Studies of the 3-phosphoglycerate kinase gene of *Penicillium chrysogenum*.

By Isobel Claire Hoskins

Bsc (Hons.) Biochemistry,
University of Sheffield.

Thesis submitted for the degree of Ph.D in the Department of Genetics, University of Leicester.

June 1991
To my family.
Studies of the 3-phosphoglycerate kinase gene

of *Penicillium chrysogenum*

Isobel Claire Hoskins

June 1991

Abstract

Studies of *Saccharomyces cerevisiae* and *Aspergillus nidulans* have shown that the 3-phosphoglycerate kinase gene (PGK) is highly expressed from a strong promoter (Holland and Holland 1978; Clements 1986). The aim of the project was to isolate and study the potentially strong PGK gene promoter of *P. chrysogenum*.

The *P. chrysogenum* PGK gene was isolated and the promoter and terminator sequences determined. The promoter contained a 40bp pyrimidine rich region in which transcription was initiated at multiple sites. Matches to both the glycolytic box and the essential region of the *Aspergillus nidulans* PGK promoter were found in the promoter by sequence comparison.

The expression of the PGK promoter was studied using a fusion between the PGK promoter and the *Escherichia coli lacZ* reporter gene. The gene fusion was transformed into *P. chrysogenum* and a transformed strain containing two copies of the fusion at the oliC locus was monitored for the reporter activity. The PGK promoter was up to three times more active during growth in media containing carbon sources metabolised by gluconeogenesis compared to those metabolised by glycolysis, and the rate at which the promoter activity increased in logarithmic growth was also greater. The expression of the *P. chrysogenum* PGK promoter in *Aspergillus nidulans* was investigated by transformation of the gene fusion into *A. nidulans* at the gutE locus. The PGK promoter retained its broad pattern of control.

A second gene fusion was made between the *P. chrysogenum* PGK promoter and the Isopenicillin-N-Synthetase gene. A transformed *P. chrysogenum* strain containing one copy of the gene fusion at the oliC locus was compared to the original strain in a batch fermentation. The transformed strain had twofold higher *IPNS* mRNA and enzyme levels during the first three days of the fermentation. The penicillin titre was slightly increased in this period.
Acknowledgments

I would like to thank my academic supervisor Dr. Clive Roberts and my Industrial supervisor Dr Alison Earl for their guidance over the last 3 years. I also thank the members of Leicester University Department of Genetics for their help and friendship. Many people advised and helped in doing the work carried out at SmithKlineBeecham and I would like to thank Dave Weyburn, Dr Linden Gledhill, and the members of the microfermenter unit in particular. Thanks too to Professor Geoff. Turner for advice and plasmids. Lastly I thank my husband Stephen for his support.
Abbreviations

ACT  acyl coenzyme A : 6-amino-penicillanic acid acyltransferase
ACV  6-(L-α-amino-adipyl)-L-cysteinyl-D-valine
ACVS  ACV synthase
6-APA  6-amino-penicillanic acid
ATP  adenosine triphosphate
bp  base pair
BSA  Bovine serum albumin
Ci  Curie
cpm  counts per minute
DNA  Deoxyribonucleic acid
dNTP  2'-deoxy(N) 5' triphosphate, N= adenosine (A), cytidine (C), guanosine (G), or thymidine (T)
EDTA  Ethylenediamine tetra-acetic acid
EGTA  Ethyleneglycol-bis(β-aminoethyl ether) tetra acetic acid
HEPES  N-2-Hydroethylpiperazine-N'-ethanesulphonic acid
IPTG  isopropyl-β-D-galactopyranoside
IPN  isopenicillin-N
IPNS  isopenicillin-N synthetase
kb  kilobase
kD  kiloDalton
kV  kilovolt
MOPS  N-2-(N-Morpholino)ethanesulphonic acid
mRNA  messenger ribonucleic acid
mw  molecular weight
nt  nucleotide
ONPG  o-nitrophenyl-β-D-galactopyranoside
paba  para amino benzoic acid
PEG  polyethylene glycol
PVP  polyvinyl pyrrolidine
RNA  ribonucleic acid
rpm  rotations per minute
rRNA  ribosomal ribonucleic acid
SDS  sodium dodecyl sulphate
SSC  saline sodium citrate
TEMED  N,N,N',N'-tetra-methylethylene diamine
tRNA  transfer ribonucleic acid
v/v  volume for volume
w/v  weight for volume
X-gal  5-bromo-4-chloro-3-indoyl β-D-galactopyranoside
μF  microfarad
# Contents

## Chapter 1: Introduction

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Gene expression in eukaryotic cells</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Gene expression in filamentous fungi</td>
<td>9</td>
</tr>
<tr>
<td>1.3 Filamentous fungal genes encoding enzymes in the glycolytic and gluconeogenic pathways</td>
<td>17</td>
</tr>
<tr>
<td>1.4 Penicillin biosynthesis</td>
<td>22</td>
</tr>
<tr>
<td>1.5 Aims of the project</td>
<td>32</td>
</tr>
</tbody>
</table>

## Chapter 2: Materials and methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Genetic materials and methods associated with <em>Penicillium chrysogenum</em> and <em>Aspergillus nidulans</em></td>
<td>33</td>
</tr>
<tr>
<td>2.1.1 Strains</td>
<td>33</td>
</tr>
<tr>
<td>2.1.2 Growth media</td>
<td>33</td>
</tr>
<tr>
<td>2.1.3 Growth and storage of strains</td>
<td>36</td>
</tr>
<tr>
<td>2.1.4 Preparation of conidiospore suspension</td>
<td>37</td>
</tr>
<tr>
<td>2.1.5 Method of colony staining for β-galactosidase production</td>
<td>37</td>
</tr>
<tr>
<td>2.1.6 β-galactosidase assays</td>
<td>38</td>
</tr>
<tr>
<td>2.1.7 Isopenicillin-N synthetase (IPNS) enzyme assays</td>
<td>41</td>
</tr>
<tr>
<td>2.1.8 Penicillin titre</td>
<td>43</td>
</tr>
<tr>
<td>2.1.9 DNA mediated transformation of <em>P. chrysogenum</em></td>
<td>43</td>
</tr>
<tr>
<td>2.1.10 DNA mediated transformation of <em>A. nidulans</em></td>
<td>44</td>
</tr>
<tr>
<td>2.2 Genetic materials and methods associated with <em>Esherichia coli.</em></td>
<td>46</td>
</tr>
<tr>
<td>2.2.1 <em>E. coli</em> strains</td>
<td>46</td>
</tr>
<tr>
<td>2.2.2 Growth media</td>
<td>46</td>
</tr>
<tr>
<td>2.2.3 Growth and storage of strains</td>
<td>47</td>
</tr>
<tr>
<td>2.2.4 Plasmids and phage</td>
<td>47</td>
</tr>
<tr>
<td>2.2.5 Transformation of JM83</td>
<td>47</td>
</tr>
<tr>
<td>2.2.6 Electroporation of TG1</td>
<td>48</td>
</tr>
<tr>
<td>2.2.7 Transformation of JM101 and JM109</td>
<td>49</td>
</tr>
<tr>
<td>2.3 Recovery of nucleic acids</td>
<td>50</td>
</tr>
<tr>
<td>2.4 DNA preparation</td>
<td>51</td>
</tr>
<tr>
<td>2.4.1 Small scale preparation of plasmid DNA from <em>E. coli</em></td>
<td>51</td>
</tr>
</tbody>
</table>
2.4.2 Preparation of cosmid DNA from dense lawns of E. coli, for colony hybridisation
2.4.3 Large scale preparation of plasmid DNA from E. coli
2.4.4 Small scale preparation of chromosomal DNA from P. chrysogenum and A. nidulans
2.5 Analysis of nucleic acids by agarose gel electrophoresis
2.6 DNA manipulation techniques
2.6.1 Digestion of DNA with restriction enzymes
2.6.2 Digestion of DNA with Bal31 exonuclease
2.6.3 Recovery of DNA fragments from agarose gels
2.6.4 Fill-in of recessed ends of DNA fragments
2.6.5 Ligation of DNA fragments
2.6.6 Removal of 5' phosphates from linear DNA
2.6.7 Site directed mutagenesis
2.7 Analysis of DNA by transfer onto nylon membranes and hybridisation using radioactive DNA probes (Southern hybridisation)
2.7.1 Southern blot filter preparation
2.7.2 Preparation of DNA for "dot" blot analysis
2.7.3 Oligonucleotide labelling of DNA fragments to be used as probes
2.7.4 DNA hybridisation of Southern blot and "dot" blot filter membranes
2.7.5 Autoradiography
2.7.6 Stripping DNA probes from the filter membrane
2.7.7 Efficiency of incorporation of radioactivity into the probe
2.8 Preparation of P. chrysogenum total cellular RNA
2.9 Analysis of RNA by transfer onto nitrocellulose membranes and hybridisation using radioactive probes
2.9.1 Northern blot filter preparation
2.9.2 Hybridisation of RNA Northern blot membranes
2.9.3 Densitometric analysis of autoradiographs of RNA filters
2.10 Analysis of RNA by protection against SI nuclease digestion
2.10.1 Probe preparation
2.10.2 Hybridisation of total RNA to the probe 68
2.10.3 SI nuclease reaction 68
2.11 Sequence analysis of DNA subcloned into M13 phage 69
2.11.1 Preparation of replicative form DNA 69
2.11.2 Preparation of recombinant single stranded M13 DNA sequencing templates 69
2.11.3 Determination of DNA sequence by the deoxyribonucleotide chain termination method 70
2.11.4 Sequencing gels 71
2.12 Computing 73
2.13 Source of materials 73
2.14 Containment and safety 73

Chapter 3: Isolation of the PGK gene of Penicillium chrysogenum 74
3.1.1 P. chrysogenum strains used and the origin of the cosmid library 74
3.1.2 Preparation of PGK coding region DNA probe from A. nidulans 75
3.1.3 Hybridisation of A. nidulans PGK probe to P. chrysogenum genomic DNA. 75
3.1.4 Screening the cosmid library of P. chrysogenum genomic DNA for the PGK gene 77
3.1.5 Verification of the selected clones 79
3.2.1 Subcloning and analysis of the putative PGK gene of P. chrysogenum 80
3.2.2 Restriction map and orientation of pPC1 81
3.2.3 Subcloning of the coding and upstream sequences of the PGK gene 82
3.2.4 Verification of PGK by sequence analysis 83
3.3 Summary 84

Chapter 4: Sequence analysis of the promoter and terminator regions of the PGK gene from Penicillium chrysogenum 85
4.1 Sequencing strategy 85
4.1.1 Construction of subclones for sequence analysis by the use of restriction sites 86
4.1.2 The deletion of further sequence by Bal31
4.2.1 Promoter sequence 90
4.2.2 Terminator sequence 92
4.3 Summary 92

Chapter 5: Analysis of the transcription of the PGK gene
5.1 Preparation of total cellular RNA 95
5.2 Determination of the 5' and 3' ends of the PGK mRNA by SI exonuclease protection studies 95
5.2.1 Determination of the 5' end of the message 96
5.2.2 Determination of the 3' end of the message 97
5.3.1 Preparation of the DNA probes used to identify different mRNAs in Northern hybridisations 99
5.3.2 The identification and estimation of the size of the PGK mRNA of P. chrysogenum 99
5.3.3 The relative abundance of PGK mRNA and IPNS mRNA 101
5.3.4 The relative abundance of PGK message on different carbon sources 102
5.4 Summary 103

Chapter 6: Construction and use of a reporter gene fusion of the P. chrysogenum PGK promoter to the E. coli lacZ gene
6.1 Transformation of P. chrysogenum N.R.R.L. 1951 with the A. nidulans PGK promoter-lacZ gene fusion 106
6.2 Construction of a vector containing a P. chrysogenum PGK promoter-lacZ fusion 108
6.3 Transformation of the gene fusion vector pOPL8 into P. chrysogenum 111
6.4 Analysis of the transformants 111
6.5 Identification of the PGK promoter-lacZ fusion mRNA and comparison to the native PGK mRNA 113
6.6 Summary 114
### Chapter 7: Investigation of the physiological control of the *P. chrysogenum* PGK promoter

7.1.1 Activity of the PGK promoter during growth in extended batch culture

7.1.2 Expression of the PGK promoter during growth and on different carbon sources

7.1.3 The effect of heat on PGK promoter activity

7.2 The expression of the PGK promoter of *P. chrysogenum* in *A. nidulans*

7.2.1 The construction of a *P. chrysogenum* PGK promoter–lacZ fusion vector for transformation into *A. nidulans*

7.2.2 Transformation of pPH16 into *A. nidulans*

7.2.3 Identification of transformed strains with probable single copies of the vector

7.2.4 Identification of Type I integrants by Southern analysis

7.2.5 Expression of the *Aspergillus* and *Penicillium* PGK promoters in *A. nidulans*

7.3 Summary

### Chapter 8: Expression of the IPNS gene under the control of the PGK promoter in a *P. chrysogenum* fermentation

8.1 Construction of the fusion of the PGK promoter to the IPNS coding region

8.1.1 Stage 1 removal of the NcoI site in PC3.4 and construction of a second NcoI site by site directed mutagenesis

8.1.2 Stage 2 Removal of the NcoI sites 3’ to the IPNS coding region

8.1.3 Stage 3 Replacement of the IPNS gene promoter with the promoter from the PGK gene

8.1.4 Stage 4 Replacement of the 3’ sequences of the IPNS gene

8.1.5 Stage 5 The transfer of the PGK promoter–IPNS gene fusion to the plasmid pPOL20

8.2 Transformation of the PGK promoter–IPNS fusion vector pPIO1 into *P. chrysogenum* strain N.R.R.L 1951
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3</td>
<td>Analysis of the transformed strains</td>
<td>138</td>
</tr>
<tr>
<td>8.4</td>
<td>Use of the transformed strain IC38 in fermentation experiments</td>
<td>139</td>
</tr>
<tr>
<td>8.4.1</td>
<td>Penicillin titre</td>
<td>139</td>
</tr>
<tr>
<td>8.4.2</td>
<td>Determination of IPNS enzyme activity</td>
<td>140</td>
</tr>
<tr>
<td>8.4.3</td>
<td>Investigation of the IPNS assay</td>
<td>141</td>
</tr>
<tr>
<td>8.4.4</td>
<td>Comparison of the mRNA abundance of the fusion gene and the native IPNS gene</td>
<td>142</td>
</tr>
<tr>
<td>8.4.5</td>
<td>Stability of the transformed strain</td>
<td>144</td>
</tr>
<tr>
<td>8.5</td>
<td>Summary</td>
<td>144</td>
</tr>
<tr>
<td><strong>Chapter 9: Discussion</strong></td>
<td></td>
<td>146</td>
</tr>
<tr>
<td>9.1</td>
<td>Analysis of filamentous fungal genes encoding glycolytic enzymes</td>
<td>147</td>
</tr>
<tr>
<td>9.2</td>
<td>Isolation of the <em>P. chrysogenum</em> PGK gene</td>
<td>150</td>
</tr>
<tr>
<td>9.3</td>
<td>Sequence analysis of the PGK promoter</td>
<td>150</td>
</tr>
<tr>
<td>9.4</td>
<td>The sequence analysis of the PGK terminator region</td>
<td>152</td>
</tr>
<tr>
<td>9.5</td>
<td>Expression of the PGK promoter</td>
<td>153</td>
</tr>
<tr>
<td>9.5.1</td>
<td>Modulation of the <em>P. chrysogenum</em> PGK promoter by growth on different carbon sources: comparison at a fixed point in growth</td>
<td>153</td>
</tr>
<tr>
<td>9.5.2</td>
<td>Modulation of the <em>P. chrysogenum</em> PGK promoter in the growth cycle and by carbon source</td>
<td>154</td>
</tr>
<tr>
<td>9.5.3</td>
<td>Modulation of the <em>A. nidulans</em> PGK promoter in the growth cycle and by growth on different carbon sources</td>
<td>155</td>
</tr>
<tr>
<td>9.5.4</td>
<td>Heterologous expression of the <em>P. chrysogenum</em> PGK promoter in <em>A. nidulans</em></td>
<td>155</td>
</tr>
<tr>
<td>9.6</td>
<td>Use of the <em>P. chrysogenum</em> PGK promoter to drive the expression of the IPNS gene of <em>P. chrysogenum</em></td>
<td>158</td>
</tr>
<tr>
<td>9.7</td>
<td>Summary and future work</td>
<td>161</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1 Gene expression in eukaryotic cells

The potential exists in eukaryotic cells for the control of gene expression to occur at many stages from the gene to the final protein product. The general mechanisms by which gene expression is controlled are thought to be universal among eukaryotes since the molecular basis for transcription and translation is very similar in many species. This discussion is therefore based on data obtained from a wide range of organisms.

Chromatin can exist in an inactive state in which transcription does not occur or as active chromatin in which genes may be transcribed. The control of the transition between these two states may affect gene expression (reviewed by Latchman 1990; Grunstein 1990). Inactive chromatin is associated with nucleosomes and arranged in higher order structures. It has decreased sensitivity to endonucleases such as DNaseI and a higher degree of DNA methylation. Active chromatin in which transcription may occur remains associated with nucleosomes but not organised into the higher order structures observed in inactive chromatin. Some depletion of the histone H1 which is thought to be important in maintaining those higher order structures is evident in active chromatin, the DNA is more sensitive to DNase I and it is less heavily methylated. Modifications of histones by acetylation and perhaps the binding of ubiquitin are also associated with active chromatin and may promote the unfolding of chromatin to allow the binding of transcription factors.

Two high mobility group, proteins, HMG14 and 17 are associated with active chromatin in higher eukaryotes. They are responsible for the DNase sensitivity patterns of specific tissues, which reflects different patterns of transcription in those tissues, and therefore these proteins may be involved in maintaining an open chromatin structure which allows transcription to occur (Weisbrod and Weintraub 1979). Although DNA associated with nucleosomes may be
transcribed, nucleosomes or the presence of histone H1 alone repress transcriptional initiation (Grunstein 1990). Thus it is envisaged that the initiation site must be free of nucleosomes before transcription factors and RNA polymerase II can bind to the promoter to form the open transcription complex and start transcription. Several general activator proteins which bind to S. cerevisiae promoters affect chromatin structure and therefore may control gene expression in yeast at this level. One of these activators is the general regulatory factor GRF2 which may prevent nucleosomes from binding to the upstream activation sequence of the divergent promoter of GAL1-GAL10 (Chasman et al. 1990).

Enhancers, DNA sequences which regulate DNA transcription upon binding protein factors, have been shown to be nucleosome free and hypersensitive to DNaseI (Gross and Garrard 1988). This suggests that the proteins binding to enhancer sequences may affect chromatin structure or that the DNA sequence itself prevents nucleosome binding.

Another mechanism to control gene expression is modification of the DNA molecule by methylation which may effect the control of gene expression (reviewed by Cedar 1988). Experiments in which methylated and non-methylated DNA was injected into fibroblasts showed that unmethylated DNA was transcribed at a basal level but that methylated DNA was not and furthermore was resistant to DNase degradation. This suggests that methylation of DNA is associated with a general mechanism to repress transcription which acts on chromatin structure. Specific repression of transcription by methylation of DNA is indicated by evidence that methylation may hinder the binding of activating proteins to promoter regions. The in vivo pattern of interactions of transcription factors with the liver specific tyrosine aminotransferase is affected by methylation of the gene (Cedar 1988). Methylation of the binding site of transcription factors E2F and SPl affected the binding of the factors but this is not true for SP1 (reviewed by Dynan 1989).

Transcription of active chromatin is regarded as the major control point of gene expression in many cases and is carried out by one of three RNA polymerases. RNA polymerase
I transcribes rRNA genes in the nucleolus, RNA polymerase II transcribes genes for proteins, and RNA polymerase III, tRNA and snRNA genes (Lewin 1987). This discussion is limited to the expression of polymerase II genes though similar principles apply to the genes transcribed by the other two RNA polymerases.

A gene transcribed by RNA polymerase II has a promoter region encompassing a series of short sequence elements to which regulatory proteins bind to control the rate of transcription of the gene. The point of initiation of transcription can be at any nucleotide but the preference is for adenyl nucleotides (Baker and Ziff 1981), and many genes have multiple transcriptional start points. Transcription of a gene may continue up to 1kb after the translational stop codon and there may also be many transcriptional stop points. Searches of the 3' ends of 100 eukaryotic genes revealed in 66 cases a consensus (PyGTGTPyPy) which could be a termination signal (Maclauchlan et al. 1985).

The primary transcript contains a leader sequence, a coding region which is interrupted by introns between 50nt to 10,000nt in size and a 3' untranslated region. Leader sequences of the RNA transcripts are 40-80 nucleotides in length.

Following transcription the primary transcript is processed to yield the mature mRNA. The first nucleotide of the transcript is capped at the 5' end by a modified guanosine residue. The 3' end of the transcript is cleaved 10 to 30 nucleotides 3' to the polyadenylation signal which is highly conserved (AAUAAA) and a polyadenosine (poly-A) tail is added to the new 3' end (Proudfoot and Brownlee 1976). Introns are removed by a splicing reaction. The nucleotide sequences at the junctions between introns and exons are conserved (Breathnach and Chambon 1980), and an internal consensus exists for formation of the lariat intermediate in the splicing reaction (Keller and Noon 1984).

The "core" of promoters of genes transcribed by RNA polymerase II extends about 200bp upstream from the transcriptional start (Montague 1987). The elements discussed below together with the transcription factors which bind to them are listed in Table 1.
Table 1  Some eukaryotic promoter motifs and the factors which bind to them

<table>
<thead>
<tr>
<th>Element</th>
<th>Position</th>
<th>Consensus</th>
<th>Factors which bind</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>core promoter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TATA</td>
<td>-30- -40</td>
<td>TATAAAA</td>
<td>TFIID</td>
<td>Breathnach and Chambon 1980</td>
</tr>
<tr>
<td>CAAT</td>
<td>-75 to -80</td>
<td>GGCCATCT</td>
<td>CTF, NFy, CBP</td>
<td>Breathnach and Chambon 1980</td>
</tr>
<tr>
<td>GC box</td>
<td>variable</td>
<td>GGGCGG</td>
<td>SP1</td>
<td>Dorn et al. 1987.</td>
</tr>
<tr>
<td>specialised elements at generally &gt; -100</td>
<td></td>
<td></td>
<td></td>
<td>Dynan and Tijan 1985</td>
</tr>
<tr>
<td>CAAC box</td>
<td></td>
<td>GCCACACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat shock</td>
<td></td>
<td>C--GAA--TTC--G</td>
<td>HSTF</td>
<td>Pelham 1985</td>
</tr>
<tr>
<td>MRE</td>
<td></td>
<td>CCTTGCAGCCCG</td>
<td>several factors</td>
<td>Stuart et al. 1984</td>
</tr>
<tr>
<td>Octamer</td>
<td></td>
<td>ATGCAAT</td>
<td>OCT and others</td>
<td>Rosales et al. 1987</td>
</tr>
<tr>
<td>CRE</td>
<td></td>
<td>TGACTCA</td>
<td>CREB</td>
<td>Montminy et al. 1986</td>
</tr>
<tr>
<td>UASg</td>
<td></td>
<td>CGGAGGACAGCTCCCG</td>
<td>GAL4</td>
<td>Johnston 1987 (review)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGACTC</td>
<td>GCN4</td>
<td>Fink 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC box</td>
<td></td>
<td>AATCCGTGACCC</td>
<td>RAPI</td>
<td>Chambers et al. 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC C A A C TT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The TATA motif is commonly found in eukaryotic promoters (Breathnach and Chambon 1980). This element is located about 30 bases (-30) from the transcriptional initiation site (+1) in most genes, excepting some highly expressed "housekeeping" genes which function in all cells in multicellular organisms (Nussinov et al. 1986). The TATA motif is involved in positioning of the transcriptional start point and mutations within it decrease transcription levels (Myers et al. 1986). Deletion of the TATA motif brings heterogeneity to the 5' ends of the mRNAs transcribed and deletion of sequences 3' to the motif moves the initiation site a corresponding distance downstream (Grosschel and Birnsteil 1980).

