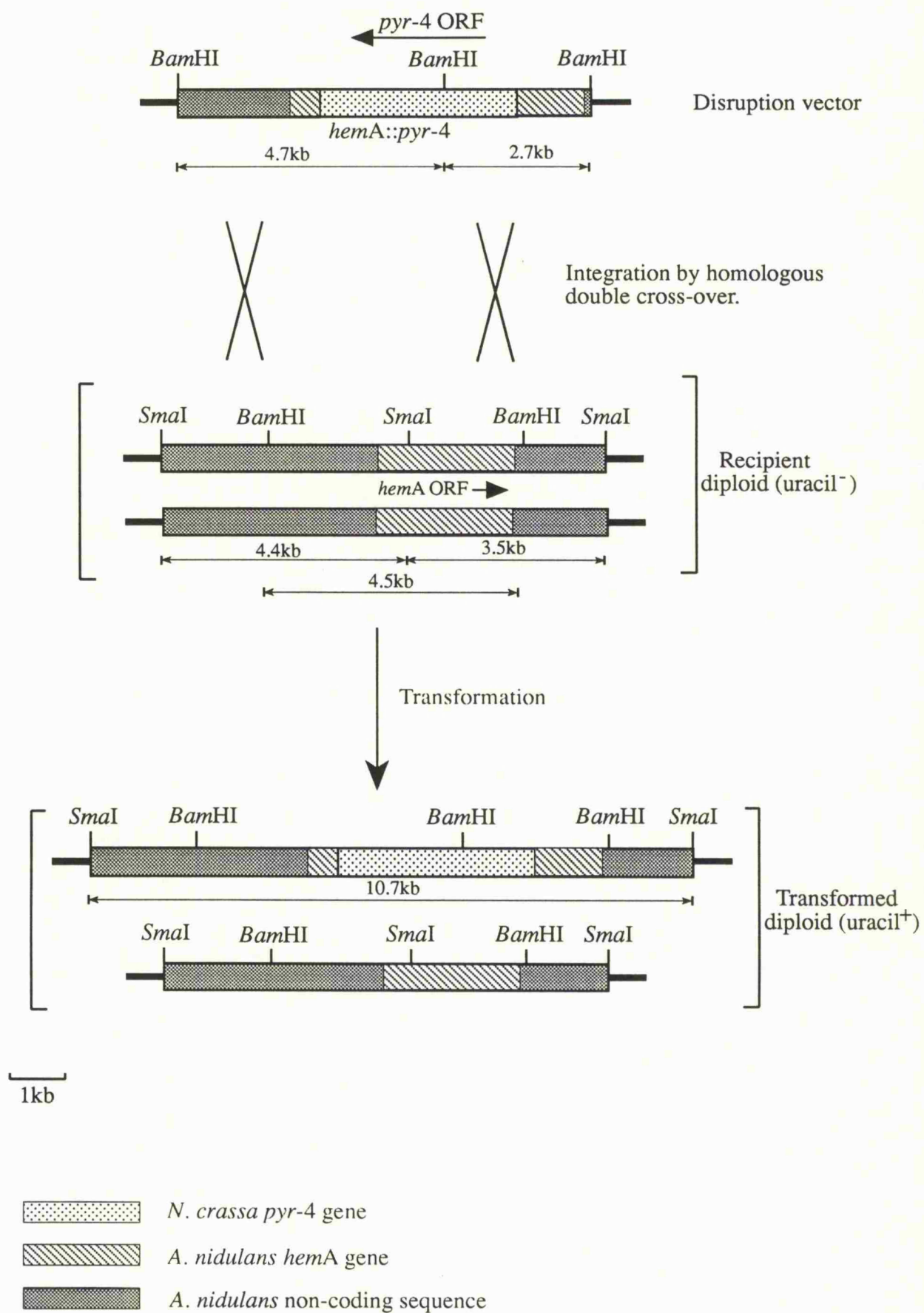
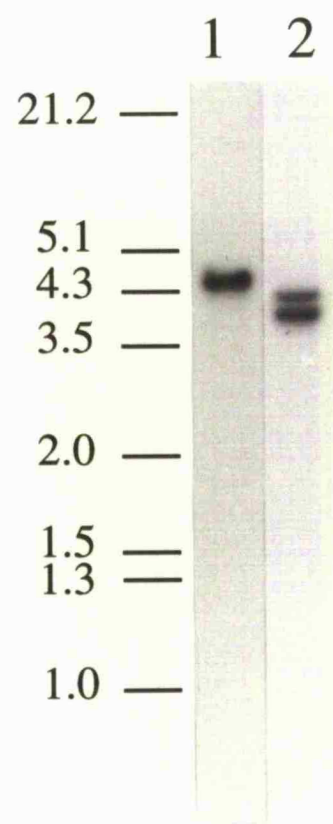


Figure 6.10. Southern blot hybridisation of the *A. nidulans* genomic DNA.

A. nidulans genomic DNA was digested with the restriction enzymes *Bam*HI and *Sma*I. The DNA was separated by gel electrophoresis and Southern blot hybridisation carried out using an [α - 32 P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* coding sequence. After washing the filter under high stringency conditions in 3x SSC, 0.2% (w/v) SDS solution, hybridisation signals were produced at 4.5kb with the *Bam*HI digested DNA (Lane 1) and at 4.4kb and 3.5kb with the *Sma*I digested DNA (Lane 2).

Phage lambda DNA digested with *Eco*RI and *Hind*III provided the molecular weight markers.

Lanes	Enzymes	Fragment sizes (kb)
1	<i>Bam</i> HI	4.5
2	<i>Sma</i> I	3.5, 4.4



6.5. Transformation into *Aspergillus nidulans* diploid strain D10.

The transformation procedure was carried out in accordance to the method described on Ballance *et al.*, 1983 (Section 2.3.2). Protoplasts were prepared by removing the mycelial cell wall with Novozym 234 and transformation of the protoplasts involved the use of polyethylene glycol and a heat shock. Previous studies have shown that transformation of *A. nidulans* with a linearised vector yields a higher frequency of transformants compared to that obtained with the circular vector (Streatfield *et al.*, 1992). In the context of this experiment protoplasts of the diploid strain were transformed in separate incubations with the disruption plasmid pALAS-pd either in its circular form; or the vector linearised by digestion at a single site; or the replacement construct released as a fragment by digestion at two flanking sites.

6.5.1. Preparation of the transforming plasmid.

The disruption plasmid pALAS-pd was recovered from a large scale caesium chloride preparation. The DNA solution was dialysed against TE buffer to remove all traces of salts which was found to inhibit transformation. The plasmid was linearised by digestion at the unique *EcoRI* restriction enzyme site. Release of the disruption fragment was obtained by digestion at the unique *EcoRI* and *XbaI* restriction enzyme sites. A total of 10µg of plasmid DNA was used for each of the enzymatic reactions and on completion the efficiency of the digestion was examined by analysis of 1µg of DNA by electrophoresis on a 1% (w/v) agarose gel. The preparations of digested DNA were used directly to transform the *A. nidulans* diploid strain D10 without further isolation of the DNA.

6.5.2. The efficiency of transformation.

Transformation reactions were carried out as described in Section 2.3.2. The protoplast preparations were adjusted to a concentration of 1×10^7 protoplasts per ml with sterile 0.6M KCl, 50mM CaCl₂ solution. Three aliquots each of 200µl of protoplast suspension were incubated separately with 20µl of vector preparations, containing 9µg DNA either in the form of circular molecule, linear molecule or released fragment (See legend Table 6.1). A fourth aliquot was incubated with 20µl of TE which was subsequently used as a negative control.

Protoplasts were mixed with molten minimal medium containing 0.6M KCl pre-cooled to 48°C, plated and incubated at 37°C for four days. Further samples of the protoplast suspension were plated at appropriate dilution as above in minimal medium containing uracil to evaluate the efficiency of regeneration to mycelium.

The efficiency at which protoplast regenerated to yield colonies was 27.5%. No colonies were observed on plates from the negative control lacking DNA, thus confirming the stability of the recipient diploid strain. A total of 38 transformed strains were recovered following incubation with the circular vector, 10 strains were recovered with the linear plasmid and 10 with the released fragment. These correspond to rates of transformation of 4.2, 1.1 and 1.5 transformants/µgDNA respectively (Table 6.1).

The transformed strains were replated for single colony isolation on selective, unsupplemented minimal medium and this was repeated until they appeared as uniform green colonies. In each case about one half of the original transformed strains yielded uniform colonies taken to represent stable strains

(Table 6.1). The remaining strains yielded colonies exhibiting morphological variation taken to represent unstable transformation events. The stable transformants were depicted as C1-19 for the circular vector, L1-5 and R1-4 for the linear vector and released fragment respectively.

Table 6.1. Transformation of the *A. nidulans* diploid strain D10.

Form of vector	Number of Transformants	Transformation Rate (transformants/ μ gDNA)
Circular	38 (19)	4.2 (2.10)
Linearised (<i>Eco</i> RI)	10 (5)	1.1 (0.56)
Released fragment (<i>Eco</i> RI/ <i>Xba</i> I)	10 (4)	1.5 (0.61) [§]

The gene disruption plasmid, pALAS-pd, was used to transform the protoplasts of the *A. nidulans* diploid strain D10 as the circular plasmid, the linearised plasmid (digested with *Eco*RI) and the released fragment (digested with *Eco*RI and *Xba*I). Transformed strains in which the *N. crassa pyr-4* gene has integrated into the genome were selected by uracil independent growth.

The numbers of stable transformed strains are indicated in parenthesis.

[§] Transformation rate is adjusted for the amount of the released fragment:-
Transformation rate x (mw of whole fragment/mw of fragment)

6.6. Southern blot analysis of transformed diploid strains.

In order to identify those transformants in which one of the *hemA* alleles has been disrupted Southern blot analysis was carried out using the genomic DNA extracted from each of the transformed strains. Transformants in which one of the *hemA* alleles has been disrupted are expected to yield the hybridisation signals predicted above (Section 6.4.3). A preliminary screen was carried out whereby genomic DNA prepared from all the stable transformed strains (Section 2.4.3) was digested with the restriction enzyme *Bam*HI. The nucleic acid was separated by electrophoresis in a 1.0% (w/v) agarose gel and transferred to Hybond-N filter prior to hybridisation with an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment, consisting of the entire *hemA* sequence, at 65°C for 18 hours. The filters were washed under high stringency conditions in 3x SSC, 0.2% (w/v) SDS for three hours (Section 2.6.1.4).

In no instances did any of the strains C1-19, transformed with the circular disruption vector, produce the predicted hybridisation signals at approximately 4.5kb (*hemA*⁺), together with a new 2.7kb band (*hemA::pyr-4*) (Figure 6.11). All the transformants contained the original 4.5kb fragment and new bands of 3.5 and 2.7kb. Whilst the 2.7kb band is expected and the predicted 4.7kb band may not have been resolved from the original 4.5kb band, it is not easy to account for the 3.5kb band. Thus although a degree of targeting by the disruption sequence is apparent, the integration event appears to be complex. It is concluded therefore that none of these strains represent a targeted gene replacement. With strains C3, C9 and C14, the intensity of the hybridisation signals and presence of further bands indicate possible multiple events during integration.