Four general transcription factors have been shown to interact at this region of the eukaryotic promoter. One of these, TFIID binds specifically to the TATA motif (Sawadago and Roeder 1985). Proteins which bind specialised promoter elements found upstream from the TATA motif interact with this complex and are described below.

Two further motifs are commonly found in the core of eukaryotic promoters. The CAAT motif is at -75 to -80 (Benoist et al. 1980) and its mutation results in a decrease in transcriptional efficiency (Myers et al. 1986).

The GC motif which is common in the promoters of housekeeping genes is found at various distances from the transcriptional initiation site, in either orientation and is often repeated. Mutation within the GC motif decreases transcription levels (Dynan and Tijan 1983).

More specialised elements which coordinate the expression of sets of genes under different conditions, for example in specific tissues or at specific times in development or in response to certain environmental stimuli occur further 5' to the transcriptional start point. The promoters of the β-globin gene family contain an essential sequence called the CAAC box found upstream from the CAAT box (Myers et al. 1986). In Drosophila development is controlled by the binding of regulator proteins containing the homeodomain to upstream sequences found in groups of genes. Two of these regulators Ftz (fushi tarazu) and engrailed bind to the sequence -TCAATTAAATGA- (Jaynes and
O’Farrell 1988). Examples of elements responsible for coordination of a response to environmental stress are the heat shock element (HSE) and the metal response element (MRE). The HSE (Pelham 1985) binds the heat shock factor and enables transcription to occur in cells in which general transcription is reduced as a result of heat shock or other stress. The MRE confers an increase in expression in response to heavy metals on genes which encode proteins for their detoxification. It is repeated twice in the human metallothionein-IIA gene promoter at −40 to −50 and −140 to −150 nucleotides from the transcriptional start site (Karin et al. 1984).

Enhancers are found in higher eukaryotes both 5’ or 3’ to their target genes and appear to coordinate tissue specific and developmental gene expression (reviewed in Hatzopoulos et al. 1988). Unlike promoter elements enhancers can exert their influence over large distances both 5’ or 3’ to the promoter on which they act, for example, the enhancer found in the second intron of the rearranged immunoglobulin μ heavy chain gene (Gillies et al. 1983, Banerji et al. 1983). There is evidence that enhancers act by binding transcription factors (Takahashi et al. 1986) and that they are as efficient when they are topologically separated from the promoter (Plon et al. 1986) or even on a different DNA molecule (Muller and Schaffner 1990). Such evidence supports a model for their action in which transcription factors which have bound to an enhancer interact either with specific factors bound to the promoter region or with general transcription factors bringing together the enhancer and the initiation site with consequent looping out of the intervening DNA. Certain upstream elements of promoters themselves can exhibit enhancer characteristics in a different situation (Serfling et al., 1985). The MRE motif described above exhibits enhancer-like characteristics when placed in a heterologous gene expression system (Karin et al. 1984).

The upstream activation sequences (U.A.S) of yeast promoters have some enhancer characteristics, such as ability to act at a distance in an orientation independent manner, however they do not act if placed 3’ to the target gene (Guarente and Hoar 1985).
Much progress has been made recently in the study of both general and specific transcriptional activator proteins which bind to promoter elements and regulate gene transcription. They share common motifs in their DNA binding regions and transcription activation regions. The DNA binding domain often contains a zinc finger motif(s) or a helix-turn-helix motif. The activation regions are often rich in acidic amino acids or rich in glutamine or proline residues (Mitchell and Tijan 1989). Some activators contain both a DNA binding domain and an activating domain and some have either one or the other. Transcriptional activator proteins are often only active as dimers. Several factors binding to a promoter region have a synergistic effect (Carey et al. 1990).

More than one transcription factor may recognise the same site, for example several factors CTF, CBP and NF-Y bind to the CAAT motif (Dorn et al. 1987). The octamer motif which is found in many types of cells is also recognised by several transcription factors (Rosales et al. 1987).

Transcription factors interact with the transcription initiation complex but not necessarily directly and may require the presence of further factors (reviewed by Ptashne 1990). For example the octamer motif binding factor OCT-1 requires the presence of a second activator such as VP16 (Tanaka et al. 1988) and the factors Ela (Martin et al. 1990), SPI and CTF require second unidentified factors (Pugh and Tijan 1990). The requirement of some transcriptional activators for coactivators may be a general feature of transcriptional activation. An example of a transcription factor which may act directly is GCN4 which was found to bind to purified Polymerase II protein (Brandl and Struhl 1989).

Control of the activity of transcription factors can occur indirectly in the expression of genes encoding the factors thus regulating the concentration of each factor or by direct regulation of the activity of the factor by protein modification (Reviewed in Latchman 1990). The expression of the gene encoding the factor C/EBP is regulated at transcription in a tissue specific manner (Xathopoulous et al. 1989). Several transcription factors, for example the homeodomain transcription factors in
Drosophila regulate their own transcription (Serfling 1989). The expression of the yeast protein GCN4 is regulated at translation in response to amino acid starvation (see below). Modifications to transcription factors which may affect their activity include phosphorylation and glycosylation. There are several examples of factors which are phosphorylated, for example GAL4 (Mylin et al. 1989). The phosphorylation of CREB protein increases its ability to activate transcription (Yamamoto et al. 1988). The SP1 factor has been shown to be phosphorylated on binding DNA by a DNA dependent kinase (Jackson et al. 1990) and is also glycosylated (Jackson and Tijan 1988). Some transcription factors are inactive until they bind the appropriate inducer for example the S. cerevisiae factor ACE1 which binds to the metallothioneine gene promoter is activated by binding copper when there are increased levels of copper in the cell (Furst et al. 1988).

Post transcriptional control of gene expression occurs in the processing of the primary RNA transcript (reviewed in Proudfoot and Whitelaw 1988). In higher eukaryotic genes alternative splicing out of introns can produce different proteins (Leff et al. 1986). There is also alternative use of poly-adenylation signals which can result in proteins of different lengths being produced when the signal is in the coding region of the transcript, for example, the mRNA for the immunoglobulin μ constant region (Early et al. 1980).

In the cytoplasm, control of gene expression can occur by selective degradation or stabilisation of mature mRNA which is controlled by signals in the 3' end of the mRNA (reviewed by Latchman 1990).

Translation begins at the first AUG codon in the mRNA in 95% of cases. The sequence enviroment around the translational start codon is conserved, especially at the third base upstream where an A or G is preferred (Kozak 1984). There are examples of control of gene expression occuring at translation, particularly when a rapid response is needed, for example the increased expression of the yeast protein GCN4 in amino acid starvation. When amino acids are abundant the translation of several short open reading frames immediately upstream of the translational start of the GCN4 gene prevents translation of the gene. When amino
acids are scarce the short reading frames are not translated and the GCN4 open reading frame translated (reviewed in Fink 1986). This mechanism is reminiscent of attenuation in the E. coli trp operon.

A final consideration concerns codon usage and the relative abundance of tRNAs. Different species show different bias in codon usage as do different genes within a species (Maruyama et al. 1986). Populations of tRNA species vary in relative concentration and so the presence of rare codons which are recognised by a relatively small population of tRNAs may slow translation of an mRNA.

In summary, expression of eukaryotic genes is controlled at many levels from changes in chromatin structure to form active chromatin capable of being transcribed to the final translation of mRNA to protein. The major point of control is at transcription where the binding of positively acting protein transcription factors to the promoter region of a gene regulate the rate of transcription. Much progress has recently been made in studying transcription factors and their interactions with the initiation complex in the nucleus.

The promoter region is a series of modular elements which bind transcription factors. Enhancers which regulate gene expression from a distance also have a modular structure and also bind regulatory proteins.

Modifications of the transcript occur in the nucleus, such as the addition of a 5' cap, a 3' poly-A tail, and the removal of introns. The regulation of these processes and of the stability of the mature mRNA in the cytoplasm can also affect gene expression.

Gene expression can also be modulated at the level of translation. In situations requiring a rapid response such as starvation there is evidence for the change in gene expression being at the level of translational efficiency. Codon usage is not random and may also be a point at which gene expression is controlled, as suggested for prokaryotes, highly expressed genes having codons which use abundant tRNAs.
1.2 Gene expression in filamentous fungi

Fungi are simple essentially unicellular eukaryotes and share many of the features of higher eukaryote gene expression. A great deal of classical biochemical genetics has been done with filamentous fungi and has revealed complex interacting systems of gene expression and regulation. The recent development of transformation procedures (reviewed by Fincham 1989) has made it possible to apply molecular genetic techniques to filamentous fungi. Transforming DNA is integrated into the genome and the site of integration can be designated to some extent by inclusion of fungal DNA on the transforming plasmid when a proportion of transformants will contain the plasmid integrated at the homologous gene. There are no substantiated reports of autonomously replicating plasmids in filamentous fungi.

Many filamentous fungi are used industrially, for example Penicillium chrysogenum for the production of penicillin, Aspergillus niger for citric acid, and Cephalosporium acremonium for cephalosporin production. Study of the gene expression in these organisms is potentially important to increase the yields of the products. Aspergillus nidulans, which is genetically well characterised is a good model for these industrial fungi and has been used in vector development.

The genome of filamentous fungi contains very little repeated sequence unlike that of higher eukaryotes. Approximately 90% of the Neurospora crassa genome is unique sequence and the greater part of the repeated sequence encodes ribosomal RNA (Krumlauf and Marzluff 1980). In Aspergillus nidulans 98% of the DNA sequence is unique and the repeated sequences are clustered in the genome suggesting that they also encode ribosomal RNA (Timberlake 1978). In filamentous fungi genes for metabolically related functions are usually unlinked but sometimes they are clustered, for example the qa and gut gene clusters encoding enzymes for quinic acid degradation of N. crassa (Giles et al. 1985), and A. nidulans, (Hawkins et al. 1988). Other examples of gene clusters include, in A. nidulans, the alc genes encoding enzymes of alcohol utilisation (Gwynne et al. 1987), the prn genes for proline breakdown (Arst 1984),
the nitrate gene cluster (Cove, 1979) together with the developmental gene cluster spoCl (Gwynne et al. 1984). In contrast some apparent gene clusters represent a region which produces a single transcript encoding multifunctional polypeptides resulting in proteins with several enzyme activities. The first example of this was the HIS-4 gene in yeast (Bigelis et al. 1977). In filamentous fungi examples of multifunctional polypeptides are the his-3 gene of *N. crassa* and the aromA gene of *A. nidulans* (Ahmed 1968; Giles 1967). The aromA gene may have evolved by multiple fusions of five genes which are not clustered in prokaryotes (Hawkins 1987).

Most studies of gene expression in filamentous fungi have been concerned with transcriptional control of genes transcribed by RNA polymerase II, though some work on the promoters of Polymerase I transcribed genes of *N. crassa* has shown them to be very like those of other eukaryotes (Tyler 1990). Discussion in this section is limited to the structure, transcription, and translation of RNA polymerase II transcribed genes.

The gross structure of filamentous fungal genes is the same as that of genes in higher eukaryotic cells. A series of modular sequence elements which may bind transcription factors form the promoter region. The 5′ untranslated leader sequences are most often about 100 nucleotides long and may occasionally contain introns (Gurr et al. 1987; Punt et al. 1988) The coding region is interrupted by small introns which are usually less than 150nt in length and there is a 3′ untranslated region containing signals for poly-adenylation and termination. Few studies have focussed on RNA processing and it is assumed that there is no major difference in this between filamentous fungi and higher eukaryotes.

Comparisons of DNA sequence have shown elements in filamentous fungal promoters which are also present in the promoters of higher eukaryotic genes. However few functional studies of promoters by deliberate modification are yet reported and it is therefore difficult to determine the significance of promoter sequences.

A TATA motif with the eukaryotic consensus has been identified in approximately one fifth of the promoters of
filamentous fungal genes at -30 to -60 bases upstream from the transcription start site +1 (reviewed in Gurr et al. 1987), for example in the promoters of the N. crassa am gene (Kinnaird and Fincham 1983) and the promoter of the tpi gene of A. nidulans (Mcknight et al. 1986). About one fifth show no TATA like sequence at all, for example the his-3 gene of N. crassa (Legerton and Yanofsky 1985). The remainder of the promoters contain an AT rich sequence in this region as in the promoters of the trpC and the PGK genes of A. nidulans (Hamer and Timberlake 1987, Clements and Roberts 1986). The promoters of the gdhA and aromA genes of A. nidulans have TATA like motifs outside this region (Gurr et al. 1987). The deletion of the TATA like motif in the promoter of the A. nidulans developmental gene abaA had no effect on the expression levels of that gene or the accuracy of initiation (Adams and Timberlake 1990). In yeast promoters TATA sequences are not necessarily located between -30 to -60 but may occur at many positions and in more than one copy (Brown and Lithgow 1987). Deletion of these sequences in the PGK gene of yeast also appears not to affect transcription (Ogden et al. 1986). The TATA region of yeast however has been demonstrated to bind the general transcription factor TFIID (Hahn et al. 1989).

Highly expressed genes of yeast often contain a 8–12 bp region rich in pyrimidines (the CT rich region) immediately 5' to the transcriptional start which may also control initiation (Dobson et al. 1982). Such pyrimidine rich regions of varied length and CT content occur in approximately one third of filamentous fungal promoters close to the transcriptional start point (Gurr et al. 1987). In the promoter of the oliC gene of A. nidulans the pyrimidine rich sequence extends over 100 nucleotides but deletions within it had little effect (G. Turner, informal communication). Deletion of the pyrimidine rich region in the promoter of the A. nidulans gene gpd, resulted in an 80% decrease in transcription and heterogeneous start sites (Punt et al. 1990). In the trpC promoter of A. nidulans the deletion of the pyrimidine rich sequence together with the TATA like motif resulted in alteration of the transcriptional initiation sites but no decrease in transcription (Hamer and Timberlake 1987).
Searches for a CAAT motif has revealed that 22 of 45 filamentous fungal genes examined had a similar element to the CAAT motif in the region -120 to -60 (Gurr et al. 1987). Examples are the PGK and amdS genes of A. nidulans (Clements and Roberts 1986; Corrick et al. 1987). No deletions of this sequence have been reported.

Elements involved in coordinate regulation of genes under common control are evident at -500 to -100, 5' to the transcriptional start point. In the qa and gut gene clusters a 16 nucleotide element is found in the promoter of each gene in the cluster (Hawkins et al. 1988). In N. crassa this sequence has been shown to bind to the activator protein qa-IF (Baum et al. 1987). A repressor gene is also found in both these systems but there is no evidence to show that the repressor protein binds to promoter DNA and it may interact directly with the activator protein (Giles et al. 1985). The amdS gene of A. nidulans is subject to activation by several regulatory proteins, encoded by the amdR, amdA, facB and areA genes (Hynes and Davies 1986). The sites of interaction of these proteins with the amdS promoter have been defined by mutation and comparison of 5' sequences of co-regulated genes, and the factors appear to bind independently to the amdS promoter (Hynes et al. 1988). The alc genes of A. nidulans, alcA and aldA are activated by the alcR regulatory gene product. Sequence analysis of the promoters of the two genes has identified a region which may, by comparison to the position of the above promoter elements, be the binding site for the alcR protein (Gwynne et al. 1987, Pickett et al. 1987).

Positively acting elements have been identified far 5' to the promoter of the am gene of N. crassa compared to the position of the above promoter elements. These elements at -2.1kb and -1.4kb have been identified as binding sites for positive regulatory proteins by deletion and DNA binding studies. The positioning of these elements suggests that they may have enhancer like qualities (Frederick and Kinsey 1990). However elements with all the characteristics of enhancers in higher eukaryotes have yet to be discovered in filamentous fungi.

Several of the genes encoding regulatory transcription factors in A. nidulans and N. crassa have been cloned (see
Table 2). The genes nit-2, cpc-1, areA (Kudla et al. 1990) qutA, qaIF, and amdR encode proteins which contain characteristic "zinc finger" DNA binding domains. However only the transcription factors qa-IF (Baum et al., 1987), cpc-1 (Ebbole et al. 1991) and Nit-2 (Fu and Marzluf, 1987) are known to bind DNA in vitro. Mutations have been found in the genes nit-2 and areA in the regions which encode the zinc finger domains of the transcription factors. These mutations change the specificity of activation of the gene products, thereby showing that the zinc finger may bind to a DNA target (Fu and Marzluf 1990; Kudla et al. 1990).

Studies with different copy numbers of portions of the A. nidulans amds promoter have been used to identify the binding sites of the transcription factors amdR and facB. Increasing the copy number of a 108bp segment of the promoter titrated the amdR and facB activation factors and prevented transcription of the other genes requiring those factors (Hynes et al. 1988). The binding site of the two activator proteins was therefore on the 108bp fragment of the amds promoter. The titration effect could be reversed for both activators by increasing the copy number of the gene encoding the activator in the mutants with increased numbers of the 108bp segment (Adrianopoulos and Hynes 1990; Katz and Hynes 1989).

The regulation of transcription of the genes encoding the above regulatory factors falls into two classes. In one class which includes the amdR and nirA genes the mRNA transcript is present at a constitutive low level, which suggests that the concentration or the activity of the protein factor encoded by the mRNA is controlled post transcriptionally. In the other class, which includes the genes gutA, qa-IF, facB, alcR, cpc-1, nit-2 and creA, transcription of the genes is induced under the appropriate conditions. In all cases the degree of induction is not great, about 5-10 fold maximum, except for qa-IF which is induced 40-50 fold (Giles et al. 1985).

The expression of the genes gutA, qa-IF, facB, alcR and cpc-1 may also be regulated autogenously by the binding of the gene product to its own promoter, thereby activating transcription. The recognition sequences for binding of the activators qa-IF and cpc-1 and the putative recognition
### TABLE 2: TRANSCRIPTION ACTIVATORS OF FILAMENTOUS FUNGI

<table>
<thead>
<tr>
<th>Name</th>
<th>Target Pathway</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus nidulans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alcR</td>
<td>alcohol breakdown</td>
<td>Lockington et al. 1987</td>
</tr>
<tr>
<td>amdR</td>
<td>lactams and omega amino acid breakdown</td>
<td>Adrianopoulos and Hynes 1990</td>
</tr>
<tr>
<td>areA</td>
<td>nitrogen regulation</td>
<td>Caddick et al. 1986</td>
</tr>
<tr>
<td>creA</td>
<td>carbon catabolite repression</td>
<td>Dowzer and Kelly 1989</td>
</tr>
<tr>
<td>facB</td>
<td>acetate utilisation</td>
<td>Katz and Hynes 1989</td>
</tr>
<tr>
<td>nirA</td>
<td>activator of nitrate and nitrite reductases</td>
<td>Burger et al. 1991</td>
</tr>
<tr>
<td>qutA</td>
<td>quinic acid catabolism</td>
<td>Beri et al. 1987</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nit-2</td>
<td>nitrogen regulation</td>
<td>Fu and Mazluf 1990</td>
</tr>
<tr>
<td>cpc-1</td>
<td>general amino acid control</td>
<td>Paluh et al. 1988</td>
</tr>
<tr>
<td>qa-IF</td>
<td>quinic acid breakdown</td>
<td>Baum et al. 1987</td>
</tr>
</tbody>
</table>
sequence for qutA are found in the promoters of the corresponding genes (Beri et al. 1987; Paluh 1988). The functional activators alcR and facB are required for induction of their respective genes (Lockington et al. 1987; Katz and Hynes 1989). Autogenous regulation of the genes described above cannot be the only mechanism by which activator function is controlled, since other post-transcriptional interactions must prevent activation of gene expression under non-inducing conditions. In essence autoregulation provides an amplification mechanism. Similar autoregulation of the expression of several eukaryotic transcription factors are documented and it may be a common regulatory mechanism (Serfling 1989).

The activity of the transcription factor cpc-1 is controlled by another factor, cpc-2. Mutation in the gene encoding cpc-2 appears to prevent the activation by cpc-1 of amino acid biosynthetic genes in response to starvation even though cpc-1 RNA is induced (Kruger et al. 1990). Therefore the activity of cpc-1 is regulated by other factors and the concentration of cpc-1 may also be autoregulated at transcription.

Filamentous fungal genes transcribed by RNA polymerase II show similarities with both yeast and higher eukaryotes. At the transcriptional start site, a few filamentous fungal genes have the consensus sequence PyAAG found in highly expressed yeast genes (Dobson et al. 1982). Two such filamentous fungal genes are argB and trpC (Upshall et al. 1986; Hamer and Timberlake 1987). Transcription continues for several hundred bases beyond the stop codon. A potential termination signal CATGGTCT similar to the consensus CCTGTCC proposed by McLauchlan et al. (1985) occurs downstream from the argB, gdhA, tpiA of Aspergillus nidulans and the acp-1, pl-1 genes of Neurospora crassa (Gurr et al. 1987). Very little analysis has been done on transcriptional termination in filamentous fungal genes.

Within the coding region intron/exon boundaries show close similarity between higher eukaryotes filamentous fungi and yeast (reviewed in Gurr et al. 1987). The 5' splice site (consensus GTANGT) shows conservation of the first two nucleotides of the intron. At the 3' splice site (consensus PyAG) the last two nucleotides of the intron are also
conserved between filamentous fungi, yeast and higher eukaryotes. The internal splicing signal in filamentous fungi although similar to that of yeast is more like that of higher eukaryotes in that it is less well conserved (consensus PyGCTAACN). The yeast signal is invariant and this may be the reason why problems have been encountered in expression of both filamentous fungal and higher eukaryotic genes in yeast due to the incorrect splicing of mRNAs transcribed from these genes. Filamentous fungi appear to be able to correctly splice higher eukaryotic mRNAs, for example the gene for human tissue plasminogen activator protein has been inserted into *Aspergillus nidulans*, the mRNA produced is correctly spliced and the active protein secreted (Upshall et al. 1987).

After the stop codon, known polyadenylation signals occur in a few genes, for example in *A. nidulans* 15 bases before the polyadenylation site of the tpi gene (Mcknight et al. 1986) and 16 bases before the major polyadenylation site of the PGK gene (Clements and Roberts 1986). In other genes AT rich sequences are often found in the 3’ region (Gurr et al. 1987).

At the translational start codon an adenine residue is conserved at position -3 in 83% of filamentous fungal genes (Gurr et al. 1987). The DNA consensus sequence for this region is TCACAATGGC (Ballance 1986). Translation begins at the first AUG in the mRNA in most of the cases which have been determined.

Codon usage in filamentous fungi is not random. Codon bias is not as great as in highly expressed yeast genes but it is more marked than that found in higher eukaryotes (Gurr et al. 1987). The degree of codon bias may affect the translation of an mRNA. Experiments were carried out on the am gene of *N. crassa* where rare codons were introduced into the coding region to replace more common ones thus decreasing the bias towards common codons. The resultant protein concentrations were 35% lower than that produced by translation of the wild type gene (Fincham et al. 1985). Filamentous fungi generally exhibit strong expression of genes from other fungal species. Most striking such heterologous genes are often normally regulated. For example the *A. nidulans* amdS gene has been transformed into *A. niger*
and it is controlled normally even though A. niger has no equivalent gene (Kelly and Hynes 1985). The \textit{\texttt{qu}}E gene of A. nidulans has been expressed in \textit{N. crassa} and is inducible by quinate. In this case the binding site for the activator protein is known and is very similar in the two species (Miett and Case 1990). The genes encoding acetyl coA synthetase from A. nidulans and \textit{N. crassa} are also expressed and regulated normally in the other species even though the promoters of the genes in the two organisms were very different (Connerton et al. 1990). The examples of heterologous expression within the filamentous ascomycete fungi indicates that the different species can recognise each other's transcription and translation signals.

Expression can also occur in fungi of a different group. The glucoamylase gene from A. niger will function in the basidiomycete \textit{Ustilago maydis} though it is not induced by starch as in A. niger (T. J. Smith et al. 1990).

To summarise, filamentous fungal genes are broadly similar in organisation to higher eukaryotic genes. Several elements involved in transcriptional control, transcript processing and translation are conserved between filamentous fungi and higher eukaryotes and higher eukaryotic genes appear to be expressed and processed correctly in these organisms. The filamentous fungi are therefore potentially very useful for the heterologous production of higher eukaryotic proteins since many of the problems encountered with use of yeast and prokaryotes do not exist.
1.3 Filamentous fungal genes encoding enzymes in the glycolytic and gluconeogenic pathways.

Several of the genes encoding enzymes in the glycolytic and gluconeogenic pathways have been cloned and sequenced, including those for triose phosphate isomerase (tpi) in A. nidulans (Mcknight et al. 1986), pyruvate kinase (pyk) in A. nidulans and A. niger (van der Graaff 1989) glyceraldehyde-3-phosphate dehydrogenase, gpd, (Punt et al. 1988), and phosphoglycerate kinase (PGK) in A. nidulans (Clements and Roberts 1985; 1986). During the course of the thesis, the cloning and sequence analysis of phosphoglycerate kinase (PGK) in P. chrysogenum (Van Solingen et al. 1988), Trichoderma viriadae (Goldman et al. 1990), and Trichoderma reesi (Vanhanen et al. 1989) have also been reported. A diagram of the glycolytic and gluconeogenic pathways is presented in Figure 1-1.

The promoters of the A. nidulans genes share some similarities in sequence motifs (Punt et al. 1988; Streatfield 1990). An 8bp consensus sequence (TG\textsuperscript{A}/TGGT\textsuperscript{G}/T\textsuperscript{C}) has been identified at variable distances 5' to the transcriptional start sites which is thought to be a binding site for a regulator of glycolytic gene expression. The genes also all have a pyrimidine rich region 5' to the transcriptional start point. Functional analysis of the gpd and PGK promoters has been reported during the course of my work and these studies are reviewed in the discussion. The PGK gene in A. nidulans is highly expressed constitutively (Clements and Roberts 1986) and since my project is concerned with PGK gene expression in Penicillium chrysogenum information on phosphoglycerate kinase gene expression is now reviewed in greater detail.
Figure 1-1: The glycolytic and gluconeogenic pathways of carbon metabolism

The enzymes catalysing the numbered steps in the pathway are listed:

<table>
<thead>
<tr>
<th>Number</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Triosephosphate isomerase</td>
</tr>
<tr>
<td>2</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>3</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>4</td>
<td>Pyruvate kinase</td>
</tr>
</tbody>
</table>
GLYCOLYSIS

- Glucose → Glucose-6-phosphate
- Glucose-6-phosphate → Fructose-6-phosphate
- Fructose-6-phosphate → Fructose-1,6-bisphosphate
- Fructose-1,6-bisphosphate → Glycerol
- Glycerol → Di-hydroxyacetone phosphate
- Di-hydroxyacetone phosphate → Glyceraldehyde 3-phosphate
- Glyceraldehyde 3-phosphate → 1,3-Phosphoglycerate
- 1,3-Phosphoglycerate → 3-Phosphoglycerate
- 3-Phosphoglycerate → 2-Phosphoglycerate
- 2-Phosphoglycerate → Phosphoenolpyruvate
- Phosphoenolpyruvate → Pyruvate
- Pyruvate → Acetyl CoA
- Acetyl CoA → Quinone acid
- Quinone acid → Acetate
The Phosphoglycerate kinase gene and enzyme

The enzyme Phosphoglycerate kinase (PGK) catalyses the interconversion of 1,3-phosphoglycerate and 3-phosphoglycerate in glycolysis and gluconeogenesis. The enzyme is monomeric with a molecular weight of about 45,000. The gene (PGK) encoding this enzyme has been cloned from several organisms including yeast (Dobson et al. 1982), A. nidulans (Clements and Roberts 1985), P. chrysogenum (Koekman et al. 1986) and Trichoderma reesi (Vanhanen et al. 1989). The amino acid sequence has been derived from the DNA sequence of the yeast and Aspergillus genes and is also known for the human and horse enzymes (Huang et al. 1980, Banks et al. 1979). Good three dimensional models exist for the yeast and horse proteins which show marked structural identity between the two species (Watson et al. 1982, Banks et al. 1979). Comparison of the protein sequences shows that there is high conservation of amino acid sequence between species, for example the A. nidulans derived sequence is 68% homologous to the yeast sequence and 64% homologous to the mammalian sequence (Clements and Roberts 1986). Studies in yeast and A. nidulans show that the gene is expressed at a high level (Holland and Holland 1978, Clements and Roberts 1986). Its expression is subject to some induction by fermentative carbon source in yeast (Stanway et al. 1987), but in A. nidulans slight induction has been observed on gluconeogenic carbon sources such as quinic acid and acetate (Clements 1986).

The yeast PGK gene encodes 416 amino acids in an uninterrupted open reading frame. Transcription begins 36 bases upstream from the start codon and continues 86 to 93 bases beyond the translational stop. No polyadenylation signal motifs have been identified 3' to the coding region. Codon bias is marked, with only 25 of 61 possible codons being used for 95% of residues. Other highly expressed yeast genes also exhibit codon bias (Dobson et al. 1982, Hitzman et al. 1982). The yeast promoter contains a pyrimidine rich region between positions -34 to -16, a CAAT motif at -93 and a TATA motif at -118 5' to the transcriptional start point, +1 (Hitzman et al. 1982; Dobson et al. 1982). The TATA motif and CAAT sequences have
found to be dispensible for PGK gene expression (Ogden 1986). There is some homology to 18S rRNA in the region of the transcriptional start point.

A yeast upstream activator sequence (UAS) is located at -497 to -366 and deletion of this sequence leads to a low level of transcription (Ogden et al. 1986). Further studies have shown that the UAS consists of two domains (Stanway et al. 1987). The region -437 to -422 encompasses an activator element (the Activator Core box) and the region -431 to -391 contains three direct repeats of the element 5'CTTCC 3' which have been shown by deletion analysis to be necessary for the high level of expression. DNA binding studies have shown that interactions with transcription factor proteins occur in this region. The AC box binds to the activator/repressor protein RAPI which also has other possible binding sites in the 5' regions of the genes PYK, ENOL, ADH1, PDC1, TPI (Chambers et al. 1988). The binding of RAPI depends on the carbon source on which the yeast is cultured and may also depend on the RAPI phosphorylation state (Chambers et al. 1989; Tsang et al. 1990). The level of RAPI mRNA is not regulated by carbon source. The region -487 to -460 contains a modulator segment which interacts with a factor originally called the Y protein but now identified as the ARS (Autonomously Replicating Sequence) binding factor I (Stanway et al. 1987; Chambers et al. 1990).

The yeast 5' sequence also contains a heat shock consensus sequence between -336 and -323 (Piper et al. 1985). A series of deletions of the promoter upstream region were used to study the action of this element cloned in a plasmid. When UAS sequences were absent from the promoter and the heat shock element present, transcription was low but when the cells bearing this plasmid were subjected to increased temperatures, transcription increased 50-100 times. If however, the UAS was present with the heat shock element, transcription levels were high and maintained at about the same level at increased temperatures. The TATA and CAAT boxes were not necessary for transcription under these conditions. It is thought that the heat shock element promotes transcription during growth at increased temperatures and the UAS elements are inactive in these conditions (Piper et al. 1988).
Fusion of the PGK promoter of yeast to the coding regions of heterologous genes on high copy number plasmids does not produce the expected high protein levels of the native gene (50-80% cell protein) but only 1-2% cell protein (Mellor et al. 1985). The stability of the gene fusion mRNA was found to be unaffected, indicating that a defect in transcription was likely. Three fusions of a heterologous gene to the PGK gene which included 37, 236 or 1230 bases of the coding region were constructed and analysis of transcription showed that up to 237 bases of coding region were required for high level transcription (Ogden et al. 1987). The presence of this internal activator decreases the potential of yeast PGK for heterologous gene expression though even expression at the level of 1-2% of cell protein may be useful.

The *Aspergillus nidulans* PGK gene has been sequenced and encodes a protein of 421 amino acids (Clements and Roberts 1986). Analysis of the sequence has shown that two 57 base introns interrupt the coding region. These fall at the beginning and end of sequence postulated to code for a region of β-sheet in the yeast and mammalian protein. Transcription starts 32 bases 5' to the initiation codon and polyadenylation occurs 23, 83 and 115 bases 3' to the stop codon with the latter site preferred. Sequences located 5' to the first and the third of these sites which could form secondary structures in the RNA occur. The size of the PGK mRNA population is 1.4-1.5kb. Bias in codon usage is less marked than in the yeast PGK gene but a pyrimidine is preferred at the third position in 85% of residues. Examination of the promoter sequence identified a TATA like motif 32 bases 5' to the transcriptional initiation site and a CAAT like motif 80 bases from this site. The presence of TATA and CAAT motifs is relatively rare in filamentous fungal promoters and the roles of these two motifs in gene expression in filamentous fungi remain to be determined (Gurr et al. 1987). There is an agreement in 12 of 15 bases at position -42 to -28 with a sequence thought to have a role in transcription in the yeast gene between positions -34 to -20 (Dobson et al. 1982). A pyrimidine rich region which can have a role in positioning the transcriptional start point occurs between the TATA motif and the start of
transcription. Analysis of the A. nidulans promoter function done by S. Streatfield (1990) is discussed in Chapter 9.

The Penicillium chrysogenum PGK gene has been previously cloned (Koekman et al. 1986) and the coding region sequence was reported during the course of my work (van Solingen et al. 1988). The gene encodes a 416 amino acid protein. The available data shows that the deduced amino acid sequence has 81% homology to Aspergillus nidulans PGK and 64% homology to the yeast PGK protein. Two small introns interrupt the P. chrysogenum PGK coding region at the same positions as the 57 base pair introns in the A. nidulans gene but of 55 and 62 bases respectively. The P. chrysogenum PGK gene is expected to be highly expressed constitutively.
1.4 Penicillin biosynthesis

Penicillin is a β-lactam antibiotic which attacks the synthesis of bacterial cell wall peptidoglycans causing lysis and cell death. Penicillin is an important commercial product, both as an antibiotic and as a chemical feed stock for the manufacture of other lactam antibiotics. It is isolated as penicillin-G (benzyl penicillin) from the asexual fungus *P. chrysogenum* grown in batch fermentation.

The immediate precursor of penicillin-G is isopenicillin-N which is synthesised by a pathway common to several antibiotic producing species of fungi and bacteria such as *Streptomyces* sp, *Flavobacterium*, *Cephalosporium acremonium*, *Penicillium chrysogenum* and *A. nidulans* (Queener and Neuss 1982). Isopenicillin-N, can undergo an acyl exchange reaction with a hydrophobic donor to produce penicillin-G, or penicillin-V in *P. chrysogenum* or *A. nidulans* which produces penicillin at low levels. Alternatively, in *Cephalosporium* spp. and *Streptomyces* sp, isopenicillin-N can be isomerised to penicillin-N which is then converted to cephalosporins and cephemycins respectively. Studies of penicillin biosynthesis have been carried out in *Penicillium chrysogenum*, *Cephalosporium acremonium*, *Aspergillus nidulans* and *Streptomyces* spp.

**Strain improvement**

The *P. chrysogenum* strains used by industry have been improved for penicillin production over a period of 50 years and the most successful method of strain improvement is that of repeated cycles of random mutation and screening to select strains with high titre (Rowlands 1983). Relatively little effort is given to directed strain improvement. The large number of translocations and mutations induced in the industrial strains of *P. chrysogenum* makes recombination between strains difficult to achieve. However use of the parasexual cycle to obtain vegetative recombination between closely related strains has increased penicillin titre. Ball and his colleagues, (1973, 1976) introduced single step mutations into closely related strains to enable mitotic recombination between them in heterozygous diploid strains. The resulting strains had an increased penicillin titre and
the work coincidentally produced a rudimentary genetic map of *Penicillium chrysogenum*. The sexual cycle combined with mutation in *A. nidulans* has been used to increase penicillin titre in this species (Simpson and Caten 1977, Simpson and Caten 1980). Protoplast fusion has allowed interspecific breeding to produce novel antibiotics (Smith, Peberdy and Macdonald 1980).

Recombinant DNA methods have the potential to improve penicillin titre very specifically, bypassing both the problems of recombination in industrial strains and the nonspecific nature of random mutation for strain improvement. In *Cephalosporium acremonium* recombinant DNA techniques were used successfully to increase the copy number of the cefEF gene, which encodes a bifunctional enzyme deacetoxycephalosporin C synthetase/deacetylcephalosporin C synthetase which was rate limiting in cephalosporin biosynthesis in the industrial strain 394-4. A strain with two copies of the gene had a cephalosporin titre 15% higher than the strain 394-4 (Skatrud et al. 1989). The targets for genetic manipulation in *P. chrysogenum* are the genes for the biosynthesis of penicillin, and also any genes encoding factors which control the flux through the penicillin biosynthetic pathway and the primary metabolic pathways which provide the precursor molecules.

**The enzymes and reactions of penicillin biosynthesis**

The pathway of penicillin biosynthesis starts from the primary metabolites L-cysteine, L-valine, derived from intermediates of glycolysis and the tricarboxylic acid cycle, and α-amino adipic acid, which is an intermediate of lysine synthesis (Figure 1-2). A tripeptide, (1-α-amino adipyl)-L-cysteinyl-D-valine (ACV) is formed from L-cysteine, α-amino adipate and D-valine by the action of a multifunctional enzyme, ACV synthase. The tripeptide is then cyclised by Isopenicillin-N-synthase (IPNS) to form Isopenicillin-N (IPN). Hydrophobic penicillins such as penicillin-G produced by *P. chrysogenum* are then formed by a side chain exchange which may involve two steps, the first being removal of the amino adipate molecule from IPN to form 6-APA (6-amino-penicillanic acid) and the second being addition of the hydrophobic side chain, which is
Figure 1-2: The pathway of penicillin biosynthesis
The pathway was taken from Demain (1983)
The enzymes catalysing the numbered steps of the pathway are listed below:-
1  $\alpha$-aminoadipyl-cysteinyl-valine synthase (ACVS)
2  isopenicillin-N synthetase (IPNS)
3  acyl transferase (ACT)
H₂N(1)\(\text{CH-(CH₂)₃-COOH}\)  
L-α-amino adipic acid (AAD)

1

H₂N\(\text{CH-(CH₂)₃-CON}\)\(^\text{SH}\)  
δ(L-α-amino adipyl)L-cysteine

1

H₂N\(\text{CH-(CH₂)₃-CON}\)\(^\text{SH}\)\(^\text{CH₃}\)  
δ(L-α-amino adipyl)L-cysteinyl-D-valine (LLD-tripeptide)

2

Isopenicillin N  
cephalosporins and cephamycins

3

Phenylacetate

Phenylacetyl-CoA  
CoA + AAA

6-APA

Phenylacetyl-CoA

Benzylpenicillin
phenylacetate for penicillin-G. The reaction is catalysed by acyltransferase ACT (Alvarez et al. 1987, Nuesch et al. 1987).

The enzymes ACVS, IPNS and ACT have been purified from a variety of organisms and the genes encoding the enzymes identified and cloned (see below).

The ACV synthase (ACVS) activity was purified from A. nidulans and is a single polypeptide chain of 220 Kd which requires ATP, and magnesium ions for in vitro activity (Van Liempt et al. 1989). The enzyme IPNS has been purified from several sources and is a single 39kd polypeptide (in fungi) which acts essentially as a dehydrogenase, removing four hydrogen ions from the tripeptide. Activity of the enzyme requires ferrous ions, ascorbate and molecular oxygen in vitro (Nuesch et al. 1987). The acyl transferase was purified from P. chrysogenum and consists of two polypeptides of 29 and 11kd. The 29kd polypeptide has been shown to have the activity converting 6-APA (6-amino penicillanic acid) to penicillin-G (Alvarez et al. 1987, Barredo et al. 1989). Several different side chains may be exchanged with amino adipate depending on their availability.

Metabolic control of penicillin production in P. chrysogenum

The availability of precursors is one level at which the penicillin biosynthetic pathway is controlled. Valine concentrations are controlled by feedback inhibition of the first enzyme of the valine specific biosynthetic pathway by valine and mutants in which the sensitivity of this enzyme to valine is reduced produce more penicillin (Goulden and Chattarway 1969). Cysteine levels depend on sulphur metabolism. P. chrysogenum obtains its sulphur for cysteine by sulphate reduction (Segal & Johnson 1963).

The compound α-amino adipate is an intermediate of lysine metabolism and thus there is competition between penicillin and lysine biosynthetic pathways for amino adipate. The metabolic control of the lysine biosynthetic pathway may be expected to also affect the rate of penicillin biosynthesis. The lysine biosynthetic pathway is controlled by feedback inhibition of the first enzyme of this pathway, homocitrate synthase by lysine. Repression of homocitrate synthase leads to reduced levels of the
intermediate α-amino adipate and therefore reduced penicillin synthesis (Luengo et al. 1980). High levels of exogenous lysine also inhibit penicillin production and addition of α-amino adipate alleviates this inhibition (Somerson et al. 1961). Some high titre strains have mutations which make homocitrate synthase insensitive to repression by lysine (Jaklitsch et al. 1987). The aminoacid α-amino adipate is also available from the later steps of penicillin biosynthesis since it is not incorporated into the final penicillin-G molecule, and is released by ACT to be recycled (Martin and Aharonowitz 1983). The recycled α-amino adipate would increase the α-amino adipate pool for which the lysine biosynthetic pathway and penicillin biosynthetic pathway compete. There is evidence that α-amino adipate may, under some conditions, be the limiting factor in penicillin biosynthesis. The incorporation of radioactive valine into penicillin was stimulated when non growing cells were supplied with α-amino adipate but not stimulated if either valine or cysteine was supplied (Martin and Aharonowitz 1983).

Penicillin biosynthesis is subject to catabolite repression by both carbon source and nitrogen source (Martin and Demain 1980, Aharonowitz 1980, Sanchez et al. 1980). Repression due to glucose may not be mediated by cAMP, but may be due to an intermediate of glucose metabolism (Martin and Aharonowitz 1983).

It has been suggested that penicillin itself as an end product inhibits penicillin biosynthesis but the mechanism is not known (Gordee and Day 1972). It has been shown more recently that stationary phase cells supplied with exogenous penicillin responded by reducing the incorporation of labelled valine into the tripeptide ACV (Revilla et al. 1978). The efficiency of removal of the product from the cell may also affect the rate of penicillin synthesis. Industrial strains produce only a little isopenicillin-N if not supplied with phenylacetate to make penicillin-G. It appears that penicillin-G is secreted easily while isopenicillin-N is not and it is this which enhances the rate of penicillin synthesis (Nuesch et al. 1987).
Genetics of penicillin biosynthesis

Both Penicillium chrysogenum and Aspergillus nidulans have been used to study the genetics of penicillin production. It is difficult to use P. chrysogenum for genetic studies since the industrial strains have been highly mutated and mitotic segregation from heterozygous diploids synthesised between strains only yields the parental genotypes. This is because recombinant strains are not viable due to the large numbers of translocations in the parental genotypes (Macdonald 1968). The genetic system in Aspergillus nidulans is well defined and segregation occurs normally.

Two types of mutant have been isolated from A. nidulans, one group (penA-C) with increased titre and a group (npeA-D) with decreased titre (Ditchburn et al. 1976; Edwards et al. 1974). The pen mutations were mapped to three linkage groups penA to group VII, penB to linkage group III, and penC to group IV (Ditchburn et al. 1976). Mutants impaired in penicillin synthesis fell into 4 classes of which npeA was the most common and is located on chromosome VI (Edwards et al.; 1974, Holt et al. 1976). Tests of dominance revealed that npe mutants are all recessive to the wild type gene and that penA mutants are also recessive. However penB mutations are dominant and penC mutations semidominant implying that the genes in which the mutations have occurred have a regulatory function. A map of these mutations has been constructed (Makins et al. 1983). The npeA mutants are unable to synthesise the tripeptide LLD-ACV and were believed to be similar to the Y mutant of P. chrysogenum mentioned below (Makins et al. 1981; Makins and Holt 1982, Normansell et al. 1980). Disomic strains have been constructed for penB by use of sod (stabilisation of disomic) mutants which contain translocations and therefore generate duplications of some chromosome regions (Upshall et al. 1979). A strain with two copies of the penB locus was constructed one of them situated in a translocation. However the penicillin titre was unaffected (O'Donnell et al. 1985).

P. chrysogenum mutants with low titre have been isolated, all of which were recessive to wild type (Normansell et al., 1980). Diploids synthesised between these mutants showed that there were 5 complementation
groups which were named V, W, X, Y and Z are located on three chromosomes, W, Y and Z being on the same chromosome. The majority of mutants belonged to group Z. The mutants were examined for ability to carry out the early steps of the biosynthetic pathway resulting in formation of the tripeptide LLD-ACV and for ability to carry out the final acyl transfer reaction. The mutant groups X, Y, and Z were deficient in tripeptide synthesis and groups V and X were deficient in the acyl transfer activity. Group W mutants contained both these activities.

Genes encoding the enzymes of penicillin biosynthesis

Since this project was started much progress has been made in cloning the genes encoding the enzymes of the first three steps of penicillin biosynthesis.

The gene \((pcbAB)\) encoding ACVS has been identified in several organisms by cross hybridisation studies (D.J. Smith et al. 1990; Hoskins et al. 1990) and its disruption in Cephalosporium acremonium leads to the loss of the ability to produce penicillin. It has been cloned from \(P.\ chrysogenum\) and the sequence determined (Diez et al. 1990).