Strains L1-5, transformed with the linear disruption vector, produced hybridisation patterns which were similar to those observed with the strains C1-19 (Figure 6.12, Lanes L1-5), and can therefore be interpreted in the same manner. Strain L5 (Figure 6.12, Lane L5) displayed a hybridisation pattern similar to strains C3, C9 and C14, which again indicate possible multiple events during integration. Southern blot analysis has shown that strains transformed with the disruption vector in the circular or linear form all resulted from complex integration events and therefore no further analysis was done with these strains.

Four stable strains, R1-4 (Figure 6.12, Lanes R1-4), were recovered by transformation with the released disruption fragment and two of these, R2 and R4, produced bands identical to those predicted for targeted gene replacement (Figure 6.12, Lanes R2 and R4). Strains R2 and R4 produce the expected hybridisation signal at 4.5kb (*hemA*⁺) together with the new 2.7kb band. The predicted 4.7kb band has been resolved and it is noticeable that the 4.5 (+4.7) kb bands are markedly more intense than the 2.7kb bands. In the case of strains R1 and R3 a new 2.7kb band was also found but in strain R1 the 4.5kb band is of similar intensity and may not be a doublet. The presence of a 5.1kb band in strain R3 is not easily accountable.

The preliminary screen has thus indicated that strains R2 and R4 appear to have been derived as a result of a targeted gene replacement. It was decided to subject these to strains to genetic analysis to test for the isolation of a *hemA*⁻ (*hemA::pyr-4*) segregant before further Southern blot analysis by digestion of the genomic DNA with *Sma*I was carried out.

Figure 6.11. Southern blot analysis of the transformed strains C1-19.

Genomic DNA was isolated from strains C1-19 transformed with the circularised disruption vector and digested with the restriction enzyme *Bam*HI. The nucleic acids were separated by electrophoresis on a 1.0% (w/v) agarose gel, and transferred to Hybond-N filter. Southern blot hybridisation was carried out using an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence.

Phage lambda DNA digested with *Eco*RI and *Hind*III provided the molecular weight markers.

Lanes

D10	Recipient diploid D10 DNA digested with <i>Bam</i> HI,
1-19	Transformed strains C1-19 DNA digested with <i>Bam</i> HI.

Transformants obtained with circularised vector



Figure 6.12. Southern blot analysis of the transformed strains L1-5 and R1-4.

Genomic DNA, isolated from strains transformed with the linearised disruption vector or the released, L1-5 or R1-4 respectively, was digested with the restriction enzyme *Bam*HI and the nucleic acid separated by electrophoresis on a 1.0% (w/v) agarose gel. The nucleic acid was transferred to Hybond-N filter and Southern blot hybridisation carried out using an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence.

Phage lambda DNA digested with *Eco*RI and *Hind*III provided the molecular weight markers.

Lanes	
D10	Recipient diploid D10 DNA digested with <i>Bam</i> HI,
L1-5	Transformed strains L1-5 DNA digested with <i>Bam</i> HI,
R1-4	Transformed strains R1-4 DNA digested with <i>Bam</i> HI.

D10 L1 L2 L3 L4 L5 R1 R2 R3 R4

21.2 —

5.1 —

4.3 —

3.5 —

2.0 —

1.5 —

1.3 —

1.0 —



6.7. Summary.

A strategy for the disruption of the *hemA* gene was proposed using a gene replacement method. An appropriate disruption vector was designed, constructed and used to transform a diploid strain of *A. nidulans*. Transformation with the circular plasmid produced 19 stable transformants (2.1 transformants/ μ gDNA), and only 5 stable transformants were obtained as a result of transformation with the linear plasmid (0.56 transformants/ μ gDNA). This is contrary to previously observations where it has been shown that plasmid linearisation results in a 50% increase in the rate of transformation (Streatfield *et al.*, 1992). In no case did Southern blot analysis show a pattern of bands consistent with replacement of one of the *hemA* allele, and the strains appear to have been derived by complex integration events.

Transformation with the released fragment produced 4 stable transformants (0.61 transformants/ μ gDNA) and two of these transformants, R2 and R4, produced signals consistent with targeted gene replacement upon Southern blot analysis.

CHAPTER 7.

RECOVERY OF THE *hemA*⁻(*hemA::pyr-4*) MUTANT STRAIN OF *ASPERGILLUS NIDULANS*.

7.1. Introduction.

The *Aspergillus nidulans* diploid strain D10 has been transformed with the *hemA* disruption vector, pALAS-pd. Preliminary Southern blot analysis indicated that two of the transformed strains, R2 and R4, are potential heterozygotes for a *hemA* mutation *hemA::pyr-4*, that is, the vector has disrupted one of the resident *hemA* genes.

This chapter describes the isolation of the *hemA* mutant strain (*hemA::pyr-4*) from the transformed diploid strains, R2 and R4 by mitotic haploidisation. Furthermore, genetic and physical analysis will be used to provide evidence that gene disruption has occurred by targeted integration.

7.2. Isolation of the segregants.

The diploid transformed strains, R2 and R4, were inoculated onto glucose malt extract medium containing the mitotic spindle inhibitor, benomyl ($0.7\mu\text{g.ml}^{-1}$) to induce mitotic haploidisation. Both strains produced vigorously growing yellow or white spored sectors when the medium is supplemented either δ -aminolaevulinic acid (δ -ALA) or uracil depending on the class of segregant to be isolated. Small samples of conidiospores were taken from the white or yellow sectors and replated onto glucose minimal medium supplemented either δ -ALA or uracil to obtain pure strains. All media were supplemented with para-aminobenzoic acid (paba), pyrodoxine (pyro).

7.2.1. Isolation of the *hemA*⁻ (*hemA::pyr-4*) segregants.

The original *A. nidulans* diploid recipient strain D10 was homozygous for *pyrG* (Figure 6.2) and therefore has a requirement for uracil. Following transformation with the disruption vector, diploids in which a single homologous recombinant event has occurred will become uracil independent due to the presence of the *pyr-4* gene (*hemA::pyr-4*), and since the *pyr-4* complements the *pyrG* mutation in *A. nidulans*. Upon haploidisation only the haploid strains carrying the *pyr-4*⁺ gene are expected to grow on medium deficient in uracil. If the disrupted *hemA* sequence from the vector has replaced one of the resident *hemA* genes in the diploid, then haploid segregants carrying the *pyr-4*⁺ gene would possess the disrupted *hemA* gene, and therefore have a requirement for δ -ALA.

Isolation of *hemA*⁻ segregants was carried out by inoculating the diploid transformants, R2 and R4, onto the haploidising medium, composed of glucose malt extract medium with benomyl ($0.7\mu\text{g.ml}^{-1}$) and supplemented with

δ -ALA. Abundant yellow or white spored sectors were obtained and spores retrieved from these sectors replated to obtain pure strains (Table 7.1). A total of 39 segregants were recovered from the transformed diploid strain R2 and these were designated R2h-1 to 39, similarly 34 segregants recovered from R4 were designated R4h-1 to 34.

7.2.2. Isolation of the *hemA*⁺ segregants.

Isolation of *hemA*⁺ segregants was carried out by inoculating the diploid transformants, R2 and R4, onto the haploidizing medium, composed of glucose malt extract medium with benomyl (0.7 μ g.ml⁻¹) and supplemented with uracil. Haploidisation on this media will allow the recovery of segregants which are uracil requiring (*pyr-4*⁻), and since δ -ALA was not required for growth they are presumed to be *hemA*⁺. Abundant white or yellow spored sectors were formed, and conidiospores from these sectors were purified by replating (Table 7.1). A total of 26 segregants recovered from the transformed diploid strain R2 and designated R2u-1 to 26, and similarly 22 segregants recovered R4 were designated R4u-1 to 22.

Table 7.1. Isolation of the *hemA*⁺ and *hemA*⁻ (*hemA::pyr-4*) segregants.

Transformed diploid strains	Number of segregants isolated.					
	<i>hemA</i> ⁺			<i>hemA</i> ⁻ (<i>hemA::pyr-4</i>)		
	white	yellow	Total	white	yellow	Total
R2	14	12	26	25	14	39
R4	18	4	22	19	15	34

The transformed diploid strains, R2 and R4, were inoculated onto glucose malt extract medium containing the mitotic spindle inhibitor, benomyl (0.7µg.ml⁻¹) to induce mitotic haploidisation and supplemented with para-aminobenzoic acid (paba), pyrodoxine (pyro) and ALA or uracil. Vigorously growing yellow and white spored sectors were produced. The conidiospores taken from these sectors were replated onto glucose minimal medium supplemented with para-aminobenzoic acid and pyrodoxine and ALA or uracil to isolate the pure strains.

7.3. Analysis of the haploid segregants.

Both classes of segregants, *hemA*⁻ (*hemA::pyr-4*) and *hemA*⁺, were tested to establish their phenotypes with respect to δ -ALA or uracil. Segregant strains exhibiting at least one nutritional requirement in addition to a recessive colour marker were taken as being haploid. The test media, glucose minimal medium, was supplemented with para-aminobenzoic acid and pyrodoxine; and the characteristics of strains for these requirements was established at a later stage (Chapter 8).

7.3.1. Analysis of the *hemA*⁻ (*hemA::pyr-4*) haploid segregants.

Spores recovered from the haploid strains R2h-1 to 39 and R4h-1 to 34 were inoculated onto test media and incubated at 37°C for three days. All grew on medium supplemented with δ -ALA but failed to grow on the unsupplemented medium or on medium with uracil. The results indicate that the strains R2h-1 to 39 and R4h-1 to 34 are all haploid, requiring δ -ALA but are uracil independent.

Two white, R2h-24 and R4h-14, and two yellow strains, R2h-35 and R4h-1, all of which were also shown to require both para-aminobenzoic acid and pyrodoxine (Chapter 8), were used for subsequent molecular analysis.

7.3.2. Analysis of the *hemA*⁺ haploid segregants.

Spores recovered from the haploid strains R2u-1 to 26 and R4u-1 to 22 were inoculated onto test media and incubated at 37°C for three days. All grew on medium supplemented with uracil but failed to grow on the unsupplemented medium or on medium with only δ -ALA. The results indicate that the strains

R2u-1 to 26 and R4u-1 to 22 are all haploid, uracil dependent and δ -ALA independent. They are the reciprocal class with respect to the *hemA*⁻ (*hemA::pyr-4*) haploids, derived from the diploid strains, R2 and R4.

Two white, R2u-14 and R4u-17, and two yellow strains, R2u-3 and R4u-6 all of which were shown to require both para-aminobenzoic acid and pyrodoxine (Chapter 8), were used for subsequent molecular analysis.

7.4. Molecular analysis of the transformed diploid strains and the haploid segregants.

Characterisation of the nature of the disruption event at the molecular level was carried out by Southern blot analysis using genomic DNA isolated from the transformed strains and their derived haploid segregants. Genomic DNA was isolated from the transformed strain, R2, the derived haploid segregants, R2u-3 and R2u-14 both of which were uracil dependent (*hemA*⁺), and the δ -ALA dependent haploid segregants, R2h-24 and R2h-35 *hemA*⁻ (*hemA::pyr-4*). Similarly, genomic DNA was isolated from the transformed strain, R4, the derived haploid segregants, R4u-6 and R4u-17 both of which were uracil dependent (*hemA*⁺), and the δ -ALA dependent haploid segregants, R4h-1 and R4h-14 *hemA*⁻ (*hemA::pyr-4*). Genomic DNA was also isolated from the recipient diploid strain, D10, and used as a control.

The DNA prepared from each strain was digested with the restriction enzymes *Bam*HI and *Sma*I in separate reactions. The nucleic acids were separated by gel electrophoresis in a 1.0% (w/v) agarose gel, transferred onto Hybond-N filter and hybridised using an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment, consisting of the entire *hemA* sequence, at 65°C for 18 hours. The filters were washed under high stringency conditions in 3x SSC, 0.2% (w/v) SDS for three hours.

7.4.1. Southern blot analysis of the transformed diploid, R2, and the derived haploid segregants.

The Southern blot hybridisation data for the transformed diploid strain, R2, and the derived haploid segregants are illustrated in Figure 7.1. The hybridisation pattern of the recipient diploid strain, D10, and the *hemA*⁺

(*pyr-4*⁻) haploids, R2u-3 and R2u-14 showed that the *hemA* DNA probe only hybridises to fragments derived from the native locus as predicted (Section 6.4.3), when the genomic DNA was digested with the restriction enzymes *Bam*HI and *Sma*I. In all cases, the strains produced the predicted signals at 4.5kb, when the DNA was digested with restriction enzyme *Bam*HI, and at 3.5 and 4.4kb when the DNA was digested with restriction enzyme *Sma*I (Figure 7.1 Lanes 1, 5, 6, 7, 8, 12 and 13). This indicates that the *hemA* DNA probe identifies the presence of a native *hemA* locus in these strains.

The hybridisation pattern of the genomic DNA isolated from the transformed diploid strain, R2, following digestion with the restriction enzyme *Bam*HI, indicate the presence of the original 4.5kb fragment and new bands at 2.7 and 6.0kb (Figure 7.1 Lane 2). Whilst the 2.7kb band is expected and the predicted 4.7kb band may not have been resolved from the 4.5kb fragment, it is not easy to account for the 6.0kb band. The hybridisation pattern of the *Bam*HI digested genomic DNA isolated from the *hemA*⁻ (*hemA::pyr-4*) haploid segregants, R2h-24 and R2h-35, indicate the presence of the 2.7, 4.7 and the 6.0kb fragments but not the original 4.5kb band (Figure 7.1 Lanes 3 and 4). The 2.7 and 4.7kb bands are expected, but the 6.0kb band is again not easily accountable.

When the genomic DNA isolated from the transformed diploid strain, R2, was digested with the restriction enzyme *Sma*I, the hybridisation pattern indicate the presence of the original 3.5 and 4.4kb bands as well as new bands at 10.7, 18.0 and 21.0kb (Figure 7.1 Lane 9). Whilst the 10.7kb band is predicted, the 18.0 and 21.0kb bands are not easily accountable. The hybridisation pattern of the *Sma*I digested genomic DNA isolated from the *hemA*⁻ (*hemA::pyr-4*) haploid segregants, R2h-24 and R2h-35, shows the presence of the 10.7, 18.0 and 21.0kb band but not the original 3.5 and 4.4kb fragments (Figure 7.1 Lanes 10 and 11).

Again, whilst the 10.7kb band is expected, the 18.0 and 21.0kb bands are not easily accountable.

The hybridisation pattern exhibited in the Southern blot data indicate that the disruption vector has integrated onto one of the *hemA* alleles within the diploid. This results in the *hemA*⁺ haploid segregant producing a hybridisation pattern identical to the recipient strain. The *hemA*⁻ (*hemA::pyr-4*) haploid segregants produced a hybridisation pattern which, although indicative of a complex integration event, confirms that the disruption vector has interrupted the native *hemA* gene.

7.4.2. Southern blot analysis of the transformed diploid, R4, and the derived haploid segregants.

The Southern blot hybridisation data for the transformed diploid strain, R4, and the derived haploid segregants are illustrated in Figure 7.2. The hybridisation pattern of the recipient diploid strain, D10, and the *hemA*⁺ (*pyr-4*⁻) haploids, R4u-6 and R4u-17 showed that the *hemA* DNA probe only hybridises to fragments derived from the native locus as predicted (Section 6.2.3), when the genomic DNA was digested with the restriction enzymes *Bam*HI and *Sma*I. In all cases, the strains produced the predicted signals at 4.5kb, when the DNA was digested with restriction enzyme *Bam*HI, and at 3.5 and 4.4kb when the DNA was digested with restriction enzyme *Sma*I (Figure 7.2 Lanes 1, 5, 6, 7, 11 and 12). This indicates that the *hemA* DNA probe identifies the presence of a native *hemA* locus within these strains.

The hybridisation pattern of the genomic DNA isolated from the transformed diploid strain, R2, following digestion with the restriction enzyme *Bam*HI, indicate the presence of the original 4.5kb fragment and a new band at 2.7kb

(Figure 7.2 Lane 2). Whilst the 2.7kb band is expected, the predicted 4.7kb band may not have been resolved from the 4.5kb band. The hybridisation pattern of the *Bam*HI digested genomic DNA isolated from the *hemA*⁻ (*hemA::pyr-4*) haploid segregants, R4h-1 and R4h-14, indicate the presence of the 2.7, 4.7kb bands, but not the original 4.5kb fragment (Figure 7.1 Lanes 3 and 4). The 2.7 and 4.7kb band are expected, however the difference in intensities between bands is not easily accountable and may represent multiple integrated copies of the vector.

When the genomic DNA isolated from the transformed diploid strain, R2, was digested with the restriction enzyme *Sma*I, the hybridisation pattern indicate the presence of the original 3.5 and 4.4kb bands as well as new bands at 4.7, 6.0 and 10.7kb (Figure 7.1 Lanes 8). Whilst the 10.7kb band is predicted, the 4.7 and 6.0kb bands are not easily accountable. The hybridisation pattern of the *Sma*I digested genomic DNA isolated from the *hemA*⁻ (*hemA::pyr-4*) haploid segregants, R4h-1 and R4h-14, shows the presence of the 4.7, 6.0 and 10.7kb bands but not the original 3.5 and 4.4kb fragments (Figure 7.1 Lanes 9 and 10). Again, whilst the 10.7kb band is expected, the 4.7 and 6.0kb bands are not easily accountable.

The hybridisation pattern exhibited in the Southern blot data indicate that the disruption vector has integrated onto one of the *hemA* alleles within the diploid. This results in the *hemA*⁺ haploid segregant producing a hybridisation pattern identical to the recipient strain. The *hemA*⁻ (*hemA::pyr-4*) haploid segregants produced a hybridisation pattern which, although indicative of a complex integration event, confirms that the disruption vector has interrupted the native *hemA* gene.

Taken together, these data demonstrate that the disruption vector has targeted to one of the two *hemA* alleles in the transformed diploid strains R2 and R4. Haploidisation of the transformed strains resulted in the isolation of the *hemA*⁻ (*hemA::pyr-4*) and the *hemA*⁺ (*pyr-4*⁻) haploid segregants. Southern blot analysis of the *hemA*⁺ haploid segregant produced a hybridisation pattern identical to the recipient strain. Southern blot analysis of the *hemA*⁻ (*hemA::pyr-4*) haploid segregants indicate that although disruption vector had targeted *hemA* locus within the genome, the *hemA* gene has been disrupted by complex integration events rather than by direct gene replacement.

Figure 7.1. Southern blot analysis of the transformed strain R2 and the derived haploid segregants.

Genomic DNA was isolated from the recipient strain D10, the transformed diploid R2, the uracil independent (*hemA::pyr-4*) and the uracil dependent (*hemA*⁺) segregants. The DNA were digested with the restriction enzyme *Bam*HI and *Sma*I and the nucleic acid separated by electrophoresis on a 1.0% (w/v) agarose gel. The nucleic acid was transferred to Hybond-N filter and Southern blot hybridisation carried out using an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence.

Phage lambda DNA digested with *Eco*RI and *Hind*III provided the molecular weight markers.

Lanes	Ploidy/Strain	Phenotype [§]	Fragment sizes (kb)	
			<u><i>Bam</i>HI digest</u>	<u><i>Sma</i>I digest</u>
1	2n D10	uracil	4.5	
2	2n R2		6.0, (4.7, 4.5), 2.7	
3	n R2h-24	ALA	6.0, 4.7, 2.7	
4	n R2h-35	ALA	6.0, 4.7, 2.7	
5	n R2u-3	uracil	4.5	
6	n R2u-14	uracil	4.5	
7	2n D10	uracil	4.5	
8	2n D10	uracil		4.4, 3.5
9	2n R2			21.0, 18.0, 10.7, 4.4, 3.5
10	n R2h-24	ALA		21.0, 18.0, 10.7
11	n R2h-35	ALA		21.0, 18.0, 10.7
12	n R2u-3	uracil		4.4, 3.5
13	n R2u-14	uracil		4.4, 3.5