The gene \(pcbC\) (IPNS) encoding the biosynthetic enzyme IPNS in \(P.\ chrysogenum\) (Carr et al. 1986, Barredo et al. 1989), A. nidulans (Ramon et al. 1987), C. acremonium (Samson et al. 1985), Streptomyces clavurigens (Leskiw et al. 1988) have been cloned and the sequence determined. The IPNS gene has no introns and the GC content of the gene is unusually high in \(C.\ acremonium\). The promoter activity has been studied in A. chrysogenum and A. nidulans (A.J. Smith et al. 1990; Gomez-Pardo et al. 1990) In A. nidulans 2kb of the IPNS promoter is sufficient to drive expression, in idiophase, of an IPNS promoter fusion to the Esherichia coli lacZ gene, which suggests that the gene is regulated transcriptionally. The role in catalysis of the two cysteines at positions 106 and 255 in the protein has been investigated by site directed mutagenesis in which one or both residues were converted into serine residues. The conclusions were that the cysteines played no direct role in catalysis or in the conformation of the protein. One of the single mutants in cys-106 and the double mutant were significantly impaired in IPNS activity and thus cys-106 may
be involved indirectly in binding the substrate or the ferrous ions (Samson et al. 1989).

The acyltransferase (ACT or penDE) gene has also been cloned and sequenced from *P. chrysogenum* (Barredo et al. 1989). The gene encodes a protein of 40kd which is cleaved post translationally to give two polypeptides of 11kd (N-terminal region of 40kd protein), and 29kd (C-terminal) respectively. The 29kd protein has been shown to catalyse the transfer of a hydrophobic side chain onto the 6-APA molecule and at a low level the replacement of the aminoadipate side chain of IPN with a hydrophobic side chain. The function of the 11kd protein is not yet defined. It is also not yet known if the two polypeptides associate in vivo.

Since my project began the genes encoding IPNS ACT and ACVS were found to be clustered on a chromosome in a region of about 18 kb (Figure 1-3) in *A. nidulans* (Macabe et al. 1989) and *P. chrysogenum* (D.J. Smith et al. 1989). In *A. nidulans* the cluster is at the npeA locus described above. The ACVS gene is transcribed in the opposite orientation to the IPNS and ACT genes in *A. nidulans*. The IPNS and ACT genes are transcribed in the same direction in *P. chrysogenum* and since the arrangement of the cluster is the same as in *A. nidulans* the ACVS gene is probably transcribed in the opposite orientation (D.J. Smith et al. 1990).

The 38kb fragment containing the cluster in *P. chrysogenum* was transformed into *Aspergillus niger* and *Neurospora crassa* which do not normally produce penicillin (D.J. Smith et al. 1990). The transformed strains produced detectable amounts of penicillin and the activities of the three enzymes were detected. This suggests that the 38kb fragment of DNA contains sufficient information to enable penicillin production to occur. There are possibly regulatory genes also present on it since the known genes in the cluster only occupy about 18kb of DNA. The genetic evidence presented above also implies that regulatory functions may be found elsewhere in the genome.

Two reports of studies of high producing strains show that two such strains have increased copies of a large DNA fragment of 38 or 35 kb which include the biosynthetic cluster (D.J. Smith et al. 1990; Barredo et al. 1989). In one
Figure 1-3: Clustering of β-lactam biosynthetic genes in prokaryote and eukaryote organisms

This Figure is adapted from D.J. Smith et al. 1990 and shows the organisation of genes encoding the enzymes catalysing β-lactam biosynthesis in the genomic DNA of the species listed below.

The stippled boxes represent regions of DNA which cross hybridise to ACVS gene specific probes generated from the *P. chrysogenum*, *Streptomyces clavurigenes* and *Flavobacterium* sp. (D.J. Smith et al. 1990)

The other boxes represent genes identified in previous studies by other groups.

A Flavobacterium sp
B *Streptomyces clavurigenes*
C *Penicillium chrysogenum*
D *Aspergillus nidulans*

ACVS gene [δ(L-α-amino adipyl)-L-cysteinyl-D-valine Synthase]

IPNS gene (Isopenicillin-N synthetase)

ACT gene (Acyl transferase)

genes encoding enzymes in cephamycin or cephalosporin biosynthesis
strain the copy number of the fragment was 8-16 times that of the copy number of the fragment in P. chrysogenum strain N.R.R.L. 1951 and in the other it was 14 times that of the low producing strain Wis-54-1255. The increased copy number resulted in increased levels of mRNA for all three genes (D.J. Smith et al. 1990). It is not known if the copies of the clusters are tandemly repeated or dispersed in the genome.

There is one report in which increased copy number of the gene cluster was not the reason for the high titre of another P. chrysogenum strain (Soliday et al. 1990). In this strain the increased titre may be due to a mutation in an undefined regulatory gene.

The mutants in penicillin production isolated for P. chrysogenum and A. nidulans were at several loci on different chromosomes. It is possible that these may be regulatory genes acting directly on the gene cluster encoding the enzymes of penicillin biosynthesis or they may be genes acting indirectly in several possible ways upon the supply of metabolic precursors.

Studies of the organisation of genes encoding penicillin biosynthetic enzymes in several species were done using heterologous DNA probes from the Flavobacterium sp SC12,154, a cephalosporin producer, P. chrysogenum and Cephalosporium acremonium ACVS genes. The studies showed that the ACVS and IPNS genes are also clustered in Cephalosporium acremonium, Streptomyces sp. and Flavobacterium in association with genes encoding enzymes involved in cephalosporin and cephamycin biosynthesis in the latter two species (D.J. Smith et al. 1990, Hoskins et al. 1990; Figure 1-3). In A. nidulans, and C. acremonium, the direction of transcription of the two genes is the same. The clustering of the genes suggests that they may have been transferred laterally between species.

In Cephalosporium acremonium the penicillin biosynthetic genes are unlinked to the Cephalosporium cef gene cluster. The absence of biosynthetic pathways for cephalosporins or cepamycins in the other fungi suggest that the cluster of genes for penicillin and cephalosporin biosynthesis were transferred from the bacteria to an ancestor of the fungus Cephalosporium acremonium in one or two steps and that
subsequently the cef genes were lost by the ancestor of P. chrysogenum and A. nidulans.

Further evidence for the transfer from bacteria to fungi of the genes for penicillin biosynthesis comes from comparative studies of the sequenced IPNS genes. Comparison of the derived fungal IPNS protein sequences have shown that there is a high degree of conservation between the fungal proteins and between the fungal proteins and Streptomyces spp. proteins. The Streptomyces clavurigerius IPNS protein has 56% homology to the fungal IPNS proteins (Leskiw et al. 1988) and the A. nidulans IPNS protein has 74% and 82% homology to the C. acremonium and P. chrysogenum proteins respectively (Ramon et al. 1987). Moreover the IPNS gene in filamentous fungi has no introns, in contrast to the observation that most filamentous fungal genes have small introns. These observations are compatible with a prokaryotic origin for the IPNS gene.

The IPNS genes have a high GC content compared to the average GC content of the corresponding fungi and is especially marked in C. acremonium (63% GC). These observations also suggest that the gene did not originate in filamentous fungi. Streptomyces spp. have an unusually high GC content in their DNA, for example S. wadayamensis has an average GC content of 71.4% (Ramon et al. 1987; Carr et al. 1986). Therefore the IPNS gene and the ACVS gene could have been transferred from a prokaryotic species with high GC content like Streptomyces to an ancestor of the filamentous fungi.

Studies of the divergence of base sequence of the IPNS genes shows that the genes in Aspergillus nidulans and Penicillium chrysogenum evolved from a common ancestor and that the gene in Cephalosporium acremonium is more distantly related (Ramon et al. 1987). These relationships agree with evolutionary relationships of the species obtained by comparison of 5sRNA base sequences (Chen et al. 1984). Therefore the IPNS gene was probably transferred to a common ancestor of the filamentous fungi. The GC content of the gene has fallen as the fungi evolved being 63% for IPNS in C. acremonium, 56% in P. chrysogenum and 52% in A. nidulans converging with the average level of about 50% for filamentous fungi (Ramon et al. 1987).
In summary: the reactions and some of the metabolic controls of the penicillin biosynthetic pathway have been elucidated. The three genes and the three enzymes involved directly in the pathway for penicillin biosynthesis have been isolated. The genes encoding the enzymes in this pathway are clustered in filamentous fungi. The genes encoding the common steps of the pathway are also clustered in prokaryotes and are believed to have been transferred laterally from bacteria to fungi. A genomic DNA fragment of 35-38 kb containing the gene cluster has been shown to be amplified in some production strains and is also sufficient to confer penicillin production on the species A. niger and N. crassa which do not naturally produce penicillin. There are as yet no reports of the isolation of regulatory genes but some may also be present on the large fragment amplified in the production strains of P. chrysogenum or at the other loci identified by mutation. The IPNS gene is the most studied of the three genes isolated.
1.5 Aims of the project

This project was funded in part by SmithKlineBeecham because they were interested in isolating potentially strong promoters from *Penicillium chrysogenum* for use in the manipulation of *P. chrysogenum* genes involved in penicillin biosynthesis. Previous work in *Saccharomyces cerevisiae* and *Aspergillus nidulans* has shown that the PGK gene was highly expressed (Holland and Holland 1978; Clements 1986) and therefore the PGK gene of *P. chrysogenum* might also be highly expressed. The initial aim of the project was to isolate the PGK gene of *P. chrysogenum* by using a PGK coding region probe from *A. nidulans* The next objective was to determine the promoter and terminator sequence in preparation for manipulation of the promoter and to identify any sequence motifs of interest. The size and abundance of the PGK mRNA was also investigated together with the transcriptional start and stop sites.

Once the promoter had been characterised the aim was to construct two gene fusions to the PGK gene promoter. The first fusion to the *Esherichia coli lacZ* reporter gene would enable preliminary study of the expression of the PGK promoter and provide the opportunity for eventual functional analysis of the promoter. The reporter function could be used also to discover whether the PGK promoter from *P. chrysogenum* would function in *A. nidulans*.

Since SmithKlineBeecham were interested in the manipulation of genes involved in penicillin biosynthesis the second gene fusion would be of the PGK promoter to the *P. chrysogenum* gene encoding Isopenicillin-N synthetase (IPNS). The effect of placing the IPNS gene under a different control and increasing the levels of IPNS enzyme on penicillin titre could thus be investigated.
Chapter 2

Materials and Methods.

2.1 Genetic materials and methods associated with *P. chrysogenum* and *Aspergillus nidulans*.

2.1.1 Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. chrysogenum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N.R.R.L. 1951</td>
<td>Wild type</td>
<td>Raper et al. 1944</td>
</tr>
<tr>
<td>OLI13</td>
<td><em>oliCl3</em>, requires nicotinamide (mutation undefined)</td>
<td>Bull et al. 1988</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QG716 (7-16)</td>
<td><em>pyrG89 pabaAl</em></td>
<td>Streatfield 1990</td>
</tr>
<tr>
<td></td>
<td><em>yA</em>; bgaA4; qutE208;</td>
<td></td>
</tr>
</tbody>
</table>

2.1.2 Growth Media.

The defined minimal medium (MM) was based on that of Pontecorvo et al. (1953), and was prepared as a 10x solution of stock salts (see below). After autoclaving, a carbon source was added aseptically from a sterile stock solution to the concentrations described below and sterile 1M MgSO$_4$ was added to a final concentration of 10mM (Armitt et al., 1976). Since *P. chrysogenum* grew very poorly on *A. nidulans* minimal medium 0.05% (w/v) of yeast extract was added as a supplement when growing *P. chrysogenum* cultures. This amount was empirically determined as the amount of yeast extract which would optimally improve growth on minimal medium without supporting significant growth on its own.

Stock salts solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Final amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO$_3$</td>
<td>6.00g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.52g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.52g</td>
</tr>
<tr>
<td>Trace elements solution</td>
<td>1.0ml</td>
</tr>
</tbody>
</table>

The pH of the solution was brought to 6.5 with NaOH.
Trace elements solution (Armitt et al., 1976).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄·7H₂O</td>
<td>1.00g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>8.80g</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.40g</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>0.15g</td>
</tr>
<tr>
<td>Na₂B₄O₇·10H₂O</td>
<td>0.10g</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄</td>
<td>0.05g</td>
</tr>
</tbody>
</table>

Minimal agar (MA) medium for plates was solidified with 1.5% (w/v) agar. Liquid minimal medium culture contained the wetting agent Tween 80 at 10⁻⁵ (v/v).

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Stock solution</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1M</td>
<td>0.02M</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2M</td>
<td>0.05M</td>
</tr>
<tr>
<td>Acetate</td>
<td>2M</td>
<td>0.1M</td>
</tr>
<tr>
<td>Quinic acid</td>
<td>20%(w/v)</td>
<td>1% (w/v)</td>
</tr>
</tbody>
</table>

Quinic acid was prepared as a 20% (w/v) stock solution and the pH adjusted to pH6.5 with NaOH. Stock solutions of 20% (w/v) quinic acid, 1M glucose, 2M glycerol, 2M acetate pH6.5 were prepared in distilled water and sterilized by autoclaving at 151bs pressure for 10 minutes.

Several complex media were used, most commonly malt extract medium.

**Malt Extract medium:**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.0g</td>
</tr>
<tr>
<td>Malt extract (oxoid)</td>
<td>20.0g</td>
</tr>
<tr>
<td>Bacto-peptone (oxoid)</td>
<td>1.0g</td>
</tr>
</tbody>
</table>

For plates (MEA) agar was added to 1.5% (w/v) as before.
The other complex growth media are fermentation media for P. chrysogenum (Smith D.J. et al. 1990).

**Glycerol molasses medium (GM)**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>7.5g</td>
</tr>
<tr>
<td>molasses (Appleford Ltd)</td>
<td>7.5g</td>
</tr>
<tr>
<td>Yeast extract (Oxoid)</td>
<td>5.0g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10.0g</td>
</tr>
<tr>
<td>Magnesium sulphate (7H₂O)</td>
<td>0.05g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.006g</td>
</tr>
<tr>
<td>Ammonium ferric sulphate 24H₂O</td>
<td>0.016g</td>
</tr>
<tr>
<td>Cupric sulphate (7H₂O) solution</td>
<td>0.1ml</td>
</tr>
<tr>
<td>Calcium sulphate</td>
<td>0.25g</td>
</tr>
</tbody>
</table>

Cupric sulphate solution was 0.1g of CuSO₄ dissolved in 100ml of distilled water. The medium was brought to pH 6.6 and for plates agar was added to 1.5% (w/v).

Modified FM seed medium was used for the germination of conidia and growth of the seed culture for fermentations.

**Modified FM seed medium**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn steep liquor (spray dried) (SB)</td>
<td>35g</td>
</tr>
<tr>
<td>Glucose</td>
<td>15g</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>5g</td>
</tr>
<tr>
<td>Rape seed oil (SB)</td>
<td>8g</td>
</tr>
</tbody>
</table>

The medium was made up in the order listed and stirred vigorously. The pH was brought to pH 5.9.
The modified final stage FM medium was that used in batch fermentations.

**Modified FM final stage medium**

<table>
<thead>
<tr>
<th>nutrient</th>
<th>amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn steep liquor (spray dried) (SB)</td>
<td>20g</td>
</tr>
<tr>
<td>Lactose</td>
<td>55g</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>10g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>7g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>4.5g</td>
</tr>
<tr>
<td>Phenoxyacetic acid</td>
<td>1g</td>
</tr>
</tbody>
</table>

The medium was made up in the order listed with vigorous stirring and was brought to pH 6.6.

Nutritional supplements were added to both defined and complex media as required; para-amoeno benzoic acid to a final concentration of 1.0mg/l, and uracil to 1.1g/l.

The antibiotic oligomycin which was used to select *P. chrysogenum* transformants was made up as a 3mg/ml stock in ethanol and stored in the freezer. For plates it was added to a concentration of 3μg/ml.

All media were sterilized by autoclaving at 151bs pressure for 15-20 minutes.

2.1.3 **Growth and storage of strains.**

Stock cultures of strains were maintained on MEA slants. Long term storage of strains was on silica gels (Roberts, 1969).

**Overnight batch culture**

All *P. chrysogenum* cultures were grown at 30°C unless stated otherwise and all *A. nidulans* cultures at 37°C. Liquid cultures were grown on a rotary shaker operating at 250 revolutions per minute in an Ehrlenmeyer flask with vertical indentations to aerate and suspend the culture. The culture volume did not exceed one fifth of the volume of the flask used.
Penicillium chrysogenum fermentations

A 1L flask containing 100ml of FM seed medium was inoculated with two to three loopsful of conidia and incubated for 24 hours on a rotary shaker at 250rpm. The culture was then transferred to a 21 fermenter containing 1.5l of the FM final stage medium. The culture was grown at 25°C, stirred at 1600rpm, air was supplied at a rate of 1.5l/minute and the pH was kept between 6 and 8.

2.1.4 Preparation of conidiospore suspension.

Dilute suspensions of conidiospores were spread on the surface of MEA plates and incubated for three to four days at 30°C (P. chrysogenum) or two to three days at 37°C (A. nidulans) to provide well conidiating confluent growth. Conidia were harvested by flooding the plates with 15ml sterile Tween-Saline solution (0.8% (w/v) NaCl; 0.025% (v/v) Tween-80) and drawing a glass spreader or a wire across the surface of the plate. The suspensions were collected, transferred to sterile disposable universal tubes and centrifuged at 330rpm to collect the conidiospores. The conidiospores were resuspended in phosphate buffer (20mM KH₂PO₄; 50mM Na₂HPO₄; 50mM NaCl; 0.4mM MgSO₄·7H₂O, final pH 7.2). The concentration of conidial suspensions were estimated by making serial dilutions and counting the conidia present using a haemocytometer examined under a light microscope.

2.1.5 Method of colony staining for β-galactosidase production.

The colony staining technique developed by Streatfield (1990) was used to detect β-galactosidase production by A. nidulans and P. chrysogenum strains.

Conidia were inoculated onto a small area of an MA glucose plates supplemented with the necessary nutrients and brought to a pH of 6.0 by 0.1M citric acid/ 0.2M Na₂HPO₄. The media also contained the chromogenic substrate X-gal at a final concentration of 40μg/ml. The plates were incubated at the appropriate temperature for 1 day, after which clean sterile 9cm filter paper discs were applied to the surface of the
medium and the plates were incubated for a further day. Subsequently, the filters were stripped from the surface of the plates, so removing conidia which would otherwise hinder observation, and the plate or the face of the filters that had been in contact with the medium were examined.

2.1.6 β-galactosidase enzyme assays

The activity of β-galactosidase was assayed spectrophotometrically by a modification of the method of Wallenfels (1962). The conversion of the substrate o-nitrophenyl-β-D-galacto-pyranoside (ONPG) to o-nitrophenol was monitored spectrophotometrically at 420nm.

Method 1 (used for the β-galactosidase assays of the samples from fermentation experiment)

Samples of mycelium from the fermentations were harvested by filtration through glass fibre filters and washed with 0.08% saline. The mycelium was then ground to a fine powder under liquid nitrogen using a pre-cooled pestle and mortar and resuspended in extraction buffer (50mM phosphate buffer, 1mM MgCl₂ pH 7.0) with 5ml of extraction buffer for every 1g fresh weight of powdered mycelium. The samples were stored on ice until all had been resuspended and then the cell debris was removed by centrifugation at 4000rpm, for 10 minutes at 4°C. 1ml of the supernatant was then transferred to an Eppendorf tube and centrifuged at 15000rpm for 6 minutes at 4°C. The supernatant was decanted and stored on ice. The assay was done within 5 hours.

A volume of 50μl of the cell extract diluted 1/10 was mixed with 1ml of the assay buffer (21.5g Na₂HPO₄·12H₂O, 5.52g NaH₂PO₄·H₂O, 0.75g KCl, 0.246g MgSO₄·7H₂O, 2.7ml β-mercaptoethanol per litre). The addition of 200μl of ONPG (4mg/ml) started the reaction which was then incubated for 15 minutes at 37°C. The reaction was stopped by the addition of 2ml of 1M Na₂CO₃ and the absorbance at 420nm measured against a blank. The blank was a reaction to which the Na₂CO₃ had been added before the ONPG. The extract volume required to give an OD of 0.4 after 30 minutes at 37°C was calculated, the reaction then done with that volume of extract and the OD measured and used to calculate the
specific activity in nmoles ONPG converted/min/mg protein

Specific activity = \( \frac{OD_{420} \times V_t}{e \times V_s \times t \times p} \)

\( OD_{420} \) = observed \( OD_{420} \) \times any dilution factors
\( V_t \) = total volume of the reaction
\( e \) = the extinction coefficient for ONPG
\( V_s \) = the sample volume
\( t \) = time of incubation
\( p \) = protein concentration of extract

The protein concentration of the extract was measured by the method of Lowry et al. (1951) and a calibration curve was constructed each time using bovine serum albumin (BSA).

Method 2 (used in all other β-galactosidase assays)

Liquid minimal media was inoculated with a suspension of conidiospores to \( 10^6 \) spores per ml, and 400ml cultures were typically grown in 21 indented flasks at the appropriate temperature a rotary shaker for 15 to 24 hrs depending upon the carbon source. Mycelium was harvested by filtration on Whatman No.1 filters, and washed with distilled water or phosphate buffer (pH 7.2). The mycelial pad was then immersed in liquid nitrogen in a pre-cooled mortar and ground to a fine powder with a cold pestle. The frozen powdered mycelium was resuspended in 1ml extraction buffer (50mM sodium phosphate pH 7.6, 1mM MgCl\(_2\)), allowed to thaw and extracted by gently shaking the slurry in a polypropylene tube on ice for about 30 minutes. The cell debris was removed by centrifugation for 10 minutes in an Eppendorf microfuge at 4°C. The supernatant was decanted and stored on ice. The extract was diluted in extraction buffer, and the enzyme assayed within a maximum of 5 hours after extraction.
The assays were performed at a constant temperature of 25°C. Reagents were added to a quartz cuvette in the following order:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction buffer</td>
<td>0.8ml</td>
</tr>
<tr>
<td>Extract (dil 1/10)</td>
<td>25-100μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 0.9ml</td>
</tr>
</tbody>
</table>

The optical density of the reaction mix was monitored for about 30 seconds to obtain a base rate before the addition of 100μl of 10mM ONPG to give a final concentration of 1mM. Different concentrations of the same sample of extract were assayed to verify that the initial rate of the reaction was proportional to the amount of cell free extract added.

The concentration of soluble protein was assayed in each cell free extract by the method of Lowry et al. (1951), and a calibration curve was constructed using BSA standards as before.

For the purposes of certain aspects of this study where relative activities were required, it was convenient to define units of β-galactosidase activity in the manner of Fantes (1972). One unit of β-galactosidase activity produced a change in optical density at 420nm of 0.001 per minute. The specific activity is defined as the activity per mg of protein assayed.

That is, specific activity = \( \frac{\text{OD change at 420nm}}{10^3} \times \frac{\text{min}}{\text{mg protein}} \)

One unit of activity is equivalent to a rate of hydrolysis of ONPG of 0.188 nmol/min.
2.1.7 **Isopenicillin N synthetase (IPNS) enzyme assays**

This assay was done as described in Smith et al. (1990) which was adapted from Ramos et al. (1985)

**Preparation of cell free extracts**

A volume of 10ml of culture was added to 100ml of cold 0.08% (w/v) sodium chloride solution and the suspension filtered through a scinttered glass disc. The mycelial pad was rinsed with a further 200ml of cold 0.08% sodium chloride and then resuspended in 10ml of extraction buffer (30mM Tris-HCl pH8.0, 1.0mM Dithiothreitol, 1mM phenylmethylsulfonyl fluoride). The mycelium was disrupted by ultra sonic vibration using a Ultrasonics model W-385 sonicator. The mycelium was subjected to 3 bursts of 15 seconds treatment with one minute between each at a power setting of 5, a cycle rate of 5 seconds, and a duty cycle of 50%. The mycelial debris was removed by centrifugation at 16000 rpm in an SS34 rotor in a Sorvall centrifuge at 4°C for 30 minutes. A volume of 2ml of the crude extract was passed down a PD10 sepharose column pre-equilibrated with extraction buffer. 1ml of the extraction buffer was passed down after the extract and the eluate discarded. Then 1.5ml of the buffer was passed down the column and the eluate collected and stored on ice.