[§] the phenotype denotes the nutritional requirement of each strain

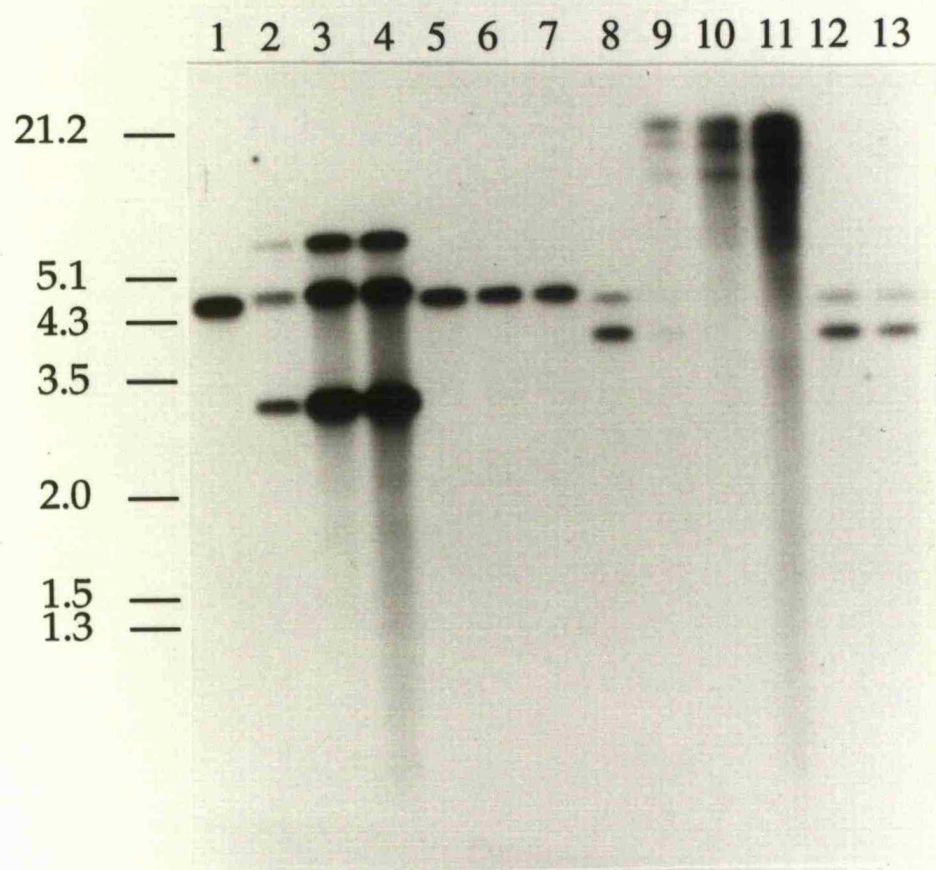


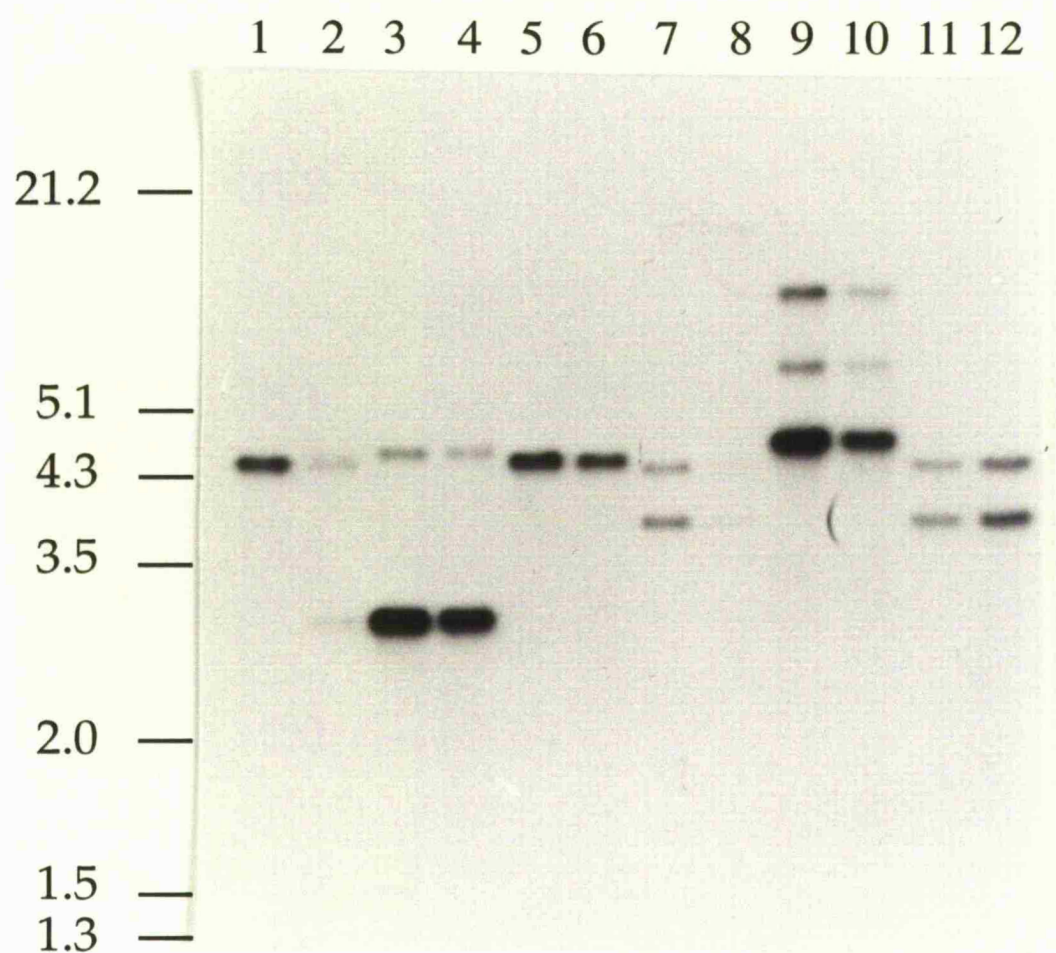
Figure 7.2. Southern blot analysis of the transformed strain R4 and the derived haploid segregants.

Genomic DNA was isolated from the recipient strain D10, the transformed diploid R4, the uracil independent (*hemA::pyr-4*) and the uracil dependent (*hemA*⁺) segregants. The DNA were digested with the restriction enzyme *Bam*HI and *Sma*I and the nucleic acid separated by electrophoresis on a 1.0% (w/v) agarose gel. The nucleic acid was transferred to Hybond-N filter and Southern blot hybridisation carried out using an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence.

Phage lambda DNA digested with *Eco*RI and *Hind*III provided the molecular weight markers.

Lanes	Ploidy/Strain Phenotype [§]		Fragment sizes (kb)	
			<u><i>Bam</i>HI digest</u>	<u><i>Sma</i>I digest</u>
1	2n	D10 uracil	4.5	
2	2n	R4	(4.7, 4.5), 2.7	
3	n	R4h-14 ALA	4.7, 2.7	
4	n	R4h-1 ALA	4.7, 2.7	
5	n	R4u-6 uracil	4.5	
6	n	R2u-17 uracil	4.5	
7	2n	D10 uracil		4.4, 3.5
8	2n	R4		10.7, 6.0, 4.7, 4.4, 3.5
9	n	R4h-14 ALA		10.7, 6.0, 4.7
10	n	R4h-1 ALA		10.7, 6.0, 4.7
11	n	R4u-6 uracil		4.4, 3.5
12	n	R4u-17 uracil		4.4, 3.5

[§] the phenotype denotes the nutritional requirement of each strain



7.5. Characterisation of the *hemA*⁻ (*hemA::pyr-4*) mutant strains.

Two pairs of independently isolated *hemA*⁻ (*hemA::pyr-4*) mutants, R2h-24 and R2h-35, from R2 and R4h-1 and R4h-14 from R4 were tested to confirm their phenotypic characteristics and to establish an absence of both δ -aminolaevulinate synthase (δ -ALAS) enzyme activity and transcript. As δ -ALAS is the first enzyme in the biosynthesis of haem, *hemA*⁻ (*hemA::pyr-4*) mutant strains may be expected to grow in the presence of δ -aminolaevulinic acid (δ -ALA) or on medium supplemented with haem and L-methionine (Section 6.2).

7.5.1. Growth properties of the *hemA*⁻ (*hemA::pyr-4*) mutant strains.

The *hemA*⁻ haploid strains, R2h-24, R2h-35, R4h-1 and R4h-14, were tested for growth on glucose minimal medium supplemented with para-aminobenzoic acid, pyridoxine and δ -ALA at concentrations, ranging from 0 - 50ng.ml⁻¹ (Table 7.2). There was no growth in the absence of δ -ALA; however as the concentration of δ -ALA increased the size of the colonies increased linearly with normal (wild type) colony morphology being observed at 50ng.ml⁻¹ δ -ALA (Table 7.2).

Similarly, the *hemA*⁻ haploid strains, R2h-24, R2h-35, R4h-1 and R4h-14, were tested for growth incubated on glucose minimal medium supplemented with para-aminobenzoic acid, pyridoxine, L-methionine (50 μ g.ml⁻¹) and haem at concentrations, ranging from 0 - 50 μ g.ml⁻¹. None of the strains showed any growth following incubation at 37°C for three days (Table 7.2).

These results indicate that the *hemA*⁻ (*hemA::pyr-4*) haploid strains are deficient in the ability to produce δ -ALA. Since δ -ALA is the first enzyme in the haem

biosynthetic pathway, these strains can be considered to represent mutants deficient in the haem biosynthetic pathway. However media supplemented with haem, the end product of the haem biosynthetic pathway, and L-methionine, an essential by-product of the pathway, did not support growth of these *hemA*⁻ (*hemA::pyr-4*) haploid strains.

Table 7.2. Growth of *hemA*⁻ (*hemA::pyr-4*) haploids strains on different concentrations of ALA.

ALA concentration (ng.ml ⁻¹)	Growth of haploids strains.				
	Wild-type	Mutant <i>hemA</i> ⁻ (<i>hemA::pyr-4</i>) haploids strains.			
	R153	R2h-24	R2h-35	R4h-1	R4h-14
50	++++	++++	++++	++++	++++
45	++++	++++	++++	++++	++++
40	++++	+++	+++	+++	+++
35	++++	+++	+++	+++	+++
30	++++	++	++	++	++
25	++++	++	++	++	++
20	++++	++	++	++	++
15	++++	++	++	+	+
10	++++	+	+	-	-
5	++++	-	-	-	-
0	++++	-	-	-	-

A. nidulans hemA⁻ (*hemA::pyr-4*) haploid strains were grown on glucose minimal medium supplemented with the concentrations of ALA shown. The plates were incubated at 37°C for three days and then observed for the presence of growth which was scored upon the size of the colonies. No growth is represented '-' and the size of colonies represented in relation to the wild type control '++++'.

7.5.2. Attempts to assay δ -aminolaevulinate synthase activity.

The assay for δ -aminolaevulinate synthase activity was based on the method used to detect the activity of δ -ALAS in the yeast, *Saccharomyces cerevisiae* (Volland and Felix, 1984). In principle, the activity of the enzyme is estimated by measuring the amount of end-product, δ -aminolaevulinic acid (δ -ALA), produced from glycine and succinyl CoA in a reaction system. The amount of δ -ALA produced is measured colorimetrically using Ehrlich reagent (Mauzerall and Granick, 1956).

The colorimetric test was used to prepare a calibration graph by measuring the absorbance at OD₅₅₅ of a range of δ -ALA concentrations (0, 10, 20, 30, 40, 50, 60, 70 and 80 μ M) which were treated with Ehrlich reagent. To 1ml of each concentration of δ -ALA, 0.2ml 30% trichloroacetic acid was added. This was followed by the addition of 0.5ml of 3.2M sodium acetate buffer, pH 4.7, containing 0.6M NaOH. A further 0.04ml of 2,4-pentanedione was added and the tubes were incubated for 10 minutes in a boiling waterbath. After cooling to room temperature, 1.5ml of modified Ehrlich mercury reagent (168ml glacial acetic acid, 40ml 70% perchloric acid, 4.0g dimethylaminobenzaldehyde, 0.7g mercuric chloride, made to a total volume of 220ml with water) was added and after 15 minutes the absorbances was measured at OD₅₅₅. A graph of absorbance (OD₅₅₅) against δ -ALA concentrations was plotted which produced a linear relationship between the two parameters (Figure 7.3). The calibration graph was then used to estimate the amount of δ -ALA produced in the reaction assay.

Cell-free extract of the wild type *A. nidulans* strain R153 was prepared as described in Section 2.1.6. Suspensions of conidiospores were inoculated to 10^6 .ml⁻¹ in 400ml minimal medium in 2 litre baffled flasks. Following

incubation at 37°C for 18 hours, the mycelium was harvested, washed with extraction buffer (100mM sodium potassium phosphate buffer pH 7.6, 0.5mM pyridoxal phosphate, 3mM MgSO₄ 2mM dithiothreitol) and ground to a fine powder under liquid nitrogen, in a mortar and pestle. The powdered mycelium was resuspended in 1ml extraction buffer and the protein was extracted by gently shaking on ice for 30 minutes. The cell debris was removed by centrifugation and the supernatant recovered and stored on ice. The concentration of the protein in the supernatant was estimated colorimetrically by comparison with a calibration graph prepared from the absorbance (OD₅₉₅) of a BSA protein standard following exposure to the Biorad protein assay reagent (Section 2.11).

The reaction mixture for the enzyme assay was made up to a total volume of 10ml with 100mM sodium potassium phosphate buffer pH 7.6, 100mM glycine, 10mM sodium succinate, 0.5mM pyridoxal phosphate, 5mM ATP, 5mM MgCl₂ 2mM dithiothreitol, 0.2mM CoA and 1 unit of exogenous succinyl-CoA synthase.ml⁻¹. The reaction mixture was added to flask and incubated at 30°C for 5 minutes to allowed equilibration. The enzymatic reaction was initiated by the addition of the crude extract with a protein concentration of 4mg.ml⁻¹ of the reaction mixture. The reaction mixture was allowed to incubate at 30°C and, at 10 minute intervals for a total of 1 hour, 1ml aliquots were withdrawn and added to 0.2ml 30% trichloroacetic acid to stop the reaction. The suspension was centrifuged to remove the precipitated proteins and 1ml of the supernatant was removed to fresh tubes for colorimetric determination of δ -ALA.

Attempts to assay the δ -ALAS activity was unsuccessful. There were no detectable δ -ALA following the enzyme assay, using crude extract prepared from wild type *A. nidulans* R153 or in the untreated crude extract. Since the colorimetric test produced a calibration graph it was assumed that the presence

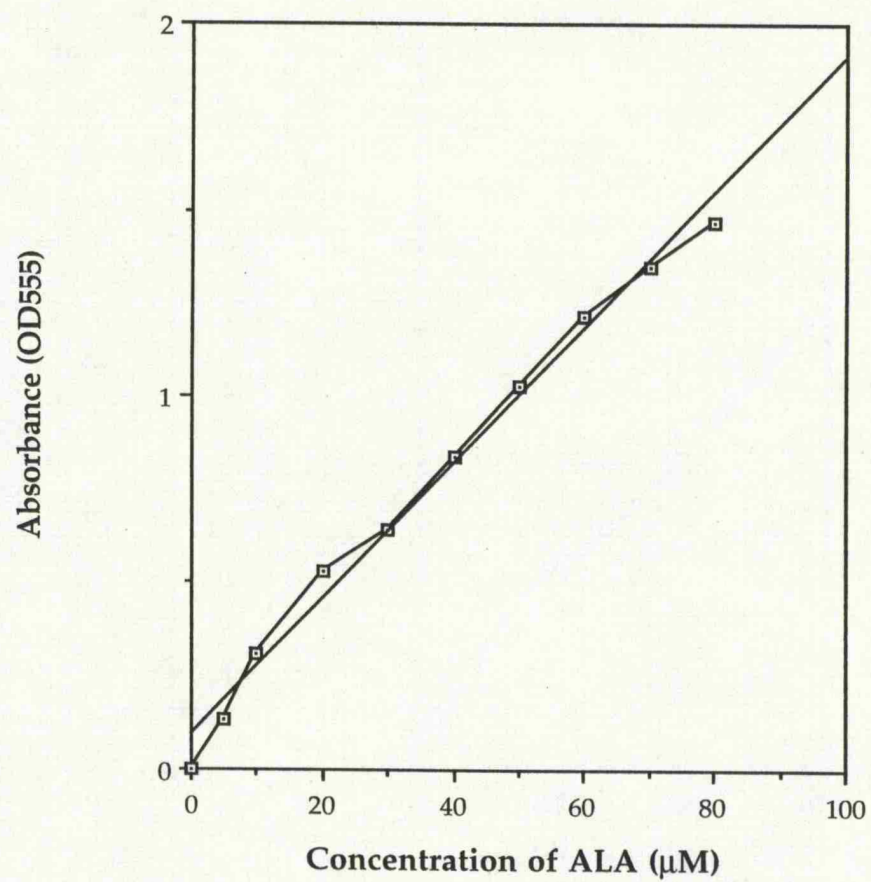
of δ -ALA was easily detectable and therefore the explanation for the lack of δ -ALAS activity in the crude extract may be due to the assay protocol being ineffective in supporting enzyme function. The lack of detectable δ -ALA in the untreated crude extract may be due to further metabolism of δ -ALA.

Further attempts to design a suitable protocol for the assay of δ -ALAS activity was not carried out. Since the aim was to establish that the *hemA*⁻(*hemA::pyr-4*) mutants lacked δ -ALAS activity, this could be carried out indirectly using northern blot analysis to show an absence of the *hemA* gene transcript in these mutants.

Figure 7.3. Calibration graph of ALA concentration against absorbance.

A range of ALA concentrations were treated with Ehrlich reagent and the absorbance at OD₅₅₅ measured and plotted against ALA concentration.

**Calibration graph of ALA concentration
against Absorbance**



7.5.3. Northern blot analysis to detect *hemA* mRNA.

Northern blot analysis was carried out on total cellular RNA isolated from the *hemA*⁻ (*hemA::pyr-4*) strains and a wild type control. The *hemA*⁻(*hemA::pyr-4*) haploids, R2h-24, R2h-35, R4h-1 and R4h-14, were grown in glucose minimal medium supplemented with δ -ALA (50ng.ml⁻¹) in a 37°C shaking incubator for 18 hours and the total cellular RNA isolated from each strain (Section 2.4.4). RNA was also extracted from the wild type *A. nidulans* strain R153 and the *hemA*⁺ haploid strain, R4u-6, which were used as a control.

The RNA, separated by electrophoresis and immobilised on Hybond-N membrane, was hybridised to a [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence (Figure 7.3). Hybridisation signals was obtained with the wild type and *hemA*⁺ controls. The size of this mRNA was estimated to be 2.3kb, consistent with it representing the *hemA* transcript. The intensity of the signal implied that the *hemA* transcript is relatively high in abundance.

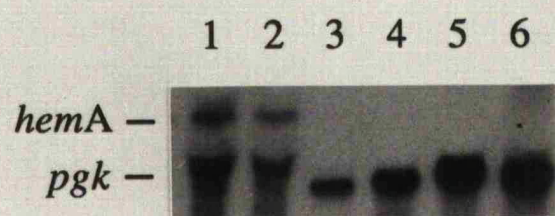
No hybridisation signals at 2.3kb were observed in the mutant strains. Since the hybridisation signal for *pgk* transcript is consistent throughout all the strains, the absence the *hemA* transcript would indicate that the *hemA*⁻ (*hemA::pyr-4*) mutant strains is unable to produce the *hemA* mRNA (Figure 7.4).

Figure 7.4. The *A. nidulans* *hemA* transcript.

Total cellular RNA isolated from the the haploid strains, wild type R153, a *hemA*⁺ segregant and *hemA*⁻ (*hemA::pyr-4*) haploids, R2Hw, R2Hy, R4Hw and R4Hy. 10µg samples were glyoxalated, to remove potential secondary structures, prior to separation by electrophoresis on a 1x MOPS agarose gel and transferred to Hybond-N filter. RNA size markers (Gibco BRL) were separated on the gel along with the total cellular RNA and used for later size estimation. The filter was incubated in a hybridisation reaction with an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence, at 65°C for 18 hours. Following hybridisation the membrane was washed using high stringency conditions in 3x SSC, 1% (w/v) SDS solution at 65°C for three hours. The membrane was also probed with the *pgk* gene to serve as an internal control.

Lanes

- | | |
|---|--|
| 1 | Wild type strain R153, |
| 2 | <i>hemA</i> ⁺ (<i>pyr-4</i> ⁻) strain R2Uw |
| 3 | <i>hemA</i> ⁻ (<i>hemA::pyr-4</i>) strain R2Hw, |
| 4 | <i>hemA</i> ⁻ (<i>hemA::pyr-4</i>) strain R2Hy, |
| 5 | <i>hemA</i> ⁻ (<i>hemA::pyr-4</i>) strain R4Hw, |
| 6 | <i>hemA</i> ⁻ (<i>hemA::pyr-4</i>) strain R4Hy, |



7.4. Summary.

The two transformed strains, R2 and R4, resulting from transformation with the released construct from the disruption vector were haploidised to isolate the *hemA*⁺ and *hemA*⁻ (*hemA::pyr-4*) segregants. Genomic DNA was extracted from all the strains and used for Southern blot analysis which indicated that in each case, one of the *hemA* gene encoding δ -aminolaevulinate synthase (δ -ALAS) has been disrupted by complex integration event rather than by directed gene replacement.

Growth tests with the *hemA*⁻ (*hemA::pyr-4*) mutant strains revealed that growth was sustained only on medium supplement with δ -ALA, furthermore, colony size was directly related to the concentration of δ -ALA. Glucose minimal medium supplemented with haem and L-methionine was unable to support the growth of the *hemA*⁻ (*hemA::pyr-4*) mutant strains.

It was not possible to establish the absence of δ -ALAS activity in the *hemA*⁻ (*hemA::pyr-4*) mutant strains since attempts to assay the activity of the enzyme were unsuccessful. However, northern blot analysis of mRNA isolated from the mutant strains revealed an absence of the *hemA* mRNA inferring an inability to transcribe the *hemA* gene or presence of an unstable transcript.

CHAPTER 8.

LOCATION OF THE *hemA* GENE IN *ASPERGILLUS* *NIDULANS*.

8.1. Introduction.

Diploid strains, R2 and R4, heterozygous for disruption of the *hemA* gene have been identified by Southern blot analysis following transformation of the *Aspergillus nidulans* diploid strain D10 with the disruption vector pALAS-np. The disrupted gene (*hemA::pyr-4⁺*) was shown to segregate from the wild-type allele *hemA⁺* when haploid strains were recovered following growth in the presence of benomyl. Since the recipient diploid strain possess genetic markers on four of its eight chromosome pairs (Figure 6.1), genetic analysis may allow the *hemA* gene to be allocated to a particular linkage group. However if the *hemA* locus is on an unmarked chromosome no specific allocation will be achieved.

8.2. Analysis of haploid segregants from the transformed strains R2 and R4.

The transformed diploid strains, R2 and R4, have been shown to generate two classes of haploid segregants, *hemA*⁺ and *hemA*⁻(*hemA::pyr-4*⁺). Since the disruption vector has inactivated one of the wild type *hemA* genes in the transformed strains, the haploid segregants carrying the *pyr-4*⁺ gene would therefore harbour the disrupted gene. This class of haploids require δ -aminolaevulinic acid (δ -ALA) for growth but do not require uracil. Conversely, haploid segregants lacking the *pyr-4*⁺ gene have the resident wild type *hemA* gene and thus do not require δ -ALA, but uracil is required for growth due to the presence of the *pyrG* mutant gene in all haploid segregants.

Haploid segregants were isolated by inoculating the transformed diploid strains, R2 and R4, onto complex medium containing glucose as carbon source, para-aminobenzoic acid (paba) and pyridoxine (pyro), and a sub-lethal concentration of benomyl to induce haploidisation (Hastie, 1970). The *hemA*⁻(*hemA::pyr-4*⁺) segregants were recovered on this medium when supplemented with δ -ALA alone, and the *hemA*⁺ segregants on the medium supplemented only with uracil.