**Isopenicillin-N Synthetase assay.**

The reagents listed below were made up daily in 30mM Tris HCl pH8.0. The stock solutions for the FeSO$_4$ and the ascorbic acid were mixed. The reagents were added to an Eppendorf tube in the volumes indicated and incubated for 30 minutes with shaking at 25°C to allow the reduction of the (L-α-Aminoadipyl)-L-Cysteinyl-D-Valine dimer (ACV) by DTT.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
<th>Volume used (μl)</th>
<th>Stock concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV</td>
<td>0.2mM</td>
<td>20</td>
<td>2.5</td>
</tr>
<tr>
<td>FeSO$_4$. 7H$_2$O</td>
<td>0.1</td>
<td>20</td>
<td>1.25</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>1.0</td>
<td>20</td>
<td>12.5</td>
</tr>
<tr>
<td>DTT</td>
<td>2.0</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>
The extract was then diluted in extraction buffer where necessary and added to the reaction tube in a total volume of 150μl. The reaction was then incubated for 15 minutes after which 230μl of methanol were added to stop the reaction. A control was included with no substrate. 100μl of the reaction was loaded into a well on a bioassay plate and incubated overnight at 50°C. Standards of 0.1 to 10μg of Isopenicillin-N made up in a 50% solution of methanol were also included on each of the plates.

**Preparation of Bioassay plates for the IPNS bioassay**

The bioassay plates were made from 250ml nutrient agar (oxoid) seeded with 500μl of a suspension of Bacillus calidolactis approximately $10^8$ to $10^9$ spores per ml. The agar was poured into a NUNC bioassay plate which had been set level and when the agar had solidified nine wells were cut out of the agar with a No.7 cork borer.

The Bacillus calidolactis was stored as a spore suspension in Ringers solution at 4°C. New suspensions were generated by plating out on sporulation medium:

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxoid bacteriological peptone</td>
<td>3</td>
</tr>
<tr>
<td>Oxoid tryptone</td>
<td>2.5</td>
</tr>
<tr>
<td>Oxoid yeast extract</td>
<td>4</td>
</tr>
<tr>
<td>Oxoid lab lemco</td>
<td>2.5</td>
</tr>
<tr>
<td>$K_2HPO_4$</td>
<td>2</td>
</tr>
<tr>
<td>$MnSO_4.4H_2O$</td>
<td>0.01</td>
</tr>
<tr>
<td>Oxoid agar No.3</td>
<td>25</td>
</tr>
</tbody>
</table>

The spores were then collected and stored frozen in aliquots. A new working stock was made by plating out the frozen cells and collecting the new growth in Ringers solution.

**Ringers solution** parts by volume:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>100</td>
</tr>
<tr>
<td>1.15% KCl</td>
<td>4</td>
</tr>
<tr>
<td>1.22% CaCl₂</td>
<td>3</td>
</tr>
<tr>
<td>2.11% KH₂PO₄</td>
<td>1</td>
</tr>
<tr>
<td>3.8% MgSO₄</td>
<td>1</td>
</tr>
<tr>
<td>0.1M phosphate buffer pH7.4</td>
<td>21</td>
</tr>
</tbody>
</table>

42
2.1.8 Penicillin titre

The penicillin titre was measured by a method in which the β-lactam ring reacts at 30°C with imadazole stabilised with mercuric chloride. A complex of penicillanic acid mercuric mercaptides is formed which is detected spectrophotometrically at 325nm.

2.1.9 DNA mediated transformation of P. chrysogenum.

The method used to transform P. chrysogenum was based on that of Bull et al. (1988). A flask containing 200ml of glucose minimal medium was inoculated with $10^6$ spores per ml and incubated overnight on a rotary shaker at 30°C. The mycelium was harvested by centrifugation at 3000 rpm in a bench top centrifuge and washed twice in 0.9M sodium chloride, an osmotic stabiliser. The mycelium was collected by centrifugation as above and resuspended in 15ml of 0.9M sodium chloride, 5mg/ml of Novozym 234 which degrades the fungal cell wall. The mycelial suspension was then shaken very gently at 30°C for 1.5 hours to allow sphaeroplasts to form. The suspension was then filtered through a nylon mesh (Gallenkamp GMX-500-v) and scinted glass filter (porosity 1) to remove mycelial debris and collect the protoplasts. The sphaeroplasts were then washed once by gentle centrifugation at 1500rpm in a Centaur bench top centrifuge in 0.9M sodium chloride and then in 1M sorbitol, 50mM CaCl$_2$. The sphaeroplasts were resuspended in the latter solution and were counted by making dilutions and examining them under a light microscope using a haemocytometer. They were resuspended at $0.5\times10^8$ to $5\times10^8$ sphaeroplasts per ml and divided into 50µl aliquots. The DNA was added to the suspenion in a maximum of 5µl of T.E. followed by 12.5µl of 25% PEG 6000, 50mM CaCl$_2$, 10mM Tris pH7.5. Following incubation on ice for 20 minutes a further 0.5ml of the 25% PEG 6000, 50mM CaCl$_2$, 10mM Tris pH7.5 was added to the mixture which was then incubated for 5 minutes at room temperature. Then 1ml of 1M Sorbitol, 50mM CaCl$_2$ was added to the transformation mixture and aliquots of the mixture overlaid in soft MEA containing 0.9M sodium chloride onto MEA plates. After incubation at 30°C for 20 hours 25ml soft
agar containing 3µg per ml of the drug oligomycin was overlaid onto the plates. Transformants were visible on the surface of the agar after 5-8 days growth at 30°C.

The efficiency of sphaeroplast regeneration was monitored by overlaying dilutions of the transformation mixture onto MEA plates as described but not adding the second overlay containing the oligomycin. Colonies were counted after two days growth.

2.1.10 DNA mediated transformation of A. nidulans.

The transformation procedure used to transform A. nidulans was adapted from that of Ballance et al. (1983) by Streatfield (1990).

Cellophane discs the size of a 9cm petri dish base and sterilised by autoclaving, were placed on the surface of 20 appropriately supplemented MEA glucose plates, and approximately 10⁶ conidiospores were spread onto each plate using a sterile glass spreader. Cultures were incubated for 15 hours at 37°C, after which the cellophane discs bearing young mycelium were divided equally between 4 clean petri dishes each containing a 15ml solution of Novozym 234 (5mg/ml) in an osmotic stabilizer, 0.6M KCl, and placed at 30°C on a slow rotary shaker for about 1 hour. The cellophane discs were then removed from the resulting sphaeroplast suspension and rinsed in 15ml of 0.6M KCl in a petri dish to remove any adhering sphaeroplasts. The suspensions of sphaeroplasts were passed through a nylon mesh (Gallenkamp GMX-500-V) and a scinttered glass filter (porosity 1) to remove cell debris. The sphaeroplasts were pelleted by centrifugation at room temperature at 1500rpm in a Centaur bench centrifuge for 5 minutes and washed three times, twice with 0.6M KCl and once with 0.6M KCl, 50mM CaCl₂. The sphaeroplasts were resuspended in 500µl of 0.6M KCl, 50mM CaCl₂, and the sphaeroplast concentration was estimated by making serial dilutions and counting the sphaeroplasts present using a haemocytometer examined under a light microscope. The volume of the suspension was then adjusted to give 1-5x10⁷ sphaeroplasts per ml. about 10 µg of DNA in 20µl of TE buffer pH8, was added to 200µl aliquots of the protoplast suspension in 20ml sterile disposable
universal tubes, followed by the addition of 50μl of 25% PEG 6000, 50mM CaCl₂, 10mM Tris.HCl, pH7.5. After 20 minutes on ice a further 2ml 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 10ml of appropriately supplemented molten minimal medium containing 0.6M KCl and 2% (w/v) agar and held at 48°C. This molten agar was poured as a top layer onto similarly supplemented minimal medium plates containing 0.6M KCl and 1.5% (w/v) agar. Plates were incubated at 37°C for 3 to 4 days.

The efficiency of sphaeroplast regeneration was assessed by adding 100μl aliquots of 10⁻², 10⁻³ and 10⁻⁴ dilutions of the final transformation mixture 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 sphaeroplasts 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 the loss of transforming DNA integrated into the genome.
2.2 Genetic materials and methods associated with *Escherichia coli*.

2.2.1 *E. coli* strains.

The *E. coli* strain strain JM83 (ara, lac-pro, strA, thi, \(\phi\)80dlacZ M15; Vieira and Messing, 1982) was used throughout in transformations of *E. coli* and strains JM101 and JM109 (supE, thi, \(\Delta\)(lac-proAB), [F' traD36, proAB+, lacI^q, lacZ, \(\Delta\)M15] Messing, 1983) for propagation of recombinant M13 phage for DNA sequencing. TGI (K12 \(\Delta\)(lac-pro), supE, thi, hsdD5, C'[traD36, proA^B+, lacI^q, lacZ, \(\Delta\)M15]) was used to propagate M13 DNA after site directed mutagenesis reactions. Sm(\(\geq\)4, endA1, hsdR'M, q7,A96, relA1, thi, \(\Delta\)(lac-proAB))

2.2.2 Media.

The growth media used are given below showing the final concentration of components.

**Luria Broth (LB).**

- Tryptone 1.0% (w/v)
- Yeast extract 0.5% (w/v)
- NaCl 0.5% (w/v)

**M9 Minimal Medium.**

- \(\text{Na}_2\text{HPO}_4\) 0.6% (w/v)
- \(\text{KH}_2\text{PO}_4\) 0.3% (w/v)
- \(\text{NH}_4\text{Cl}\) 0.1% (w/v)
- Glucose (autoclaved separately) 0.4% (w/v)
- MgSO\(_4\) (autoclaved separately) 1mM
- CaCl\(_2\) (autoclaved separately) 0.1mM
- Thiamine (filter sterilized) 10\(\mu\)g/ml

**wb medium** (for DNA mediated transformation).

- Bacto yeast extract 0.05% (w/v)
- Bacto tryptone 0.02% (w/v)
- MgSO\(_4\) .7H\(_2\)O 0.02% (w/v)

pH 7.6 with KOH.

**BL medium**

- BBL trypticase peptone 1% (w/v)
- sodium chloride 0.5% (w/v)
Agar plates were prepared by solidifying liquid media with 1.5% (w/v) Oxoid or Biolife agar, and soft agar was prepared by solidifying liquid media with 0.6% (w/v) of the same agar. The antibiotics ampicillin and chloramphenicol were added to media as required to final concentrations of 25μg/ml. Ampicillin stock solutions were filter sterilized, and chloramphenicol solutions were prepared in ethanol.

2.2.3 Growth and storage of strains.

Plasmid bearing strains were grown in and maintained on media containing the appropriate antibiotic and were incubated at 37°C. Cultures were maintained on Luria agar plates at 4°C when in current use, and frozen at -20°C in Luria broth containing 20% (v/v) glycerol for long term storage.

2.2.4 Plasmids and phage.

The origin of plasmids used for the construction of vectors and preparation of gene probes are given below.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPGK2</td>
<td>Clements and Roberts, 1985</td>
</tr>
<tr>
<td>pAL3.3</td>
<td>Hawkins et al., 1985</td>
</tr>
<tr>
<td>pAN923-41A, -42A, -43A</td>
<td>van Gorcom et al., 1986</td>
</tr>
<tr>
<td>pPOL20</td>
<td>Prof G. Turner's group</td>
</tr>
<tr>
<td></td>
<td>(unpublished)</td>
</tr>
<tr>
<td>pCYX4</td>
<td>Smith et al. 1990</td>
</tr>
</tbody>
</table>

2.2.5 DNA mediated transformation of E. coli strain JM83

During the construction of recombinant plasmid vectors, competent cells were prepared from the E. coli strain JM83 and were transformed according to the method of Hanahan (1983). In order to prepare competent cells, a single isolated colony of JM83 was used to inoculate 10ml of Ψb medium (see above) and incubated at 37°C for 3 hours with vigorous shaking. A volume of 1ml of the culture was used
to inoculate 100ml of ψb medium, which was incubated at 37°C with vigorous shaking until the culture reached an optical density of about 0.5 at 550nm. The culture was chilled on ice for 5 minutes, dispensed into 50ml sterile polypropylene tubes and the cells pelleted at 6,000rpm in a SS34 rotor in a Sorvall centrifuge, for 15 minutes at 4°C. The cells were then resuspended in two-fifths of their original volume of sterile TFBI solution (30mM potassium acetate, 100mM RbCl₂, 10mM CaCl₂, 50mM MnCl₂, 15% glycerol (v/v), pH5.8 with 0.2M acetic acid), and left on ice for 5 minutes. The cells were recovered by centrifugation as above and gently resuspended in one twenty fifth of their original volume of TfbII solution (10mM MOPS, 75mM CaCl₂, 10mM RbCl₂, 15% glycerol (v/v), pH6.5 with KOH). The cells were left on ice for 15 minutes, aliquoted into 200µl samples, frozen in liquid nitrogen and stored at -70°C.

The cells were thawed and then left on ice for 10 minutes before use. Up to 50ng of DNA in a 40µl volume was added to the thawed cells, and the mixture left on ice for 30 minutes. The cells were then heated at 42°C for 90 seconds and then returned to ice for 90 seconds. Four volumes of ψb medium was added to the mixture and the cells were incubated at 37°C for 50-60 minutes, after which they were spread onto Luria agar plates containing the appropriate antibiotics and 40µg/ml each of IPTG and X-gal if appropriate and incubated at 37°C overnight.

2.2.6 Electroporation of E. coli strain TGI

The method of electroporation was described by Dower et al. (1988). 1ml of an overnight culture of TGI was used to inoculate 300ml Luria broth which was incubated at 37°C with vigorous shaking until the culture reached an optical density of about 0.6 at 600nm. The culture was chilled on ice and four serial washes conducted in decreasing volumes of ice-cold sterile distilled water until the cells were finally resuspended in a volume of 500µl. DNA in a volume of 5µl water was added to 40µl of cells in an electroporation cuvette and a pulse (1.5kV, 25µF) delivered. 1ml of the regenerating medium, SOC (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 20mM MgCl₂, 20mM glucose), was
immediately added to the cells. Aliquots of the cell were then overlaid in soft BTL agar including 200\(\mu\)l of exponentially growing cells, onto BLA agar and incubated at 37°C overnight.

2.2.7 DNA mediated transformation of JM101 and JM109

A single colony of JM101 or JM109 taken from a M9 minimal medium plate was used to inoculate 3ml of Luria broth. The culture was grown overnight standing at 37°C. 1ml of the overnight culture was then used to inoculate 30ml of luria broth which was then incubated with vigorous shaking at 37°C for about 90 minutes, until the O.D. of the culture was 0.5 to 0.7 at 550nm. The culture was divided into sterile Eppendorf tubes and the cells pelleted by centrifugation at 12000 rpm in a microfuge for 12 seconds. The cells were resuspended in 450\(\mu\)l of cold 10mM MOPS, pH 7.0, 10mM rubidium chloride and pelleted again as above. They were then resuspended in 450\(\mu\)L of a second solution containing 10mM MOPS pH6.5, 10mM rubidium chloride, 50mM calcium chloride, and placed on ice for 90 minutes. Next, the cells were pelleted by centrifugation as above and gently resuspended in 150\(\mu\)l of the second solution (pH6.5) and 3\(\mu\)l of dimethylsulphoxide added. A maximum of 200ng of the DNA to be transformed was then added to the mixture and the mixture incubated on ice for 30 minutes. The cells were heated at 55°C for 30 seconds and immediately returned to ice for two minutes. A volume of 300\(\mu\)l of fresh cells were then added to the transformation mixture and if appropriate 30\(\mu\)l of IPTG (25mg/ml) and 30\(\mu\)l of X-gal (25mg/ml). Aliquots of the cells were mixed with 3ml of BL soft agar and overlaid onto BL plates. A control of 10ng of m13mpl9 was included as a control in the procedure. The plates were incubated at 37°C overnight and stored at 4°C.
2.3 Recovery of nucleic acids.

Proteins were extracted from nucleic acid solutions by the addition of an equal volume of phenol/chloroform (100g phenol, 100ml chloroform, 4ml isoamyl alcohol, 0.1g 8-hydroxyquinolene) saturated with 10mM Tris.HCl, pH7.5. The mixture was agitated on a Vortex mixer for 1 minute if it was plasmid DNA or RNA or gently shaken if it was genomic DNA and then the layers were separated by brief centrifugation. The upper (aqueous) phase was collected taking care not to disturb the interphase.

Nucleic acids were recovered from aqueous solutions after the addition of 0.1 volumes of 3M sodium acetate pH5.2, by precipitation with 2 volumes of cold ethanol. The solution was mixed and chilled at -20°C or -80°C for periods of 30 minutes to overnight depending on the circumstances. The precipitated nucleic acid was collected by centrifugation at 12000xg for 15 minutes at 4°C, rinsed in cold 70% (v/v) ethanol, dried under a vacuum and resuspended in the appropriate volume of sterile distilled water or TE buffer (10mM Tris.HCl, pH 8.0, 1mM EDTA).
2.4 DNA preparation.

2.4.1 Small scale preparation of plasmid DNA from E. coli

The method used followed a modification of the procedure of Birnboim and Doly (1979). Overnight cultures of the plasmid carrying strain of E. coli were grown in Luria broth containing the appropriate antibiotic. A 1.5 ml sample in an Eppendorf tube was centrifuged for 2 minutes to pellet the cells, and the medium removed by aspiration. The bacterial pellet was resuspended in 100 μl of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl pH 8.0), and left at room temperature for 5 minutes, after which 200 μl of a freshly prepared solution of 0.2 M NaOH, 1% SDS (w/v) was added. The solutions were mixed by inverting the tube gently several times and then the tube was transferred to ice for 5 minutes. 150 μl of solution III (3 M sodium acetate pH 4.8 with glacial acetic acid made up as follows to 60 ml of 5 M sodium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of H2O were added) was then added and the mixed by vortexing the inverted tube for 10 seconds. The tube was placed on ice for 10 minutes, and then centrifuged for 5 minutes in an Eppendorf centrifuge at room temperature. The supernatant was transferred to a new Eppendorf tube, extracted with phenol/chloroform and the DNA precipitated with ethanol at room temperature for 2 minutes. The DNA was resuspended in TE pH 8 buffer containing 20 μg/ml of DNAase-free RNAase which was prepared by dissolving RNase in TE pH 8.0 at 20 mml and then boiling the RNAase solution for 10 minutes.

2.4.2 Preparation of cosmid DNA from dense lawns of E. coli for colony hybridisation

The cosmid library DNA contained in E. coli bacterial cells was prepared for screening by a method adapted from Hanahan and Meselson (1980). Nitrocellulose filters were placed onto Luria agar plates containing the appropriate antibiotic and bacterial cells were spread onto the filters at a high density, to give up to 3000 small discrete colonies (diameter 0.1-0.2 mm) per filter after overnight incubation at 30°C.

The colonies on the filters were replicated onto two
further nitrocellulose filters and the colonies on all the filters then allowed to regrow at 37°C. One plate was then stored at 4°C and the cosmid DNA in the cells on the other filters amplified by transferring the filters to Luria agar plates containing 250μg/ml of chloramphenicol and incubating the plates for 20 hours. Cosmid DNA was then prepared by alkaline lysis. The filters bearing the colonies were placed on blotting paper which had been soaked in 0.5M sodium hydroxide, 1.5M sodium chloride for 10 minutes. They were then blotted dry and transferred to blotting paper soaked in 1M Tris pH7.5 for 10 minutes dried as before and finally placed on blotting paper soaked in 0.5M Tris pH7.5, 1.5M sodium chloride for 10 minutes. It was important not to allow the lysis solutions to flood the top side of the filter. After lysis of the bacterial cells the filters were soaked in 2xSSC (0.3N sodium chloride, 0.03M trisodium citrate) and the colonies removed by rubbing the filter with a gloved finger. The filters were well rinsed in 2xSSC and then dried at room temperature and then baked at 80°C for two hours.

2.4.3 Large scale preparation of plasmid DNA from E. coli.

The method used for the large scale preparation of plasmid DNA was essentially a scaled up version of the method of Birboim and Doly (1979), with the additional steps of chloramphenicol induced amplification of plasmid copy number, and purification by caesium chloride gradient centrifugation.

Initially, 10ml of Luria broth containing the appropriate antibiotic was inoculated with an E. coli strain carrying the required plasmid, and was incubated at 37°C with vigorous shaking until the culture reached an optical density of about 0.6 at 600nm. The whole culture was then used to inoculate 400ml Luria broth containing the appropriate antibiotic, and this was then incubated at 37°C with vigorous shaking until the culture reached an optical density of 0.4 at 600nm, after which chloramphenicol was added to a final concentration of 170μg/ml, and the culture incubated for another 12 to 16 hours.

The cells were pelleted by centrifugation at 4000 rpm in
GS3 rotor in a Sorvall centrifuge for 10 minutes at 4°C, resuspended in 12ml of solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH8.0) and left at room temperature for 5 minutes. To this solution 24ml of freshly prepared solution II (0.2M NaOH, 1% SDS (w/v)) was added, the solution gently mixed and held on ice for 5 minutes. Then 12ml of ice-cold solution III (sodium acetate made as described for the small scale preparation above), was added, mixed vigorously and left on ice for 15 minutes to precipitate the chromosomal DNA and proteins. The chromosomal DNA and proteins were separated by centrifugation at 10,000rpm in an SS34 rotor for 10 minutes at 4°C. The remaining nucleic acids were then precipitated by the addition of 0.6 volumes of propan-2-ol, mixing and incubation at room temperature for 15 minutes. The precipitate was collected by centrifugation at 10,000 rpm for 10 minutes at room temperature, and the pellet was rinsed with cold 70% ethanol, dried under vacuum and finally resuspended in 6ml TE buffer.

The volume of the nucleic acids was accurately measured and one gram of AR grade caesium chloride was dissolved in the solution for every ml of its volume. 0.1ml of a 10mg/ml solution of ethidium bromide was added to the solution. The refractive index of the solution was estimated and adjusted to 1.386 by the addition of further CsCl₂ or T.E. if necessary. The solution was transferred to a Beckman polyallomer "Quickseal" tube and the tube topped up with a 1g/ml CsCl₂, T.E solution. The tube was heat sealed and centrifuged at 15°C at 55000 rpm overnight in a Beckman 75Ti rotor. The plasmid band was collected and the ethidium bromide removed by repeated extraction of the solution with sodium chloride saturated propan-2-ol. Four volumes of sterile distilled water were then added and the DNA precipitated by addition of two volumes of ethanol.

The concentration and quality of plasmid preparations was determined by measuring the optical density of the nucleic acid solution at 260nm and 280nm. An O.D. of 1 at 260nm corresponds to approximately 50μg/ml DNA, and a 260nm to 280nm ratio of 1.8 indicates a pure preparation of DNA (Maniatis et al., 1982).
2.4.4 Small scale preparation of chromosomal DNA from 
*P. chrysogenum* and *A. nidulans*.

Chromosomal DNA was prepared from a dense suspension of conidiospores by a method adapted by Raj Beri from Morris (1978). Conidiospores were harvested from the surface of a single MEA plate as described above (Section 2.1.4). The conidiospores were washed by two serial centrifugations in cold sterile 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 Macartney bottle, containing 3g acid washed sterile 0.45mm glass beads and vigorously agitated for 2 minutes on a Vortex mixer when the formation of empty conidia could be seen under a light microscope. A further 0.8ml of spermidine lysis buffer was then added to the preparation and the supernatant dispensed into two Eppendorf tubes. Each tube was incubated at 60°C for 20 minutes with 67μl of a 10% solution of SDS (w/v). Next, 222μl of 4M sodium acetate pH6 was added to each tube, mixed and held on ice for 30 minutes. The precipitated cell debris was removed by centrifugation at 12000xg in a microfuge at room temperature for 5 minutes and the supernatant recovered. The supernatant was then incubated with proteinase K at a final concentration of 200μg/ml and DNAase-free RNAase at a final concentration of 100μg/ml at 37°C for 1 hour. The DNA was then precipitated by the addition of 0.1 volumes of 4M sodium acetate pH6 and 0.6 volumes of propan-2-ol. The nucleic acids were recovered by centrifugation at 12000rpm in an Eppendorf microfuge for 5 minutes. The pellets were rinsed with 70% ethanol, dried under vacuum and pooled by resuspending in 400μl of TE buffer. The solution was then extracted twice with phenol/chloroform, and once with chloroform, after which the DNA was recovered by precipitation as before with sodium acetate and propan-2-ol, washed with 70% ethanol and resuspended in 50μl TE buffer.

DNA preparations were stored at 4°C and typically yielded 20 to 50μg of high molecular weight genomic DNA.
2.5 Analysis of nucleic acids by agarose gel electrophoresis.

Horizontal agarose gels were prepared and run in electrophoresis buffer (40mM Tris base, 5mM sodium acetate, 2mM EDTA; pH 8 with glacial acetic acid) containing ethidium bromide at 0.5µg/ml. The concentration of the agarose used was in the range 0.4 to 2.0% (w/v) depending upon the size of the DNA fragments to be separated.

Molecular weight marker DNA standards were provided by HindIII digestion of λ DNA. DNA samples were mixed with 0.1 volumes of loading buffer (25% Ficoll, 0.001% Orange C, 200mM EDTA) and electrophoresis continued until the orange dye had travelled to almost the end of the gel.

DNA binding ethidium bromide in agarose gels was visualized using a short wavelength ultra-violet transilluminator, and photographed using a polaroid MP-3 camera and a Kodak Tmax 100 film (10.2 x 12.7cm).
2.6 DNA manipulation techniques.

2.6.1 Digestion of DNA with restriction enzymes.

DNA samples were routinely incubated with a 2 to 5 fold excess of restriction enzymes (manufacturer's definitions) to ensure total digestion of DNA. Typical reaction mixtures (10µl to 20µl total volume) consisted of 0.2 to 1µg DNA, the appropriate restriction enzyme buffer (BRL "React" buffers) and were incubated at 37°C for a minimum of 1 hour.

Digestion of DNA with more than one restriction enzyme

If the DNA was digested with two enzymes that act in the same buffer double digestions were done with both enzymes simultaneously. If there was a common buffer in which both enzymes could act at reasonable efficiency that was used. When one enzyme required the buffer React 3 and the other React 2 and the former enzyme could be inactivated by heat the following procedure was done. The DNA was digested with the first enzyme which was then inactivated by heating to 70°C for 10 minutes. The solution was adjusted to React 2 by diluting it two fold and adding 1/10 volume of React 1 and the second incubation done. If the enzymes would only act in different buffers or were not inactivated by heat then the digestion was done sequentially and the DNA was recovered between the incubations as described in section 2.3.

2.6.2 Digestion of DNA with Bal31 exonuclease.

Bal31 digestions were done on plasmids linearised by digestion with the appropriate restriction enzyme. The plasmid DNA was recovered from the restriction digestion reaction by extraction with phenol chloroform followed by precipitation with ethanol. The DNA pellet was resuspended in 50µl of a solution of 500µg/ml bovine serum albumin. An equal volume of 2x reaction buffer (24mM CaCl$_2$, 24mM MgCl$_2$, 0.4M NaCl, 40mM Tris-HCl pH8, 2mM EDTA) was added to the DNA and the solution incubated at 30°C for 3 minutes, after which 1 Unit of Bal31 was added and samples withdrawn from 30°C incubation at one minute intervals. The reactions were terminated by adding EGTA to a final concentration of 20mM and placing the tube on ice.
2.6.3 Recovery of DNA fragments from agarose gels.

Method A "Freeze-squeeze" method

The DNA fragments were separated on a low melting point agarose gel by electrophoresis. The desired fragment was excised from the gel and the gel slice submerged in equilibration buffer in an Eppendorf tube. The gel slice was shaken in the buffer for 30 minutes after which the buffer was removed and the agarose sliced up and placed into 0.5ml Eppendorf tubes which had been pierced at the base and plugged with siliconised glass wool. The Eppendorf tubes containing the agarose were then frozen at \(-80^\circ\text{C}\) for about an hour. Immediately on recovering the tubes they were placed into 1.5ml Eppendorf tubes and centrifuged at high speed in a microfuge. The fluid which was filtered through the glass wool plug was collected and the DNA purified by phenol extraction and recovered by ethanol precipitation.

Method B "Glass milk" method

This method was described in Vogelstein and Gillespie 1979. The desired DNA fragment was excised from the agarose gel with the minimal amount of agarose and the minimal exposure to long wave UV light. The agarose block was chopped up and placed into an Eppendorf tube to which 500\(\mu\)l to 1ml of saturated sodium iodide was added to dissolve the agarose. The tube was shaken to encourage the agarose to dissolve. For every 100ng of fragment estimated to be in the agarose block 10\(\mu\)l of "glass milk" was added. This was made of very finely ground glass suspended in distilled water. The suspension was left at room temperature for five minutes and the glass with the DNA adsorbed to it recovered by a 5 second spin at 12000 rpm. The glass and DNA was washed three times in a solution of 50% ethanol, 5mM tris pH 7.2, 50mM sodium chloride, 0.5mM EDTA which was freshly made. After the last wash the glass/DNA was resuspended in 10\(\mu\)l of T.E. pH 8 or water for every 10\(\mu\)l of the glass milk that had been added earlier. The suspension was incubated at 45°C to 55°C for 5 minutes in which time the DNA dissociated from the glass. The glass was then removed by a 30 second centrifugation at 1200 rpm and the supernatant containing the DNA decanted.
2.6.4 Fill-in of recessed ends of DNA fragments.

Filling in of 3' recessed ends of DNA fragments generated by restriction endonuclease digestion was done by redissolving the recovered DNA in 16μl sterile distilled water adding 2μl 10x "fill-in" buffer (500mM Tris-HCl pH 7.2, 100mM MgSO₄, 10mM DTT, 500μg/ml BSA), 2μl of 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 E. coli 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.

2.6.5 Ligation of DNA fragments.

Double stranded restriction endonuclease fragments recovered from agarose gels were ligated to similarly recovered plasmid DNA molecules in a 10 to 100μl reaction volume. When DNA fragments had been generated by digestion of plasmids with two different restriction endonucleases the ratio of insert to vector DNA molecules was equimolar. However, excess of insert was usually used when the DNA fragment had been generated by a single restriction endonuclease. This alleviated the problem of recircularization of the vector molecule. The total concentration of vector plus insert DNA was usually 1 to 20ng/μl, and this was incubated in ligation buffer (BRL 5x ligation buffer) overnight at 15°C after the addition of 1 Unit of T4 DNA ligase.

2.6.6 Removal of 5’phosphates from linear DNA

Calf intestinal alkaline phosphatase was used occasionally to reduce the degree of recircularization of vector molecules in ligation reactions. The endonuclease digested plasmid DNA was extracted with phenol/chloroform and precipitated with ethanol. The DNA pellet was resuspended in 18μl water and 2μl of the reaction buffer (React 9, 50mM Tris pH 8.5, 5mm MgCl₂) followed by one unit of calf intestinal phosphatase (manufacturer's conditions). The reaction was incubated for 30 minutes at
37°C. At this time a further unit of alkaline phosphatase was added to the reaction which was then continued for a further 30 minutes. The phosphatase was inactivated by extraction with phenol/chloroform, the DNA recovered by ethanol precipitation, and resuspended in the appropriate volume of TE buffer.

2.6.7 Site directed mutagenesis (SDM)

SDM was done using an Amersham Kit. The method is based on work by Ekstein's group (Nakamaye et al. 1986). The oligonucleotide was dissolved at a concentration of 500 units/ml and was 5' phosphorylated using T4 polynucleotide kinase. It was then annealed to 1μg of the single stranded template DNA. A second strand (the mutated strand) was synthesised using the Klenow fragment of E. coli DNA polymerase dTTP, dATP, dGTP and thio-dCTP. In order to join the 3' and 5' ends of the newly synthesised strand a ligation reaction with T4 ligase was done overnight at 14°C. The remaining single stranded DNA was removed by addition of sodium chloride to the sample to a concentration of 0.6M and filtration of the solution through nitrocellulose. At this stage the DNA in the sample was double stranded and had the desired mutation on one strand only. The next step was to remove the DNA strand containing the unwanted sequence and replace it by the desired sequence. The original unchanged DNA strand was cleaved by incubation with NciI, which will not attack the desired mutant strand because of the thionucleotides incorporated into it at the NciI cleavage sites. The DNA was then incubated with exonuclease III which digested the nicked strand of the DNA leaving a small region intact. The exonuclease III was inactivated by heating the reaction to 70°C for 10 minutes. The small region of the original DNA strand was then used to prime synthesis of a new strand from the remaining strand, which contained the mutation, using Klenow fragment and dNTPs. The DNA was then incubated with DNA ligase to join the ends of the newly synthesised DNA strand. Both DNA strands now carried the mutant sequence encoded by the oligonucleotide. One quarter of the DNA was transformed into E. coli TGI as described in section 2.2.6.
2.7 Analysis of DNA by transfer onto nylon membranes and hybridisation using radioactive DNA probes (Southern hybridisation).

2.7.1 Southern blot filter preparation

The method used to transfer DNA from an agarose gel to a membrane for hybridisation is based on that of Southern (1975). Following agarose gel electrophoresis, non-essential areas of the gel were removed and the DNA was denatured by immersing the gel in 1.5M NaCl, 0.5M NaOH for 30 minutes at room temperature with gentle shaking. The gel was then immersed in a neutralising solution of 1M Tris-HCl pH8, 1.5M NaCl for a further 30 minutes. A plate was laid across a tray filled with 6xSSC (20xSSC is 3M sodium chloride, 0.3M trisodium citrate) such that the plate was 2 to 3cm above the surface of the liquid. A strip of Whatman 3MM paper was placed over the plate and in contact with the 6xSSC in the tray so as to act as a wick for the uptake of the solution. The gel was laid top face down on the 3MM paper, taking care to avoid trapping air bubbles between the gel and the 3MM paper. First a Hybond-N nylon filter and then 2 pieces of 3MM paper cut to size slightly larger than the gel, soaked in 6xSSC were laid on top of the gel, and any air bubbles between the gel and the nylon filter removed. A stack of paper towels was placed on the 3MM paper, covered with a glass plate, and compressed with a 500g weight. Transfer of DNA was continued overnight. the position of the loading slots in the gel were marked, and the filter was then washed in 6xSSC, blotted dry on 3MM paper, wrapped in Saran wrap and placed on a short wavelength ultra-violet transilluminator for 30 seconds to bind the single stranded DNA to the filter.
2.7.2 Preparation of DNA for "dot-blot" analysis.

An equal volume of small scale preparations of DNA in TE buffer was added to an equivalent amount of 2x denaturation solution (2M NaCl, 0.2M NaOH, 20mM EDTA). A piece of Hybond-N nylon filter was placed on impervious paper and an aliquot of each denatured DNA preparation pipetted onto the filter to form dots of approximately equal size. After the dots had dried at room temperature the filter was washed in 6x SSC, blotted dry, wrapped in Saran wrap, and the DNA bound to the filter by exposure to short wavelength ultraviolet as for Southern blots above.

2.7.3 Oligonucleotide labelling of DNA fragments to be used as probes.

The method used to radioactively label DNA fragments was based on that of Feinberg and Vogelstein (1984). The DNA fragment (50-500ng) to be used as probe was suspended in a volume of 16µl sterile distilled water and denatured by heating at 100°C for 5 minutes directly before use. The following reagents were then added to a screw capped Eppendorf tube in the order shown:

- OLB buffer
- BSA (DNAse free 10mg/ml)
- α-32P-dCTP (10mCi/ml)
- Klenow fragment of E. coli DNA polymerase 2 Units (0.5µl)

The reaction mixture was either incubated at 37°C for 30 minutes or at room temperature for at least 5 hours, after which the reaction was stopped by the addition of 100µl of stop buffer (20mM NaCl, 20mM Tris-HCl pH 7.5, 2mM EDTA, 0.25% SDS (w/v), 15M dCTP).

OLB is made up from three solutions:
- 2 volumes solution A: 1.25M Tris-HCl pH8, 125mM MgCl₂, 0.18% (v/v) β-mercaptoethanol, 0.5mM dATP, 0.5mM dGTP, 0.5mM dTTP
- 5 volumes solution B: 2M HEPES (pH6.6 with NaOH)
- 3 volumes solution C: Hexadeoxynucleotides suspended in 3mM Tris-HCl pH7.7, 0.2mM EDTA at 90 OD units per ml
2.7.4 DNA hybridisation of Southern blot and dot blot filter membranes.

The filters were cut into convenient sized strips and all reactions carried out in hybridisation boxes made of perspex and fitted with a rubber seal. The filters were prehybridized by incubation with gentle agitation at 65°C for at least 1 hour in 25ml FHM solution (15mM Na$_2$H$_2$PO$_4$ pH7, 0.27M NaCl, 1.5mM EDTA, 0.5% (w/v) Marvel milk, 1% (w/v) SDS, 6% (w/v) PEG 6000). The radioactively labelled probe solution was heated at 100°C for 5 minutes to denature the DNA and then added directly to the prehybridisation mix. Hybridisations were generally conducted with gentle agitation at 65°C overnight.

Unbound labelled DNA was removed from the filters by washing four times in 0.1xSSC, 0.1% (w/v) SDS for 30 minutes at 65°C. The filters were then dried at room temperature, mounted onto 3MM paper and covered with Saran wrap.

2.7.5 Autoradiography.

The filters were placed in a cassette against FUJI RX-100 X-ray film backed by an intensifying screen, and the film was exposed at -80°C for as long as necessary to obtain a clear signal.

2.7.6 Stripping DNA probe from the filter membrane.

When it was necessary to hybridize DNA on a filter membrane with a second DNA probe, the first probe was removed by incubation for 30 minutes at 45°C in 0.4M NaOH, followed by a similar incubation in 0.1xSSC, 0.1% (w/v) SDS, 0.2M Tris-HCl pH7.5. The filter was then incubated at 65°C in distilled water for 1 hour. If the filter was still significantly radioactive the procedure was repeated. Removal of radioactive isotope was confirmed by autoradiography.
2.7.7 Efficiency of incorporation of radioactivity into the probe.

The incorporation of radioactive $^{32}$P into the probe DNA was estimated by taking 1μl of the probe preparation after adding stop mix, diluting it in 11μl of sterile distilled water and placing a spot of 5μl onto two pieces of DE-81 paper. The spots were allowed to dry for a few minutes and then one was washed six times for 5 minutes each in a solution of 0.5M disodium hydrogen phosphate. The paper was then washed once in distilled water and in ethanol and then allowed to dry. The radioactivity of the two pieces of DE-81 paper was then compared by Cherencov counting in a scintillation counter (United Technologies Packard 2000cA Tri-card liquid scintillation counter) and the percentage incorporation calculated.
2.8 Preparation of *P. chrysogenum* total cellular RNA.

The method used for RNA extraction was based on that of Chomczynski and Sacchi (1987).

In work with RNA all solutions and plastic disposable tubes and tips where possible were treated with 0.1% diethylpyrocarbonate for 12 hours before autoclaving. All glassware and ceramics were baked for 4 hours at 250°C before use. The mycelium was processed as soon as possible after harvesting and was kept on ice if there was any delay. To avoid RNase contamination gloves were worn.

*P. chrysogenum* cellular RNA was usually prepared from young (15 hour) growing mycelium, which was harvested and immediately washed in ice cold water, rapidly frozen in liquid nitrogen and powdered under liquid nitrogen using a pestle and mortar.

For each 0.1g wet weight of mycelium, the powder was resuspended in 1ml of extraction buffer (4M guanidinium thiocyanate, 25mM sodium citrate pH7, 0.5% (w/v) sarkosyl, 0.1M β-mercaptoethanol), and 0.1 volumes of 2M Na acetate pH4 was added. Immediately, 1ml phenol (phenol with 0.1% 8-hydroxyquinoline equilibrated with 10mM Tris HCl pH 8) and 0.2ml chloroform (25ml of chloroform mixed with 2ml of isoamyl alcohol) were added, the mixture was shaken vigorously and held on ice for 15 minutes. The aqueous phase was recovered by centrifugation in corex glass tubes for 10 minutes at 10000rpm, and RNA precipitated by adding 0.6 volumes of isopropanol, holding at -20°C for 1 hour and repeating the centrifugation. The pellet so obtained was then resuspended in 0.3ml extraction buffer, precipitated again with isopropanol, ethanol washed and dried, and resuspended in water and stored at -20°C.

For long term storage, 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate pH5.2 were added to the RNA solution, which was then stored at -20°C. The RNA precipitate was recovered by centrifugation and was resuspended in water as required.
2.9 Analysis of RNA by transfer on to nitrocellulose membranes and hybridisation using radioactive probes.

2.9.1 Northern blot filter preparation

To 4.8μl of 1mg/ml RNA the following reagents were added in the order given: 10μl deionized formamide, 2μl of 10xMOPS buffer (0.2M MOPS, 0.05M sodium acetate, 0.01M EDTA, pH7) and 3.2μl formaldehyde. The sample was incubated at 65°C for 5 minutes, immediately cooled on ice, and 0.1 volumes loading buffer (25% Ficoll, 0.001% Orange C, 200mM EDTA) and 1μl of 1mg/ml ethidium bromide were added. The samples were then loaded onto a 1% agarose gel made by dissolving 1g agarose in 74.1ml of water to which 10ml of electrophoresis buffer which contained 200mM MOPS, 50mM sodium acetate pH7.0, and 10mM EDTA had been added. The molten agarose was cooled to 65°C, 17.9ml of 38% (w/v) formaldehyde added and the gel immediately poured. Electrophoresis was at 100V for about 3 hours with circulating buffer, after which the RNA was immediately transferred onto nitrocellulose in a similar manner to that described in 2.7.1 except that the transfer solution was 20xSSC and the nitrocellulose was soaked in 20xSSC before laying it on the gel. Following transfer, the RNA was bound to the filter by baking the filter between sheets of 3MM paper at 80°C for 2 hours.

To provide a molecular weight marker for the gel, 10μg of phage λ DNA digested with the restriction endonuclease HindIII was denatured by incubating for 5 minutes at 65°C in 0.15M NaOH, 0.01M EDTA pH8. Loading buffer and ethidium bromide were added to the single stranded DNA as for the RNA samples, and the marker was loaded in a lane set well to one side of the gel. Following electrophoresis the portion of the gel with marker was excised and washed in four changes of water and two changes of 0.1M ammonium acetate. The single stranded DNA was then stained with ethidium bromide by gently shaking the gel fragment for 1 hour in a solution of 0.5μg/ml ethidium bromide in 0.1M ammonium acetate and then partially destained in the same buffer lacking ethidium bromide. The marker lane was visualized under short wavelength ultra-violet illumination and photographed.
2.9.2 Hybridisation of RNA Northern blot membranes.

Prior to hybridising RNA to single stranded DNA probe prepared as described in Section 2.7.3., the filters were incubated with gentle agitation at 42°C for about 6 hours in 20mls of a solution of 50% formamide, 6xSSC, 0.25% Marvel milk (treated with DEPC). Radioactively labelled single stranded probe DNA (prepared as described in Section 2.7.3) was then added to the solution and the filters incubated overnight at 42°C with gentle agitation.

Unbound labelled DNA was removed from the filters by washing four times in 0.1xSSC, 0.1% (w/v) SDS for 30 minutes at 50°C. The filters were then dried at room temperature, mounted onto 3MM paper, covered with Saran wrap, and exposed to X-ray film as described for the autoradiography for Southern blot filters in Section 2.7.5.

When it was necessary to hybridize RNA on a filter membrane with a second DNA probe, the first probe was removed by incubation for 1 to 2 hours at 65°C in a solution containing 5mM Tris pH8, 0.2mM EDTA, 0.05% sodium pyrophosphate, 0.002% each of BSA, Ficoll and PVP. Removal of the radioactively labelled probe was confirmed by autoradiography.

2.9.3 Densitometric analysis of autoradiographs of RNA filters.

Profiles were obtained of autoradiographic images of RNA filters by scanning the developed X-ray films with an LKB Ultrascan XL laser densitometer. In order to give a linear response to radioactive decay, the X-ray film was made sensitive by low level exposure to light prior to autoradiography.
2.10 Analysis of RNA by protection against S1 nuclease digestion

S1 nuclease analysis was done with total cellular RNA and a radioactive probe made from an M13 single stranded clone by an adaption of the method of Burke (1984)

2.10.1 Probe preparation

Template DNA was prepared by a scaled up version of the method described in section 2.10.3. About 400ng of template was incubated with 4ng of universal primer and 3μl of 10x Klenow reaction buffer (100mM Tris 50mM MgCl₂ pH 8.5) in a total volume of 10μl at 60°C for 30 minutes. The radioactive second strand was synthesised by adding 16μl of dNTP solution (80mM each of dATP, dTTP, dGTP, 1.5mM dCTP, 10mM Tris pH7.5, 1mM EDTA), 2μl of α-32P-dCTP (3000Ci/mmol, 10mCi/ml) and 1μl of the Klenow fragment of E. coli DNA polymerase. The reaction was incubated for 15 minutes at 37°C. After this time 2.5μl of dCTP solution (0.5mM dCTP in 10mM Tris pH7.5, 1mM EDTA) was added to the reaction which was then incubated for 15 more minutes.