The two groups of haploid segregant isolated were classified on test media with respect to the other genetic markers in the diploid strain. The markers *pabaA1* (chromosome I) and *pyroA4* (chromosome IV), were determined by testing for growth on a medium lacking para-aminobenzoic acid or pyridoxine respectively. The *qutE208* (chromosome VIII) mutation was determined by inability to grow with quinic acid as sole carbon source. The colour markers *yA*, yellow conidia (chromosomes I) and *wA3*, white conidia (chromosomes II) were scored by direct observation. Since the *wA3* mutation is epistatic to the

yA locus, the segregation of chromosomes I in white spored haploid strains was determined by the *pabaA1* marker (Table 8.1).

The data (Table 8.1) shows that the *hemA* alleles assort randomly with respect to each of the four markers identifying chromosomes I (*pabaA1*), II (*wA3*), IV (*pyroA4*), and VIII (*gutE208*). Therefore the *hemA* gene must be located on one of the unmarked chromosomes, namely II, V, VI or VII.

Table 8.1. Analysis of haploid segregants from the transformed strains R2 and R4.

Haploid segregants were recovered by inoculating transformed diploids, R2 and R4, onto complex medium with glucose as a carbon source and supplemented with uracil or ALA in the presence of benomyl to induce mitotic haploidisation. The haploid segregants isolated were screened on test media to classify them for the genetic markers present in the diploid strain. Uracil independence demonstrates the presence of the *N. crassa pyr-4⁺* gene.

Diploid strain	ALA dependence ^a	Number of haploid segregants in each class with respect to the chromosomal markers					
		Chromosome I <i>pabaA1/pabaA1</i> ⁺	II <i>wA3/wA3</i> ⁺	IV <i>pyroA4/pyroA4</i> ⁺	VIII <i>qutE208/qutE208</i> ⁺		
R2	<i>hemA</i> ⁺ (ura ⁻)	19	5	14	12	13	5
	<i>hemA</i> ⁻ (ura ⁺)	23	10	21	12	18	15
R4	<i>hemA</i> ⁺ (ura ⁻)	15	8	17	4	10	7
	<i>hemA</i> ⁻ (ura ⁺)	27	7	19	15	19	11
Total	<i>hemA</i> ⁺ (ura ⁻)	84	30	71	43	60	38
	<i>hemA</i> ⁻ (ura ⁺)	67	14	54	29	76	114

^a Uracll-independence demonstrates the presence of the *N. crassa pyr-4*⁺ gene

8.3. Location of the *hemA* gene by use of a "master strain" for mapping.

A series of haploid "master strains" of *A. nidulans* have been constructed which contain a marker on each of the eight linkage groups (Pontecorvo and Kafer, 1958). Such strains can be used to map new genes to chromosomes by mitotic haploidisation of a heterozygous diploid synthesised between a master strain and a second haploid containing the mutant gene (Forbes, 1959).

To identify the chromosome containing the *hemA* locus it was necessary to construct a diploid strain using a *hemA*⁻ haploid strain and a "master strain". Unfortunately the *hemA*⁻ mutant strains described above contain nutritional markers which are also present in all of the "master strains" available, and thus it was not possible to construct the necessary heterozygous diploid for mitotic haploidisation. Similarly none of the "master strains" contain the *pyrG* mutation which would have allowed the disruption of the *hemA* locus by selection for the *N. crassa pyr-4* gene marker. It was therefore necessary to induce a further *hemA*⁻ mutation by transformation of a suitable haploid *pyrG* strain, WA55 (*pyrG89, pabaA1; qutE208*). The most convenient "master strain" for combination with the new *hemA*⁻ mutant strain was R65. This strain possess genetic markers on each of its eight chromosomes and has the genotype *suadE yA adE20; wA3; galA1; pyroA4; facA303; sB3; nicB8; riboB2*. The second component was a WA55 *hemA*⁻ haploid strain in which the wild type *hemA* gene had been disrupted using the released fragment of vector pALAS-pd (Section 6.3.1).

8.3.1. Isolation of the *hemA*⁻ mutant in strain WA55.

The uracil requiring *A. nidulans* haploid strain WA55 was transformed to uracil independence using the gene disruption vector pALAS-p, containing the *pyr-4* gene of *N. crassa* following the procedure described in Section 6.3.

Protoplasts of strain WA55 were prepared and the final concentration adjusted to 1×10^7 protoplasts per ml with sterile 0.6M KCl, 50mM CaCl₂ solution. Two samples were used containing 200μl protoplast suspension. A total of 10μg of plasmid pALAS-pd (Figure 6.5) was digested with the restriction enzymes *EcoRI* and *XbaI* to release the *hemA::pyr-4*⁺ fragment and following incubation the efficiency of the digestion was examined by analysis of 1μg of DNA by electrophoresis on a 1% (w/v) agarose gel. The remainder of the reaction products were added to one sample of protoplasts and an equal volume of TE to the second as a negative control. The protoplasts were mixed with molten glucose minimal medium containing 0.6M KCl, supplemented with para-aminobenzoic acid and δ-aminolaevulinic acid, cooled to 48°C. The selective plates were poured immediately and incubated at 37°C for four days.

The efficiency at which protoplast regenerated to yield colonies was 32.5%. No colonies were observed on plates from the negative control lacking DNA, thus confirming the stability of the recipient strain. The transformed strains were replated for single colonies on selective glucose minimal medium, supplemented with δ-ALA. The experimental sample yielded 16 transformed strains of which two produced uniform morphological green colonies and the remainder proved unstable. These two strains were shown to have a requirement for δ-ALA and are uracil independent and therefore may be regarded as possible *hemA*⁻(*hemA::pyr-4*) haploid mutants. The strains were designated WA55-*hemA*-1 and WA55-*hemA*-2.

8.3.2. Southern blot analysis of the transformed haploid strains.

The haploid strains, WA55-*hemA*-1 and WA55-*hemA*-2, appear to be the required mutants since they grow on glucose minimal medium supplemented with δ -ALA alone and do not require uracil. Southern blot analysis was used to verify that the *hemA* locus has been disrupted.

Genomic DNA was prepared from the two stable transformants (Section 2.4.3) and subjected to restriction digestion by *EcoRI* or *SmaI* in separate reactions. The digests were separated by electrophoresis in a 1.0% (w/v) agarose gel and transferred to Hybond-N prior to hybridisation with an [α -³²P]dCTP radiolabelled 2.5kb *Sau3A/BamHI* DNA fragment, consisting of the entire *hemA* sequence, at 65°C for 18 hours. The filters were washed at high stringency conditions in 3x SSC, 0.2% (w/v) SDS solution at 65°C for three hours (Section 2.6.1.4).

Southern blot analysis of genomic DNA samples from the recipient haploid strain WA55 show hybridisation to fragments derived from the wild type *hemA* locus as demonstrated previously (Section 6.2.3). Restriction of the genomic DNA with the enzyme *BamHI* produced a single hybridisation of 4.5kb while restriction with *SmaI* produced two bands of 3.5 and 4.4kb (Figure 8.1, Lanes 1 and 4).

In the event of targeted gene replacement of the *hemA* locus by the disruption fragment, *hemA::pyr-4*, it is predicted that Southern blot analysis would result in two hybridisation singles at 2.7 and 4.7kb when the genomic DNA is digested with the restriction enzyme *BamHI*, and a single hybridisation at 10.7kb upon digestion with the restriction enzyme *SmaI* (Section 6.4.3).

However analysis of the transformed haploids, WA55-*hemA*⁻¹ and WA55-*hemA*⁻², resulted in a complex hybridisation pattern. Genomic DNA restricted with the enzyme *Bam*HI produced three bands of 2.7, 4.7 and 6.0kb (Figure 8.1, Lanes 2 and 3). Whilst the 2.7 and 4.7kb band are expected, it is not easy to account for the 6.0kb band. When the genomic DNA was digested with the restriction enzyme *Sma*I hybridisation signals at 10.7, 12.0 and 21.0kb are observed. (Figure 8.1, Lanes 5 and 6). Again, whilst the 10.7kb band is expected, the 12.0 and 21.0kb bands are not easily accountable.

Both haploid transformants produced similar sets of hybridisation bands which would imply that inactivation of the *hemA* gene by integration of the disruption vector has been by a similar mechanism in each case. The simplest explanation of the complex data is that multiple integration events have occurred at the *hemA* locus. However since the transformed haploid strains have a requirement for δ -ALA, it is evident that the resident *hemA* gene has been inactivated in both case.

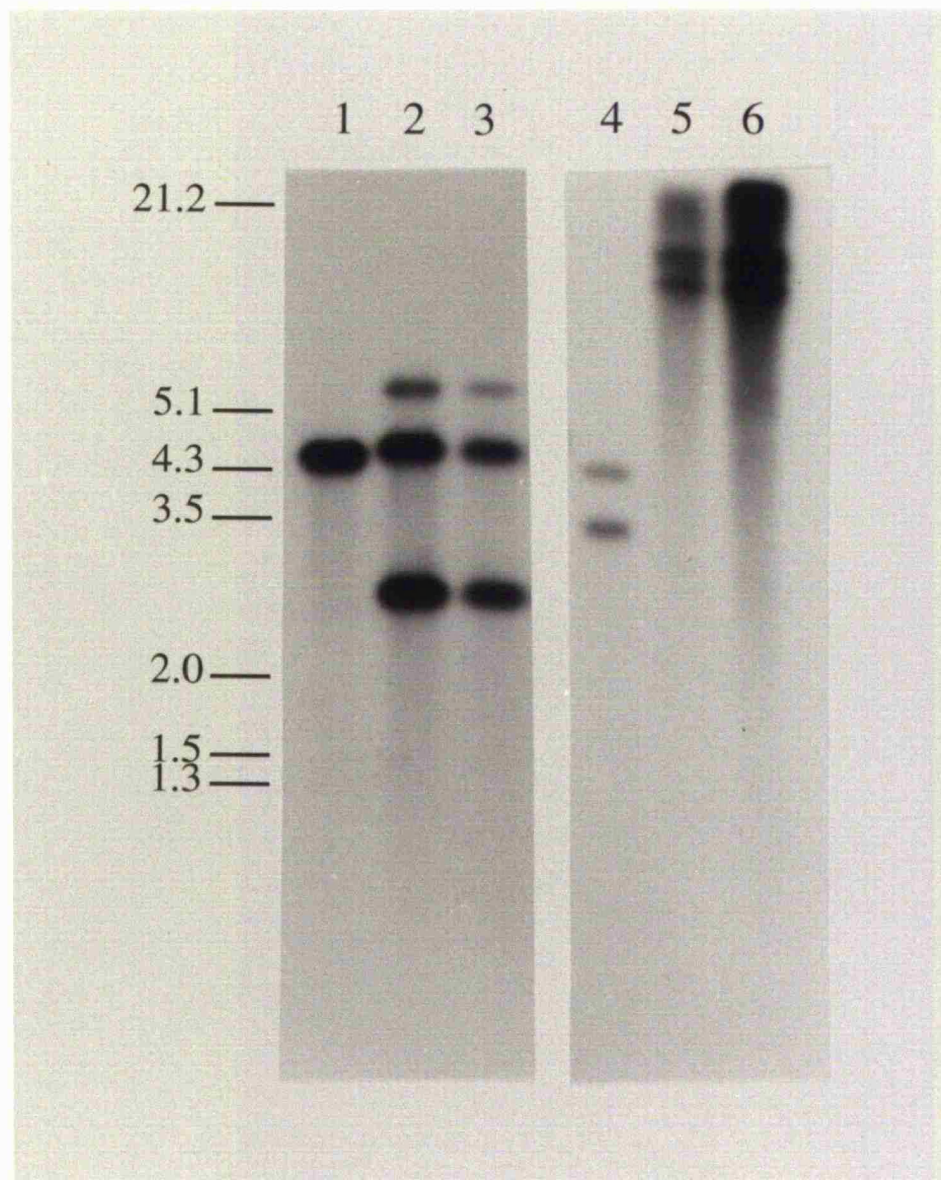
Figure 8.1. Southern blot analysis of the transformed haploid strains.