The fragment required was isolated by restriction enzyme digestion of the double stranded labelled DNA. The volume of the solution was made up to 100μl including 2μl of the appropriate enzyme and 10μl of the appropriate reaction buffer. The reaction was incubated for 1 hour at 37°C. The DNA was recovered after phenol/chloroform extraction by precipitation with ethanol. The DNA pellet was resuspended in 18μl of sterile distilled water and denatured by addition of 2μl of 1.5M NaOH, EDTA. 2l of loading dye were then added and the DNA loaded onto a 1% agarose gel. Electrophoresis was carried out for 1 hour and then the position of the desired band was determined by brief (5 minutes) autoradiography. The band was excised from the gel, the DNA collected by placing the agarose in a dialysis bag with 200-400μl of electrophoresis buffer and electroeluting for one hour. The buffer was then collected from the dialysis bag and the DNA precipitated at -80°C after extraction with an equal volume of chloroform using 5μg of carrier tRNA and 0.01M magnesium chloride to aid precipitation. The DNA was recovered by centrifugation and resuspended in 10μl of T.E.
pH8. The incorporation of radioactivity was determined by counting 1μl of the sample in a scintillation counter by Cherencov counting. The remainder of the solution was divided equally between the number of reactions to be done, usually two or three including the control reaction.

2.10.2 Hybridisation of the total RNA to the probe

Between 30 and 50μg of total cellular RNA was added to the probe in the experimental samples and the same amount of yeast tRNA added to the control sample. The nucleic acids were precipitated together at -80°C for 30 minutes and recovered by centrifugation. The nucleic acid pellet was resuspended in 2μl sterile distilled water and then 20μl of hybridisation buffer was added (80% formamide (deionised) 40mM PIPES pH 6.4, 400mM sodium chloride, 1mM EDTA pH8. The mixture was heated for five minutes at 80°C and then left at 30°C overnight.

2.10.3 SI nuclease reaction

The following reagents were added in order to the hybridisation reaction:
150μl 2x SI buffer (0.56M sodium chloride, 0.1M sodium acetate pH4.5, 9mM zinc sulphate)
3μl single stranded calf thymus DNA solution at 2mg/ml
147μl sterile distilled water.
300 units SI nuclease.

The reaction was incubated at room temperature for 1-2 hours and then the reaction stopped by the addition of 80μl of STOP buffer, (4M ammonium acetate, 20mM EDTA, 40μg/ml of tRNA) and the nucleic acids precipitated using 1ml of ethanol at -80°C for one hour. The DNA pellet was recovered and resuspended in 8μl of T.E. loading dye was added and the sample electrophoresed on an acrylamide gel as described for sequence determinations below. A set of sequencing reactions were also electrophoresed in order to estimate the size of any fragments protected from digestion by SI nuclease.
2.11 Sequence analysis of DNA subcloned into M13 phage.

2.11.1 Preparation of replicative form vector DNA.

M13 phage (Messing, 1983) were picked as a fresh plaque and used to inoculate 20mls of Luria broth seeded with one drop of an overnight culture of the E. coli strain JM101. The cells were grown with vigorous agitation at 37°C for 7 to 8 hours, pelleted by centrifugation, and the supernatant recovered. A flask containing 400ml of Luria broth was inoculated with 4ml of a rapidly growing culture of JM101 in Luria broth and 4ml of the supernatant recovered above and grown overnight at 37°C. The cells were recovered by centrifugation and replicative form DNA isolated by the method of alkaline lysis as described in Section 2.4.1.

2.11.2 Preparation of recombinant single-stranded M13 DNA sequencing templates.

Phage particles from colourless plaques were transferred with sterile toothpicks into 250μl aliquots of phage buffer (6mM Tris-HCl pH7.2, 10mM MgSO₄, 0.005% (w/v) gelatin), 50μl of which were then used to infect 2ml volumes of a JM101 culture (one drop of an overnight culture into 20mls of Luria broth). The cultures were incubated at 37°C with vigorous shaking for 6 to 8 hours, after which the cells were spun down by centrifugation in an Eppendorf microfuge for 5 minutes, and 1.2ml of the supernatant containing M13 phage was recovered. Phage particles were precipitated by the addition of 300μl of a solution of 20% (w/v) PEG 6000, 2.5M NaCl and holding at room temperature for 30 minutes. The phage were pelleted by centrifugation in an Eppendorf microfuge for 10 minutes. The supernatant was removed by aspiration and the phage pellet resuspended in 200μl of sterile distilled water. DNA was extracted from the virus particle by twice extracting with phenol/chloroform and once with chloroform. The DNA was recovered by ethanol precipitation and resuspended in 30μl of water.
2.11.3 **Determination of DNA sequence by the dideoxyribonucleotide chain termination method.**

Sequencing of M13 recombinant clones was based on the dideoxyribonucleotide chain termination method of Sanger et al. (1977), as modified by Biggin et al. (1983) and further adapted by A. Hawkins (University of Newcastle-upon-Tyne). Typically, 5μl of sequencing template (Section 2.11.2) was annealed to the 1μl of 17mer sequencing primer in 10mM Tris, 5mM MgCl₂ pH8.5 at 65°C for 1 hour. Either commercial universal primer was used, or a specific primer synthesized locally by the method of Matthes et al. (1984) with modifications to the wash cycle as described by Sproat and Gait (1985). Locally synthesized primers were ethanol precipitated twice before use.

For each template four tubes were prepared containing 2.5μl of the annealed mixture plus 2μl of either a G, A, T or C nucleotide mix shown below.

<table>
<thead>
<tr>
<th>Nucleotide mixtures for sequencing reactions (μl volumes)</th>
<th>G</th>
<th>A</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5mM dGTP</td>
<td>25</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>0.5mM dTTP</td>
<td>500</td>
<td>500</td>
<td>25</td>
<td>500</td>
</tr>
<tr>
<td>0.5mM dCTP</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>25</td>
</tr>
<tr>
<td>10mM ddGTP</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10mM ddATP</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10mM ddTTP</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>10mM ddCTP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>TE buffer</td>
<td>1000</td>
<td>1000</td>
<td>500</td>
<td>1000</td>
</tr>
</tbody>
</table>

Next, 1μl of Klenow (5 Units μl⁻¹, and 1μl of α⁻³⁵S-dATP (10Ci/ml) was added to each tube. The solutions were mixed gently and then incubated at 37°C for 20 minutes. This was followed by the addition of 2μl of sequence chase mixture (0.5mM each of dGTP, dATP, dTTP and dCTP) to each reaction and a further 20 minute incubation at 37°C.

The reactions were stopped by the addition of 4μl of formamide dye mix (stock solution containing 10ml deionized formamide, 10mg xylene cyanol-FF, 10mg bromophenol blue, 0.2ml 0.5M EDTA pH8), and the reactions were placed in a boiling water bath for 5 minutes in order to denature the DNA molecules before loading them onto a sequencing gel.
2.11.4 Sequencing gels.

The standard reagents used, sufficient to prepare one gradient gel are given below:

<table>
<thead>
<tr>
<th></th>
<th>0.5x mix</th>
<th>2.5x mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>2.05g</td>
<td>0.46g</td>
</tr>
<tr>
<td>Bis acrylamide</td>
<td>0.11g</td>
<td>0.02g</td>
</tr>
<tr>
<td>Urea</td>
<td>18.0g</td>
<td>4.0g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.4g</td>
<td></td>
</tr>
<tr>
<td>10x TBE</td>
<td>1.8ml</td>
<td>2.0ml</td>
</tr>
<tr>
<td>Water to a final volume of</td>
<td>36.0ml</td>
<td>8.0ml</td>
</tr>
</tbody>
</table>

10x TBE: 1M Tris-borate pH8.3, 20mM EDTA

Preparation and running of 36x20cm 6% polyacrylamide gels were as follows:

The solutions were dissolved with stirring and moderate heating, and the following added:-

<table>
<thead>
<tr>
<th></th>
<th>0.5x mix</th>
<th>2.5x mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% Ammonium persulphate</td>
<td>72μl</td>
<td>16μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>72μl</td>
<td>16μl</td>
</tr>
</tbody>
</table>

The gradient gels were poured as follows: 4mls of the 0.5x mix was drawn up in a glass 10ml pipette followed by 6mls of the 2.5x mix. The mixture was poured down the centre of prepared sequencing gel plates carefully, avoiding air bubbles, and was topped up with 0.5x mix down each side of the gel mould. The glass plates were then laid horizontally, a "shark’s tooth" comb inserted with the flat face down the gel, and the gel firmly clamped with "bulldog" clips. Once the gel was set, the comb was taken out, the slot washed out with 0.5x TBE, and the comb reinserted with the teeth piercing the gel to a distance of about 1mm.

The samples were loaded, each in a separate slot, and electrophoresed at 1300V with 0.5x TBE running buffer in the upper reservoir and 1x TBE running buffer in the lower.
reservoir, until the leading dye was about to run off the gel. The glass plates were disassembled and gels were then fixed in 10% acetic acid 10% methanol for 15 minutes, transferred onto Whatman 3MM paper, dried using an ATTO gel drier, and exposed to X-ray film at room temperature for as long as necessary to obtain a clear signal.
2.12 Computing.

The analysis of DNA sequences, in particular to identify restriction endonuclease sites and consensus elements, was conducted using programs provided by the University of Wisconsin Computer Group, and run on the University of Leicester DEC VAX cluster.

This thesis was compiled using the WORD-11 word processing package which was run on the DEC VAX cluster.

2.13 Source of materials.

Reagents and chemicals were of analytical grade and most were supplied by Fisons plc. and Sigma Chemical Co. Ltd., U.K. Chemicals obtained from other sources are listed below:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radionucleotides</td>
<td>Amersham, England</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>BDH Ltd., England</td>
</tr>
<tr>
<td>TEMED</td>
<td>Bio-Rad, Richmond, USA</td>
</tr>
<tr>
<td>C.I. alkaline phosphatase</td>
<td>Boehringer Mannheim GmbH</td>
</tr>
<tr>
<td>HGT agarose</td>
<td>FMC BioProducts, USA</td>
</tr>
<tr>
<td>Restriction enzymes</td>
<td>GIBCO BRL Ltd., Scotland T4</td>
</tr>
<tr>
<td>DNA Ligase</td>
<td></td>
</tr>
<tr>
<td>Deoxyribonucleoside triphosphates</td>
<td></td>
</tr>
<tr>
<td>T4 DNA kinase</td>
<td></td>
</tr>
<tr>
<td>IPTG</td>
<td>Novo Biochem</td>
</tr>
<tr>
<td>X-gal</td>
<td></td>
</tr>
<tr>
<td>NovoZym 234</td>
<td>Novo Biolab., Denmark</td>
</tr>
<tr>
<td>Klenow fragment</td>
<td>Pharmacia Ltd., England</td>
</tr>
<tr>
<td>ACV</td>
<td>SmithKline Beecham, U.K.</td>
</tr>
<tr>
<td>IPN</td>
<td></td>
</tr>
</tbody>
</table>

2.14 Containment and safety.

All experiments conducted in the work described in this thesis were done with reference to the Genetic Manipulation Advisory Group's guidelines on safety and containment conditions for such work. All procedures fall within the category of good microbial practice.
Chapter 3

Isolation of the PGK gene of Penicillium chrysogenum.

The Penicillium chrysogenum PGK gene was expected to be very similar in DNA sequence to the PGK gene previously isolated from Aspergillus nidulans since the amino acid sequence of the PGK protein is strongly conserved in evolution (Chapter 1 section 3). Preliminary Southern analysis showed that defined restriction fragments of P. chrysogenum DNA hybridised to a coding region PGK probe from A. nidulans (Streatfield unpublished). Therefore to isolate the P. chrysogenum PGK gene the coding region PGK probe from A. nidulans was used to screen a cosmid library of P. chrysogenum genomic DNA for positively reacting clones by colony hybridisation. Further analysis of such clones identified those containing the PGK gene without rearrangement compared to genomic DNA and the final verification was obtained by sequence analysis of the putative gene. A similar strategy was used to isolate cosmids which may contain the catabolic dehydroquinase gene from P. chrysogenum by using the corresponding QUTE gene from A. nidulans as a probe (Appendix 1).

3.1.1 P. chrysogenum strains used and the origin of the cosmid library

The P. chrysogenum strain N.R.R.L. 1951 (Raper et al. 1944) was used to prepare genomic DNA to test the hybridisation of the A. nidulans PGK probe to P. chrysogenum DNA. The cosmid library used to isolate the PGK gene from P. chrysogenum was constructed from the genomic DNA of P. chrysogenum strain OLI13, an oligomycin resistant mutant of the nicotinamide requiring strain HP60 which is a derivative of N.R.R.L. 1951 (Bull et al. 1988).

The cosmid library containing P. chrysogenum genomic DNA was constructed by Professor G. Turner's group. Genomic DNA from strain OLI13 was partially digested with Sau3A and fragments of an average size of 30kb were inserted into the BamH1 site of the cosmid vector pCAP2 (Figure 3.1). The resultant molecules were packaged in phage lambda proteins.
and introduced into the E. coli strain DH5. I was given an aliquot of these cells frozen at -70°C.

3.1.2 Preparation of PGK coding region DNA probe from A. nidulans

A 1.5kb Aspergillus nidulans PGK coding sequence probe was isolated from the plasmid pPGK2 (Clements 1986; Figure 3-2), which bears the entire A. nidulans PGK gene. Approximately 100µg of this plasmid was digested with the restriction enzymes HindIII and PstI together and the products separated by electrophoresis in a 1% low melting point agarose gel. The 1.5kb DNA fragment was excised in an agarose block and the DNA extracted by the method described in Chapter 2 section 6.3A and resuspended in 20µl of TE. About 10µg was recovered. Samples of 100-500ng of the fragment, labelled by the random oligonucleotide priming method (Chapter 2 section 7.3), were used in each hybridisation reaction with chromosomal or cosmid DNA and 50-100ng used for hybridisations to plasmid DNA. The 1.5kb. nidulans PGK gene probe contains all of the coding region of A. nidulans PGK excepting the first five codons of the gene together with 150 nucleotides of sequence 3' to the stop codon (Figure 3-2).

3.13 Hybridisation of A. nidulans PGK probe to P. chrysogenum genomic DNA

To verify that P. chrysogenum genomic DNA would hybridise to the A. nidulans PGK probe just described and to establish the hybridisation stringency required for a good signal, a Southern analysis (Chapter 2 section 7) was done with P. chrysogenum genomic DNA using the A. nidulans PGK probe. Separate samples of 5µg P. chrysogenum N.R.R.L 1951 genomic DNA were incubated overnight with the restriction enzymes EcoRI, HindIII, or BamHI. A sample of 5µg A. nidulans genomic DNA digested with BamHI was included as a positive control. After separation by electrophoresis on a 0.7% agarose gel the digestion products were transferred to Hybond-N membrane and placed in a solution containing 1.5xSSPE (0.015M sodium phosphate buffer pH7.0, 0.27M sodium
chloride, 1.5mM EDTA), 0.5% Marvel milk, 1% SDS, 6% PEG 6000 and hybridised with 400ng of A. nidulans PGK probe at 65°C. Four 30 minute post hybridisation washes were done at 65°C in 0.1% SDS, 0.1xSSC after which the filters were dried and exposed to X-Ray film. The positive control yielded a 9.2kb band, close to the expected size of 9.4kb, which hybridised to the A. nidulans PGK probe (Streatfield unpublished). For each P. chrysogenum genomic DNA sample one band hybridised to the radioactive A. nidulans PGK probe (Figure 3-2). The size of the hybridising fragments of P. chrysogenum genomic DNA were 9.4kb for the EcoR1 digested DNA, 9.4kb for the BamH1 digested DNA and 1.5kb for the HindIII digested DNA. Therefore P. chrysogenum genomic DNA contains a sequence which hybridises to an A. nidulans PGK gene probe under conditions of high stringency.
**Figure 3-1: A restriction map of the cosmid pCAP2**

The origin of the DNA sequences in the cosmid vector pCAP2 used in the construction of a *Penicillium chrysogenum* genomic library is indicated:

- Phage λ cos site
- pBR325 DNA
- A. nidulans DNA
- N. crassa DNA

**Restriction enzyme sites**

H=HindIII  
P=PstI  
E=EcoRI  
S=SalI  
Xa=XbaI  
N=NcoI  
Bg=BglII  
B=BamHI
pCAP2

8.53kb

ori

amp

pyr4

tet

ansi

Bg

P

E

H

H

B

H

P

Bg
Figure 3-2: Hybridisation of the A. nidulans PGK gene probe to P. chrysogenum genomic DNA

A: The origins of the DNA sequences and the position of the A. nidulans PGK gene in the plasmid pPGK2, (Clements 1986) are indicated:

\[
\begin{align*}
\text{pUC13 DNA} & \quad \downarrow \\
\text{A. nidulans DNA} & \quad \rightarrow \\
\text{PGK coding sequence} & \quad \uparrow \\
\text{extent of PGK gene probe} & \quad \swarrow
\end{align*}
\]

The direction of transcription of the PGK gene is indicated by an arrow.

B: The autoradiograph shows the hybridisation of the A. nidulans PGK probe to P. chrysogenum genomic DNA. The products of digestion of 5μg of P. chrysogenum genomic DNA with EcoR1, BamH1 or HindIII were separated by electrophoresis on a 0.7% agarose gel and transferred to Hybond-N and hybridised to 400ng of radioactively labelled A. nidulans PGK coding region probe isolated from plasmid pPGK2 shown above. The hybridisation was done as described in Chapter 2 section 7.

The size markers (M) are the DNA fragments generated by the digestion of phage Lambda DNA with HindIII.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Source of DNA</th>
<th>Restriction enzyme</th>
<th>Band size (kb) Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A. nidulans</td>
<td>EcoR1</td>
<td>9.2</td>
<td>9.3</td>
</tr>
<tr>
<td>2</td>
<td>P. chrysogenum</td>
<td>EcoR1</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>P. chrysogenum</td>
<td>BamH1</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>P. chrysogenum</td>
<td>HindIII</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>
3.1.4 Screening the cosmid library of \textit{P. chrysogenum} genomic DNA for the PGK gene.

The minimum number of cosmid clones of average insert size 30Kb required to isolate with a probability of 0.99 a 3kb region containing the PGK gene of \textit{P. chrysogenum} was estimated using the method described by Clark and Carbon (1975). The genome size for \textit{P. chrysogenum} was not available size of twice the \textit{A. nidulans} genome was assumed. The number of clones required was about 9,000 (Appendix 2) and about 12,000 colonies were screened.

Aliquots of frozen cells of the cosmid library were diluted in Luria broth and spread onto nitrocellulose filters on L-agar/ampicillin plates to give an estimated 2,000 small discrete colonies per plate following overnight growth at 30°C. Six plates yielding an even distribution of colonies were chosen to provide the master filters. These filters were replicated and the replicas processed as described in Chapter 2 section 4.2 to yield cosmid DNA bound to the nitrocellulose filters. The cosmid DNA was hybridised to 500ng of the 1.5kb \textit{A. nidulans} PGK probe DNA at 65°C and post hybridisation washes done with 1xSSC, 0.1xSDS at 60°C, lower stringency conditions than used in the test hybridisation.

Six positive signals from the hybridisation which appeared in identical positions on the duplicate filters (Figure 3-3) were selected and a small patch of nitrocellulose cut from around the area corresponding to each signal on the master plate. The cells on each piece of nitrocellulose were resuspended in a small volume of L-Broth and aliquots were spread onto L-agar plates to yield a few hundred colonies per plate for a second screen which was done in a similar way to that described above except that duplicate filters were made directly from the colonies on the agar plates. After the second screen several separate colonies from the area of a positive signal were isolated and restreaked in a grid pattern on an L-agar plate. Groups of colonies corresponding to each of four signals were transferred to a grid in this manner. Two copies of the grid were made on nitrocellulose filters and after growth,
amplification and DNA release the filters were again hybridised to the PGK probe.

In this way four independently isolated clones which hybridised to the PGK gene were recovered. The clones were further purified by twice replating for single colony isolation and the homogeneity checked by screening twenty colonies from each of the positive strains. Three of the four strains containing cosmids designated P1-3 yielded positive signals for all 20 of the colonies hybridised to probe, but the fourth showed no hybridisation and was discarded.
The cosmid library of *P. chrysogenum* genomic DNA was hybridised to a radioactively labelled *A. nidulans* PGK probe, a 1.5kb HindIII-PstI fragment of pPGK2 (Figure 3-2), radioactively labelled with $\alpha^{32}P$-dCTP. The hybridisation was done as described in Chapter 2 section 7 except that the post hybridisation washes were done at 60°C in 1xSSC, 0.1% SDS. The two autoradiographs are of duplicate nitrocellulose filters. The arrows indicate signals which occur in identical positions on both filters and therefore represent positive clones. The circled dots are orientation markers.
3.1.5 Verification of the selected clones

Cosmid DNA for Southern analysis was prepared by alkaline lysis from 3ml cultures of the three positive strains containing P1-3. About a microgram of the cosmid DNA was digested with EcoR1 (Chapter 2 section 6.1), the products separated by electrophoresis on a 0.7% agarose gel, transferred to Hybond-N and hybridised to the 1.5kb PGK probe. A single EcoR1 fragment from each cosmid of approximately 9kb hybridised to the probe (Figure 3-4). The pattern of bands generated by the digestion with EcoR1 of cosmid DNAs P2 and P3 were identical and the third cosmid had some bands in common with them.

The structure of the region of the cosmids P1 and P2 which hybridised to the A. nidulans PGK probe was compared to the structure of the region around the PGK gene in P. chrysogenum genomic DNA by Southern analysis. Samples of approximately 1μg of the cosmid DNA and 5μg of P. chrysogenum genomic DNA were digested with the enzymes EcoRI, BamHI, XhoI, XbaI and PstI both singly and in all pairwise combinations. The double digests were done together in the same buffer except those with EcoRI or BamHI and XbaI, XhoI, or PstI where the digestions were sequential since EcoRI and BamHI require React 3 and the other enzymes React 2 (Chapter 2 section 6.1). The products of the digestions were separated on a 0.7% agarose gel, transferred to a nitrocellulose filter and hybridised to the A. nidulans PGK probe.

Cosmid P1 generated a different hybridisation pattern to P. chrysogenum genomic DNA treated in the same way and therefore the region to which the probe hybridised in the cosmid was not the same as the region in the genome which hybridised to the probe. The cosmid P2 yielded a pattern very similar to P. chrysogenum genomic DNA, showing that the region hybridising to the probe was the same in cosmid P2 as in genomic DNA (Figures 3-5 and 3-6). The cosmid P1 may differ because of rearrangements occurring in the construction of the library or because the PGK gene is close to one end of the inserted genomic DNA and the neighbouring cosmid DNA changed the sizes of the hybridising bands. The insert in P2 was about 36kb as deduced from the restriction digest of P2 with EcoRI. Cosmid P2 was taken for further analysis.
Figure 3-4: Hybridisation of the *A. nidulans* PGK probe to cosmid DNA of three positive clones isolated from the cosmid library.

A: The photograph shows the DNA fragments generated when samples of the three cosmids (P1-3) were incubated with EcoR1, the products separated by electrophoresis on a 0.7% agarose gel and stained with ethidium bromide at 0.5 μg/ml. The DNA size markers (M) are as described in Figure 3-2. The order of the samples is as follows:-

<table>
<thead>
<tr>
<th>Lane</th>
<th>Source of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cosmid P1</td>
</tr>
<tr>
<td>2</td>
<td>cosmid P2</td>
</tr>
<tr>
<td>3</td>
<td>cosmid P3</td>
</tr>
</tbody>
</table>

B: The autoradiograph shows the hybridisation under the conditions described in Chapter 2 section 7, of the 1.5kb *A. nidulans* PGK coding region probe (Figure 3-2) to cosmid DNA fragments transferred to Hybond-N from the gel shown above. The hybridising band in each of the three lanes is 9.6kb in size.
Figure 3–5: Hybridisation patterns of the *A. nidulans* PGK probe to restriction fragments generated from *P. chrysogenum* genomic DNA, cosmid P1 and P2 DNAs

Samples of *P. chrysogenum* genomic DNA, cosmid P1 and cosmid P2 DNA were digested with BamHI, PstI, XbaI or XhoI. The digestion products were separated on 0.7% agarose gels by electrophoresis, transferred to Hybond-N and hybridised to the *A. nidulans* PGK coding region probe (Figure 3-2) as described in Chapter 2 section 7. The size markers (M) are as described in Figure 3-2.