Genomic DNA was isolated from the recipient haploid strain WA55, and the transformed haploid strains W1 and W2. The DNA was digested with the restriction enzyme *Bam*HI or *Sma*I and the nucleic acid separated by electrophoresis on a 1.0% (w/v) agarose gel. The nucleic acid was transferred to Hybond-N filter and Southern blot hybridisation carried out using an [α -³²P]dCTP radiolabelled 2.5kb *Bam*HI/*Sau*3A DNA fragment consisting of the entire *hemA* sequence.

Phage lambda DNA digested with *Eco*RI and *Hind*III provided the molecular weight markers.

Lanes	Strains	Phenotype§	Fragment sizes (kb)	
			<u><i>Bam</i>HI digest</u>	<u><i>Sma</i>I digest</u>
1	WA55	uracil	4.5	
2	WA55- <i>hemA</i> -1	ALA	6.0, 4.7, 2.7	
3	WA55- <i>hemA</i> -2	ALA	6.0, 4.7, 2.7	
4	WA55	uracil		4.4, 3.5
5	WA55- <i>hemA</i> -1	ALA		21.0, 12.0, 10.7,
6	WA55- <i>hemA</i> -2	ALA		21.0, 12.0, 10.7,

§ the phenotype denotes the nutritional requirement of each strain



8.4. Construction of heterozygous diploid strains between "master strain" R65 and WA55-*hemA*⁻¹ and WA55-*hemA*⁻².

The transformed haploid strains, WA55-*hemA*⁻¹ and WA55-*hemA*⁻², containing the disrupted *hemA* gene were used to construct heterozygous diploids by combination with the haploid master strain, R65. Heterokaryons were established between the strains on unsupplemented glucose minimal agar (Section 2.1.5). Suspension of conidiospores from the established heterokaryons were then plated in the same medium and a number of green spored prototrophic strains were obtained. These strains were tested for segregation of the recessive colour markers *yA* and *wA3* on complex medium containing benomyl, and a single strain from each combination retained as the required heterozygous diploid. The diploid strains were designated WA55-*hemA*⁻¹:R65 and WA55-*hemA*⁻²:R65 (Figure 8.2).

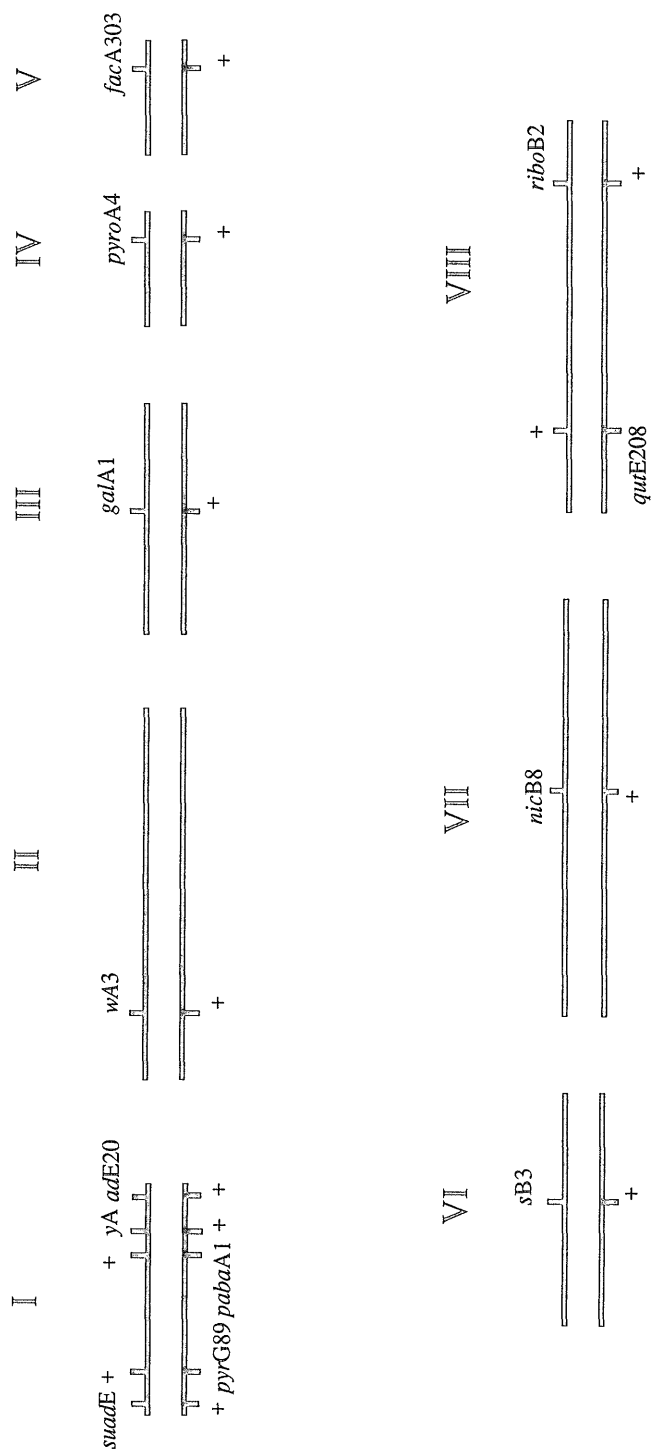
**Figure 8.2. Genetic map of the heterozygous diploid strains
WA55-*hemA*-1:R65 and WA55-*hemA*-2:R65.**

The diploid strains, WA55-*hemA*-1:R65 and WA55-*hemA*-2:R65, possess genetic markers all of its eight linkage groups. The strains produce green conidia and yield abundant recessive colour marker segregant upon growth in the presence of benomyl.

The relative lengths of the chromosomes are deduced from the linkage data (Clutterbuck, 1982).

Upper line - "Master strain" R65

Lower line - disrupted strain (W1/W2)



8.5. Analysis of haploid segregants from the diploid strains

WA55-*hemA*⁻1:R65 and WA55-*hemA*⁻2:R65.

The diploid strains, WA55-*hemA*⁻1:R65 and WA55-*hemA*⁻2:R65 each generates two classes of haploid segregants for the *hemA* locus, *hemA*⁺ or *hemA*⁻ (*hemA*::*pyr*-4⁺). Haploid segregants were isolated by inoculating the diploid strains, WA55-*hemA*⁻1:R65 and WA55-*hemA*⁻2:R65, onto complex medium containing glucose as carbon source, para-aminobenzoic acid (paba), pyridoxine (pyro), nicotinic acid (nic) riboflavin (ribo) and thiosulphate (s), and a sub-lethal concentration of benomyl to induces haploidisation. The *hemA*⁻ (*hemA*::*pyr*-4⁺) segregants were recovered on this medium supplemented with δ -ALA alone, and the *hemA*⁺ segregants on the medium supplemented only with uracil. The *hemA*⁺ and *hemA*⁻ (*hemA*::*pyr*-4⁺) segregants isolated in this manner were classified for their genetic markers as described.

Initial genetic analysis (Section 8.2) showed that the *hemA* locus assorted independently with respect to the *wA3* marker on linkage group II. This observation allows a strategy in which only white spored segregants were isolated from each of the diploid strains. This was because it was necessary to recover haploid segregants known to contain the *pyrG* mutation so that the presence of the *pyr*-4⁺ gene could be determined by uracil independence. Since *pyrG* is coupled to *yA*⁺ (green) in the heterozygous diploid (Figure 8.2), yellow segregants would all be *pyrG*⁺ and the potential green spored (*yA*⁺) segregants could not be distinguished from the original diploid. White spored haploid segregants can be classified for chromosome I because the *pabaA1* marker is coupled to the *pyrG* mutation.

The white spored haploid segregants isolated were screened on test media to classify them with respect to their genetic markers in the diploid strain (Table

8.2. The markers *pabaA1* (chromosome I) *pyroA4* (chromosome IV), *sB3* (chromosome VI), *nicB8* (chromosome VII) and *riboB2* (chromosome VIII) mutations were determined by testing for growth on a medium lacking para-aminobenzoic acid, pyridoxine, thiosulphate, nicotinic acid or riboflavin respectively. The markers *galA1* (chromosome III) and *facA303* (chromosome V) were determined by inability to grow with galactose or acetate, respectively, as sole carbon source.

The data (Table 8.2) shows independent assortment of the δ -ALA requirement with respect to the seven markers identifying chromosomes I (*pabaA1*), II (*wA3*), III (*galA1*), IV (*pyroA4*), V (*facA303*), IV (*sB3*) and VIII (*riboB2*). However in the case of linkage group VII, only two phenotypic classes were observed demonstrating that vector sequence integration has occurred on linkage group VII. The *hemA* gene locus therefore has been allocated to chromosome VII of *A. nidulans*.

Table 8.2. Analysis of haploid segregants from the heterozygous diploid strains WA55-*hemA*⁻¹:R65 and WA55-*hemA*⁻²:R65.

Haploid segregants were recovered by inoculating the heterozygous diploid strains, WA55-*hemA*⁻¹:R65 and WA55-*hemA*⁻²:R65, onto complex medium with glucose as a carbon source, supplemented with either uracil or ALA in the presence of benomyl to induce mitotic haploidisation. The haploid segregants isolated were screened on test media to classify them for the genetic markers present in the diploid strain. Uracil independence demonstrates the presence of the *N. crassa pyr-4*⁺ gene.

Diploid ALA strain ^a	dependence ^b	Number of haploid segregants in each class with respect to the chromosomal markers														
		Chromosome I	III	IV	V	VI	VII	VIII								
		<i>pabaA1/pabaA1</i> ⁺	<i>galA1/galA1</i> ⁺	<i>pyroA4/pyroA4</i> ⁺	<i>facA/facA</i> ⁺	<i>sB3/sB3</i> ⁺	<i>nicB8/nicB8</i> ⁺	<i>riboB2/riboB2</i> ⁺								
W1	<i>hemA</i> ⁺ (ura ⁻)	9	0	1	8	5	4	2	7	2	7	9	0	1	8	
	<i>hemA</i> ⁻ (ura ⁺)	11	5	6	3	8	7	4	7	4	4	7	0	11	5	6
W2	<i>hemA</i> ⁺ (ura ⁻)	3	3	0	1	2	2	1	2	1	2	1	3	0	2	1
	<i>hemA</i> ⁻ (ura ⁺)	13	5	8	7	6	8	5	6	7	7	6	0	13	7	6
Totals	<i>hemA</i> ⁺ (ura ⁻)	12	22	14	12	24	22	14	17	19	15	21	12	24	15	21
	<i>hemA</i> ⁻ (ura ⁺)	24	36													

^a W1 and W2 represents the diploid strains WA55-*hemA*¹:R65 and WA55-*hemA*²:R65 respectively
^b Uracil-independence demonstrates the presence of the *N. crassa pyr-4*⁺ gene or native the *pyrC89*⁺ gene

8.6. Summary.

Disruption of the *hemA* gene in the diploid strain D10 failed to locate the *hemA* locus to a specific chromosome since only four of the eight linkage groups were marked genetically. A strategy was therefore devised to induce further *hemA* mutation in a haploid strain of *A. nidulans*. Two of these new *hemA* disrupted mutants were combined with a multiply marked "master strain" and the *hemA* gene unambiguously located to chromosome VII of *A. nidulans*.

Since the *hemA* locus is located on chromosome VII, assortment of chromosomes I (*pyrG*) and VII (*hemA::pyr-4⁺*), will be expected to yield uracil dependent and independent strains in the ratio of 1:3 (Figure 8.3). Uracil independent strains may arise from combination of the *pyrG⁺* gene with the *hemA⁺* gene or with the *hemA⁻(hemA::pyr-4⁺)* gene, and the *pyrG⁻* gene (chromosome I) with the *hemA⁻(hemA::pyr-4⁺)* gene. Uracil dependent strains may arise from the combination of the *pyrG⁻* gene with the *hemA⁺* gene.

During the selection procedure only white spored segregants were isolated from the diploids so that the presence of the disrupted *hemA⁻(hemA::pyr-4⁺)* gene could be determined by uracil independence. That is, only strains that were either δ -ALA requiring and uracil independent, or uracil requiring and δ -ALA independent were recovered for analysis. Therefore the uracil independent strains arising from a combination of the *pyrG⁺* gene (chromosome I) and the *hemA⁺* gene (chromosome VII) were eliminated by the selection procedure, thus the ratio of uracil dependent to independent strains recovered is expected to be 1:2. It is satisfying that the observed ratio amongst a total of 36 haploid strains recovered, was 12 uracil dependent and 24 uracil independent strains.

Figure 8.3. Assortment of chromosomes I and VII.

The prediction for the assortment of chromosomes I (*pyrG*) and VII (*hemA::pyr-4⁺*) are shown in detail. Following segregation, the ratio of uracil dependent to independent strains is expected to be 1:3. However, due to the elimination of one of the uracil independent classes, the selection procedure resulted in the recovery of uracil dependent to independent strains in the ratio of 1:2.

Assortment of chromosomes I and VII.

Phenotype	^S No of strains recovered
uracil ⁺	0
uracil ⁺	14
uracil ⁻	12
uracil ⁺	10
Total	36

Ratio of uracil⁺ to uracil⁻ strains = 24 : 12 (2 : 1).

^S the phenotype denotes the requirement of uracil only.

CHAPTER 9.

DISCUSSION.

The general aim of this study was to add to the understanding of the regulation of haem biosynthesis in an obligate aerobe, the filamentous fungus *Aspergillus nidulans*. In practice, the work has concentrated primarily on the first step of the biosynthetic pathway, the production of δ -aminolevulinic acid (δ -ALA), catalysed by the nuclear encoded enzyme, δ -aminolevulinate synthase (δ -ALAS). The gene encoding δ -ALAS (*hemA*) was isolated on a 4.5kb *Bam*HI fragment from an *A. nidulans* genomic library in the phage vector λ EMBL3 and Southern blot analysis indicates that *A. nidulans* possesses a single copy of the gene.

Since mutant strains of *A. nidulans* deficient in δ -ALAS activity have not been previously identified, the secondary aim of this project has been to isolate and characterise a *hemA*⁻ mutant strain of *A. nidulans* and to assign the *hemA* locus to a particular chromosome.

9.1. Cloning and nucleotide sequence of the δ -aminolevulinate synthase (*hemA*) gene of *Aspergillus nidulans*.

The *A. nidulans hemA* gene, encoding δ -ALAS, has been cloned and sequenced. The coding region of the *hemA* gene is interrupted by a 64bp intron located between 355 and 419nt in the gene; that is between amino acids 119 and 120 of

the protein. The size of the intervening sequence is consistent with those of other filamentous fungi (Ballance, 1986; Gurr *et al.*, 1987).

The deduced amino acid sequence reveals a 58% sequence identity to that of the *HEM1* gene of the yeast *Saccharomyces cerevisiae* and identity ranging from 40 - 54% with other δ -ALAS proteins. Over the C-terminal domain, thought to be important for the catalytic function of the enzyme (Cox *et al.*, 1991), the sequence is highly conserved with identity ranging from 52 - 64% with other δ -ALAS proteins. The amino-terminal region of the sequence contains motifs which are consistent with a function in mitochondrial targeting.

A single *A. nidulans hemA* mRNA, the size of which is approximately 2.3kb, was found to be in ample abundance and in similar amounts to the highly expressed phosphoglycerate kinase (*pgkA*) gene (Section 7.5.3). S1 nuclease analysis of the 5' terminus of the *hemA* mRNA revealed a major transcriptional start site at -55nt with respect to the start (+1) of the open reading frame and a minor site at -92nt. When primer extension analysis was carried out on the upstream untranslated mRNA sequence, only the minor site, -92nt was detected. However the potential formation of secondary structures within the leader sequences region of the mRNA may have inhibited primer extension mapping, and it is likely that both translation start sites are used. Similar contradictions between the two methods of mapping have been found for the *HEM1* gene in *S. cerevisiae* (Urban-Grimal *et al.*, 1986; Keng and Guarente, 1987). The length of the upstream untranslated mRNA sequence is typical of a length for other δ -ALAS genes and also the general run of mRNA from filamentous fungi (Ballance, 1986).

9.2. Disruption of the δ -aminolevulinate synthase (*hemA*) gene of *Aspergillus nidulans*.

In the yeast, *Saccharomyces cerevisiae*, gene disruption is routinely achieved with as little as 0.3kb homologous sequence flanking the selective marker (Orr-Weaver *et al.*, 1981; Orr-Weaver *et al.*, 1983; Rothstein, 1983). Homologous recombination is improved by linearising the disruption molecule. In *Aspergillus nidulans* transformation occurs at a lower frequency than in yeast and again homologous recombination can be increased using a linear vector, however a greater amount of homologous flanking sequence is required (May *et al.*, 1989; Wernars *et al.*, 1987; Miller *et al.*, 1985; Osmani *et al.*, 1985). Disruption in *A. nidulans* is often part of a complex set of integration events which involves a number of separate or multiple integrations (Streatfield and Roberts, 1993).

Isolation of an *hemA* mutant in *A. nidulans* involved the disruption of the *hemA* in a diploid strain to enable the *hemA*⁻ mutant to be recovered and then isolated by mitotic haploidisation. The native *A. nidulans hemA* gene was replaced with the homologous cloned sequence, interrupted by a selectable marker. Transformation with the disruption sequence produced 4 stable transformants (0.61 transformants/ μ gDNA), two of which produced signals, upon Southern blot analysis, consistent with targeted gene replacement. Transformation with the linear plasmid did not increase the frequency of transformation in *A. nidulans*. This is contrary to previous observations where the frequency of transformation was increased approximately 50 fold by linearisation of the vector molecule (Streatfield *et al.*, 1992). However in both cases disruption was not the result of direct gene replacement but complex integration events.

The *hemA*⁻ (*hemA::pyr-4*) mutant strain has an absolute requirement for δ -ALA (50ng.ml⁻¹). Glucose minimal medium supplemented with haem and L-methionine was unable to support the growth of the *hemA*⁻ (*hemA::pyr-4*) mutant strains, contrary to the situation in the yeast *S. cerevisiae* (Gollub *et al.*, 1977). Although the enzyme assay was unsuccessful, northern blot analysis of cellular mRNA isolated from the mutant strains revealed absence of the *hemA* mRNA, inferring either an inability to transcribe the *hemA* gene or the presence of an unstable transcript.

The construction of a suitable *A. nidulans* diploid between the *hemA*⁻ mutant and a multiply marked "master strain", followed by genetic analysis employing mitotic haploidisation demonstrated that the *hemA* gene is unambiguously located to chromosome VII of *A. nidulans*.

9.2. Summary and future work.

The *Aspergillus nidulans* *hemA* gene, encoding δ -aminolevulinatase synthase (δ -ALAS), has been cloned and sequenced. Mutant strains presumed deficient in δ -ALAS activity have been isolated which were shown to have derived as a result of the disruption of the *A. nidulans* *hemA* gene. Molecular and genetic analysis have demonstrated that the *hemA* gene is present as a single copy, located on chromosome VII of the *A. nidulans* genome. The isolation of *hemA* gene allows the possibility for manipulation of the biosynthesis of haem thereby enabling the investigation of the role of haem in respiratory control of this obligate aerobe.

Similarities between the *hemA* gene of *A. nidulans* and the *HEM1* gene of *Saccharomyces cerevisiae* suggests that the δ -ALAS protein is targeted to the mitochondria where it is processed to produce the mature protein. The region responsible for mitochondrial targeting could be located by modifying the 5' *hemA* sequences, fusing the modified sequenced to a reporter gene and introducing the sequence into *A. nidulans*. Both the targeting region and the post translational splice site could be located using this exercise.

The work of this thesis has primarily concentrated upon the sequencing of the *hemA* gene. Regulatory studies in sequenced δ -ALAS genes in obligate aerobes suggests that the gene is regulated by the product of the pathway, haem, however, initial regulatory studies of the *hemA* gene suggests that the gene constitutively expressed (Bradshaw *et al.*, 1992). Further analysis the 5' region of the gene would clarify whether this constitutively expressed gene is typical of that which occur in the yeast, *S. cerevisiae* where the *HEM1* gene is constitutively expressed as a result of a composite of activation by haem and repression by dextrose, with additional layers control serving to counteract

these regulatory effects of haem and dextrose (Keng and Guarente, 1987). Such analysis could be performed by the fusion of the *hemA* sequence to reporter genes. Following manipulation of the promoter region the function of the 5' sequences could be analysed after the reintroduction of the modified construct into *A. nidulans*.

The *hemA* auxotrophic mutant *A. nidulans* strain can be used as a tool in which manipulating the levels of haem biosynthesis, by varying the levels of δ -ALA. The effect on the protein binding haem can be observed, in particularly those involved in respiratory control in this obligate aerobe

Disruption of the *hemA* gene produced an δ -ALA auxotrophic mutant of *A. nidulans*. Unlike the *S. cerevisiae* δ -ALA auxotrophic mutant, haem was unable to support the growth of the *A. nidulans* mutant. This mutant strain provides the opportunity to discover if a growth medium can be designed to enable recovery of haem deficient mutants in general in *A. nidulans*. Such mutants would be invaluable in the study of the genetics and regulation of the haem biosynthetic pathway in the obligate aerobe.

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