Autoradiograph A shows the result for *P. chrysogenum* genomic DNA digested with XbaI, XhoI and PstI. *P. chrysogenum* genomic DNA digested with BamHI gives a 9.8kb band when hybridised to the *A. nidulans* PGK probe (Figure 3-2).

Autoradiograph B shows the result for cosmids P1 and P2. The table shows the size of the hybridising bands.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Source of DNA</th>
<th>Enzyme</th>
<th>Size of hybridising band (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td><em>P. chrysogenum</em> genomic DNA</td>
<td>Xhol</td>
<td>5.1</td>
</tr>
<tr>
<td>A2</td>
<td>&quot; &quot;</td>
<td>Xbal</td>
<td>4.8</td>
</tr>
<tr>
<td>A3</td>
<td>&quot; &quot;</td>
<td>Pstl</td>
<td>14.5</td>
</tr>
<tr>
<td>B1</td>
<td>Cosmid P1</td>
<td>Xhol</td>
<td>5.4</td>
</tr>
<tr>
<td>B3</td>
<td>&quot; &quot;</td>
<td>Xbal</td>
<td>7.6</td>
</tr>
<tr>
<td>B5</td>
<td>&quot; &quot;</td>
<td>Pstl</td>
<td>12.5</td>
</tr>
<tr>
<td>B7</td>
<td>&quot; &quot;</td>
<td>BamHI</td>
<td>17.5</td>
</tr>
<tr>
<td>B2</td>
<td>Cosmid P2</td>
<td>Xhol</td>
<td>5.3</td>
</tr>
<tr>
<td>B4</td>
<td>&quot; &quot;</td>
<td>Xbal</td>
<td>4.5</td>
</tr>
<tr>
<td>B6</td>
<td>&quot; &quot;</td>
<td>Pstl</td>
<td>16</td>
</tr>
<tr>
<td>B8</td>
<td>&quot; &quot;</td>
<td>BamHI</td>
<td>11.5</td>
</tr>
</tbody>
</table>
Figure 3-6: Further hybridisation of genomic DNA, and cosmid P1 and P2 DNA to the A. nidulans PGK probe

Samples of P. chrysogenum genomic DNA, cosmid P1 and cosmid P2 DNAs were digested with combinations of pairs of enzymes of the set EcoRI, BamHI, PstI, XhoI, and XbaI. The products were separated by electrophoresis on 0.7% agarose gels, transferred to Hybond-N and hybridised to the 1.5kb A. nidulans PGK probe (Figure 3-2) as described in Chapter 2 section 7. The size markers (M) are as described in Figure 3-2.

Autoradiograph A shows the pattern of hybridising bands obtained for P. chrysogenum genomic DNA, Autoradiograph B shows the pattern obtained for P1 cosmid DNA. Autoradiograph C shows the pattern obtained for P2 cosmid DNA.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Genomic DNA</th>
<th>Band Size (kb)</th>
<th>Cosmid P1</th>
<th>Band Size (kb)</th>
<th>Cosmid P2</th>
<th>Band Size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI/BamHI</td>
<td>1</td>
<td>9.4</td>
<td>1</td>
<td>10.6</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>BamHI/PstI</td>
<td>7</td>
<td>10.5</td>
<td>2</td>
<td>9.2</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>EcoRI/XhoI</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>5.5, 4.6</td>
<td>3</td>
<td>5.1, 0.55</td>
</tr>
<tr>
<td>PstI/XhoI</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5.4, 3.8</td>
<td>4</td>
<td>5.1, 0.55</td>
</tr>
<tr>
<td>PstI/XbaI</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>5.4</td>
<td>5</td>
<td>4.4</td>
</tr>
<tr>
<td>XhoI/XbaI</td>
<td>10</td>
<td>1.7</td>
<td>6</td>
<td>1.6</td>
<td>6</td>
<td>1.6, 0.55</td>
</tr>
<tr>
<td>BamHI/XbaI</td>
<td>8</td>
<td>4.3</td>
<td>7</td>
<td>5.6</td>
<td>7</td>
<td>4.4</td>
</tr>
<tr>
<td>BamHI/XhoI</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>5.1, 0.55</td>
</tr>
<tr>
<td>EcoRI/PstI</td>
<td>4</td>
<td>9.4</td>
<td>9</td>
<td>4.7</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>EcoRI/XbaI</td>
<td>2</td>
<td>4.2</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td>4.4</td>
</tr>
</tbody>
</table>
3.2.1 Subcloning and analysis of the putative PGK gene of P. chrysogenum.

In order to further define the PGK gene of P. chrysogenum it was necessary to subclone it into a suitable vector and pUC19 (Yanisch-Perron et al. 1985) was chosen for this purpose. The size of the P. chrysogenum PGK gene including 5' and 3' regions was estimated to be 3Kb. Therefore the 9.8 Kb EcoRI fragment of P2 was chosen as the fragment to be subcloned because it would probably contain the entire PGK gene and it was also well separated from other EcoRI digestion products of P2 after electrophoresis on a 0.7% agarose gel.

Approximately 3-4μg of cosmid P2 was incubated with EcoRI and the products separated by electrophoresis on a 0.7% agarose gel. The 9.8kb band was excised and purified as described and approximately 1μg recovered. About 600ng of the vector pUC19 was also digested with EcoRI and the linear molecules purified by phenol/chloroform extraction and ethanol precipitation. Three ligation reactions (Chapter 2 section 6.5) were set up with approximately 10ng of linearised vector and about 40, 80, or 200ng of the 9.8kb fragment. A control ligation reaction was also set up with 40ng of linearised vector alone. The ligation products were transformed into the E. coli strain JM83 which was then spread onto L-agar/ampicillin plates containing X-gal and IPTG (Chapter 2, section 2.5). Transformed cells containing plasmids with inserts would produce colourless colonies when spread on this medium because the presence of the insert would inactivate the β-galactosidase gene of pUC19. Transformed bacterial cells containing plasmids without inserts would produce blue colonies because the β-galactosidase gene on pUC19 would still be active. Transformation of JM83 with uncut vector and the products of the control ligation reaction of the vector alone gave blue colonies showing that the transformation had worked and that the linearised vector could religate. The products of each of the other ligation reactions gave a mixture of blue and white colonies. Seventeen white colonies containing pUC19 plasmids with inserts were isolated and their plasmid DNA tested for the 9.8kb fragment by restriction analysis using
EcoRI. One plasmid with the required insert named pPC1 was used for further analysis.

3.2.2 The restriction map and the orientation of pPC1

A large scale preparation of plasmid pPC1 DNA was made and the DNA purified on a CsCl gradient (Chapter 2, section 4.3). Single and double digests of 1.2μg samples of this DNA were done with EcoRI, XhoI, XbaI, or HindIII. The published sequence of part of the P. chrysogenum PGK gene showed that the restriction enzyme Kpnl has a restriction site within the PGK coding region (Koekman et al. 1986). Therefore pPC1 was also incubated with Kpnl alone and with Kpnl and HindIII together. The double digestion was done sequentially, the DNA being extracted with phenol/chloroform and recovered by precipitation as described between incubation with each enzyme. The digestion products were separated by electrophoresis on a 0.7% agarose gel, photographed and transferred to Hybond-N. The fragments of the digested plasmid which contained the putative PGK gene were identified by Southern analysis using the A. nidulans PGK gene probe. The data and map of restriction sites obtained is presented in Figures 3-7 and 3-8 respectively. The results show that pPC1 contains a 1.5kb region which hybridises to the PGK probe of A. nidulans with flanking regions 4.5Kb and 3.5Kb in size.

The orientation of the putative PGK gene within pPC1 was determined by hybridisation of the digested DNA, used in the previous experiment, to 5′ and 3′ portions of the A. nidulans PGK probe. The A. nidulans probe fragment contains a BglII site which is asymmetrically placed. Digestion of the 1.5 kb probe fragment with BglII yields a fragment of 690bp which is the 5′ part of the coding region, and a 820bp fragment which is the 3′ part (Figure 3-2). The two fragments were separated by electrophoresis on a 1% agarose gel, excised and purified in the same way as the original probe fragment. Approximately 50-100ng of each fragment were used in hybridisations with the digested DNA. Figure 3-9 shows the results obtained and the deduced direction of transcription of the PGK gene, is included in Figure 3-8.
Figure 3-7: Restriction analysis and Southern hybridisation of plasmid pPCl.

A: The photograph shows the DNA fragments generated by digestion of pPCl with the enzymes, EcoRl, Hindlll, Xhol, Xbal, and Kpnl singly and in pairs. The DNA fragments were separated by electrophoresis on a 0.7% agarose gel. The sizes of each restriction fragment are shown in the table. The DNA size markers (M) are as described in Figure 3-2.

B: The autoradiograph shows the pattern of hybridisation, to the entire 1.5kb A. nidulans PGK probe of the above DNA, after its transfer to a Hybond-N membrane. The hybridisation conditions were as described in Chapter 2 section 7.4. Those bands which hybridise to the PGK probe are indicated by underlining in the table.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Enzymes</th>
<th>Observed fragment sizes (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EcoRl</td>
<td>9.4, 2.6</td>
</tr>
<tr>
<td>2</td>
<td>HindIII</td>
<td>5.7, 2.4, 1.6, 1.5, 0.59</td>
</tr>
<tr>
<td>3</td>
<td>Xbal</td>
<td>4, 3.8, 2.3, 1.7</td>
</tr>
<tr>
<td>4</td>
<td>Xhol</td>
<td>5, 4.2, 2.2, 0.55</td>
</tr>
<tr>
<td>5</td>
<td>EcoRl/HindIII</td>
<td>3.9, 2.6, 1.6, 1.45, 0.4</td>
</tr>
<tr>
<td>6</td>
<td>EcoRl/Xbal</td>
<td>4, 2.6, 2.3, 1.9, 1.7</td>
</tr>
<tr>
<td>7</td>
<td>EcoRl/Xhol</td>
<td>5, 2.6, 2.2, 1.5, 0.55, 0.4</td>
</tr>
<tr>
<td>8</td>
<td>Xbal/HindIII</td>
<td>4.3, 2, 1.6, 1.45, 1.2, 0.9, 0.6</td>
</tr>
<tr>
<td>9</td>
<td>Xbal/Xhol</td>
<td>3, 2.3, 1.9, 1.7, 1.35, 1.25, 0.55, 0.4</td>
</tr>
<tr>
<td>10</td>
<td>Xhol/HindIII</td>
<td>3.2, 3, 1.8, 1.6, 0.7, 0.5, 0.4</td>
</tr>
<tr>
<td>11</td>
<td>Kpnl</td>
<td>6.6, 3.1, 1.1, 0.74</td>
</tr>
<tr>
<td>12</td>
<td>Kpnl/HindIII</td>
<td>5.7, 2, 1.3, 0.9, 0.6</td>
</tr>
</tbody>
</table>
Figure 3.8: Restriction map of \textit{P. chrysogenum} genomic DNA around the PGK gene cloned in pPC1

This figure shows the restriction map deduced from the data presented in Figure 3.7. It also shows the origins of the DNA fragments subcloned into phage M13 mp19 to form PC2.1, PC2.2, PC3.2 and PC3.4. The method used to establish the orientation of the subclones is described in section 3.2 of the text.

\begin{itemize}
  \item E=EcoRI
  \item H=HindIII
  \item K=KpnI
  \item Xa= XbaI
  \item Xo=XhoI
\end{itemize}

---

- pUC19 DNA
- \textit{P. chrysogenum} DNA
- region hybridising to PGK
- direction of transcription
- M13 DNA
- multiple cloning site
- primer binding site
**Figure 3.9: Orientation of the putative *P. chrysogenum* PGK gene**

**Autoradiograph A** shows the hybridisation of the DNA on the nylon filter used to deduce the restriction map of pPC1 (Figure 3-7) to a radioactively labelled DNA probe consisting of 690 nucleotides of the 5' part of the *A. nidulans* PGK gene (Figure 3-1). The hybridisation was done as described in Chapter 2 section 7.

**Autoradiograph B** shows the hybridisation of the same DNA to a similarly labelled DNA probe containing 820 nucleotides of the 3' part of the *A. nidulans* PGK probe under the same conditions.

The DNA size markers (M) are as described in Figure 3-2.

The Table shows the sizes of the fragments hybridising to each probe. The deduced direction of transcription is shown on the restriction map in Figure 3-8. The lane marked with an asterisk illustrate the difference in hybridisation of the two probes.

<table>
<thead>
<tr>
<th>lane</th>
<th>Enzyme</th>
<th>5' probe</th>
<th>3' probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EcoRI</td>
<td>9.4</td>
<td>9.4</td>
</tr>
<tr>
<td>2</td>
<td>HindIII</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>XbaI</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>4*</td>
<td>XhoI</td>
<td>5, 0.55</td>
<td>0.55</td>
</tr>
<tr>
<td>5</td>
<td>EcoRI/HindIII</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>EcoRI/XbaI</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>7*</td>
<td>EcoRI/Xhol</td>
<td>5, 0.55</td>
<td>0.55</td>
</tr>
<tr>
<td>8</td>
<td>XbaI/HindIII</td>
<td>1.45</td>
<td>1.45</td>
</tr>
<tr>
<td>9*</td>
<td>XbaI/XhoI</td>
<td>1.7, 0.55</td>
<td>0.55</td>
</tr>
<tr>
<td>10*</td>
<td>XhoI/HindIII</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>11*</td>
<td>KpnI</td>
<td>0.74</td>
<td>3.1</td>
</tr>
<tr>
<td>12*</td>
<td>KpnI/HindIII</td>
<td>0.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>
3.2.3 Subcloning of the coding and upstream sequences of the PGK gene

The HindIII fragments of pPC1 which contain the coding and 5' sequences of the putative PGK gene of *P. chrysogenum* (Figure 3-8) were subcloned into M13mp19 in preparation for sequence analysis (Norrander et al. 1983). The 1.5 kb fragment was expected to yield coding region sequence immediately which would confirm the cloned DNA as being the PGK gene.

The 1.5 kb fragment was separated by electrophoresis from the 1.6 kb fragment on a 1% agarose gel and was excised and recovered. The double stranded replicative form (RF) DNA of M13mp19 was also digested with HindIII and the linear molecules recovered. Two ligation reactions, the first containing an estimated 50ng of 1.5Kb fragment with 20ng of vector and the second a positive control as before containing an estimated 20ng of vector alone, were set up. The products were transformed into *E. coli* JM101 (Chapter 2 section 2.7) and overlaid onto BLA plates containing X-gal and IPTG which allowed selection of M13mp19 clones containing inserts.

Single stranded and RF DNA was prepared from six isolated clones and screened for the 1.5kb insert by electrophoresis of the single stranded DNA, the required clones being subjected to restriction analysis of the RF DNA with HindIII.

The orientation in the vector of the 1.5 Kb inserts was determined by annealing single stranded DNA from pairs of the clones. Samples of 2.5µl (several hundred nanograms) of each of the DNAs were annealed in pairs at 60°C for one hour in 10mM Tris, 5mM Magnesium chloride, pH8.5. The products were separated on an agarose gel and retardation of one pair of samples compared to all the others was observed. The single stranded DNAs in the two samples had annealed to one another and therefore contained the 1.5kb insert in opposite orientation with respect to the M13mp19 vector. These two clones were selected for sequence analysis and were designated PC2.1 and PC 2.2 (Figure 3.8).

To obtain the 1.6Kb HindIII fragment, containing the 5' flanking sequences, uncontaminated by the 1.5 kb HindIII fragment the plasmid pPC1 was digested with HindIII and XhoI.
simultaneously, because XhoI cleaves the 1.5 Kb fragment. The products were separated by electrophoresis on a 0.7% agarose gel. The intact 1.6Kb fragment was excised from the gel and purified. The vector M13 mpl9 was digested with HindIII and purified as above. Three ligation reactions were set up containing approximate amounts of 10ng or 30ng of vector with 50ng of the 1.6kb insert and the positive control of 10ng of vector alone. The ligation products were introduced into the E. coli strain JM101 and after overnight growth five white plaques were isolated and amplified. Double stranded RF DNA was prepared from the cultures and tested for the presence of a 1.6Kb insert by restriction analysis with HindIII. Those clones which contained the 1.6Kb insert were then analysed further by restriction analysis with XbaI to establish the orientation of the inserts. A pair of clones with inserts of opposite orientation were selected for sequencing. These clones designated PC3.2 and PC3.4 contain the 1.6kb promoter region immediately upstream of PGK (Figure 3.8).

Double stranded DNA was prepared from all four of the clones for further manipulation and single stranded phage DNA prepared for sequencing using the universal primer.

3.2.4 Verification of PGK by sequence analysis

The M13 clone PC2.1 was thought to contain the coding region of PGK close to the binding site for the universal primer, therefore to verify that the cloned DNA contained the PGK gene, the sequence of part of the P. chrysogenum DNA present in PC2.1 was determined (Chapter 2, section 11). Sequence was obtained for one strand up to 210 bases from the HindIII site in PC 2.1 in the multiple cloning site adjacent to the to the binding site for universal primer. This sequence was compared to the sequence published for PGK in P. chrysogenum and A. nidulans (Van Solingen et al. 1988, Clements and Roberts 1986). The similarity was 98% compared to the published coding sequence of P. chrysogenum and 74% similar to the A. nidulans PGK sequence but the comparison ran over part of an intron so the similarity of sequence between the A. nidulans gene and the P. chrysogenum gene is greater than 74% in the coding region of the gene (Figure 3-10).
Figure 3-10: Sequence comparison of the cloned *P. chrysogenum* PGK gene

**A: Comparison to the *P. chrysogenum* published sequence**

The top line is the sequence obtained from the M13 clone PC2.1 and the bottom line is the published coding sequence of PGK in *P. chrysogenum* (van Solingen et al. 1988). The GAP program in the University of Wisconsin molecular biology package was used to establish sequence identity.

UPPERCASE LETTERS— exon  
lowercase letters— intron  
| identity of sequence

**B: Comparison to the *A. nidulans* sequence.**

The top line is the sequence data obtained from the M13 clone PC2.1 and the bottom line is the PGK coding sequence from *A. nidulans* (Clements and Roberts 1986). The GAP program from the University of Wisconsin molecular biology package was used to align the sequences.
A

1 ..........................gagcttcattacttgcagcc 20
51 GCGTGTCTTGATCCGGgtagcttcatttgcagcc 100
21 cctagacctgctactgagatagGTGACTTCATAGGCTCCCTCGATGAGA 70
101 cctgaaccctgctactgagatagGTGACTTCATAGGCTCCCTCGATGAGA 149
71 ACAAGAAGCTTACCA.ACCCAGCGTATCGTGCCCTGCCACCAGT 119
150 ACGAGAAGCTTACCAACCCAGCGTATCGTGCCCTGCCACCATC 199
120 AAGTATGCACTCGACACCGGCGGCAAGGCCGTTGTGCTCATGTCCCACCT 169
200 AAGTATGCACTCGACACCGGCGGCAAGGCCGTTGTGCTCATGTCCCACCT 249
170 GGGTCGCCCCTGATGGCAAGGCCCAACCAGATACAGCTTTGA....... 210
250 GGGTCGCCCCTGATGGCAAGGCCCAACCAGATACAGCTTTGAGGCCCTG 299

B

1 ....gagcttcattacttgcagccctagacctgctactgagatagGTGAC 48
751 ataaacgccccctaatgacccctacagctactagGTGAC 800
49 TTCAATGCCTCCCCCTCGATGAGAAGACAAGACAAGTTA........CCAACCCCA 91
801 TTCAATGCACTCGACACCGGCGGCAAGGCCGTTGTGCTCATGTCCCACCTCA 850
92 GCGTATGTCGTCGGCTGCCACCATGAAAGTGATGCACTCGACACCGGTG 141
851 GCGTATGTCGTCGGCTGCCACCATGAAAGTGATGCACTCGACACCGGTG 900
142 CCAAGGCCGGTTGCTACATGTCGCCACCTGGGCTGCCGATGGGCAAGGTG 191
901 CCAAGGCCGGTTGCTACATGTCGCCACCTGGGCTGCCGATGGGCAAGGTG 950
192 AACCCAAAGTACAGCTTGA.......................... 210
951 AACCCAAAGTACAGCTTGAAGGCCGGTTGCTGCCGATGGGCAAGGTG 1000
3.3 Summary

The *P. chrysogenum* PGK gene was isolated by colony hybridisation of a cosmid library containing *P. chrysogenum* genomic DNA to a *A. nidulans* PGK gene probe. The 9.8 Kb fragment containing the putative PGK gene was subcloned from the positively reacting cosmid clone into pUC19 to give pPC1. The plasmid pPC1 was subjected to restriction analysis and the putative gene was localised by Southern analysis to a region in the centre of the 9.8kb fragment. The orientation of the putative gene was determined by Southern analysis of the plasmid pPC1 using 5’ and 3’ portions of the *A. nidulans* PGK gene as the radioactive probe. A 1.5Kb HindIII fragment containing the coding region of the putative gene was subcloned into the vector M13mp19 and sequence analysis of part of the 1.5kb insert identified the gene as PGK. The sequence obtained had 74% similarity to the 5’ coding sequence of the *A. nidulans* PGK gene and 98% similarity to the simultaneously published complete sequence of the *P. chrysogenum* PGK gene (van Solingen et al. 1988). The 5’ flanking DNA of the *P. chrysogenum* PGK gene was also cloned into M13mp19 in preparation for sequence analysis of the promoter region.
Chapter 4

Sequence analysis of the promoter and terminator regions of the PGK gene of Penicillium chrysogenum.

The previous chapter described the isolation of the P. chrysogenum PGK gene and this chapter describes the sequence analysis of the promoter and the terminator regions of this gene. The sequence of the coding region alone has been published (van Solingen et al. 1988) and therefore was not repeated except to verify that the isolated gene was PGK (Chapter 3).

The coding region and the promoter region of PGK exist on adjacent HindIII restriction fragments of 1.5 and 1.6kb respectively. These fragments were used to construct the pairs of M13mp19 recombinant phage PC3.4, PC3.2 containing the promoter and PC2.1, PC2.2 containing the remainder of the gene in each orientation (Chapter 3, section 2.3), and were the basis of the strategy for the sequence determination (Figure 4-1). The DNA sequence was determined by the dideoxy method (Sanger et al. 1977) using the universal primer except for one region for which a synthetic primer was made.

4.1 Sequencing strategy

A number of restriction enzyme sites within the promoter and terminator regions were used to delete segments from the P. chrysogenum PGK promoter DNA in the replicative form (RF) dsDNA of the four recombinant phage described above. In addition Bal31 exonuclease digestion was used to remove further segments of DNA to obtain access to additional sequence information (Figure 4-1).
Figure 4-1: Construction of subclones for sequence analysis.

This Figure shows the restriction map of the PGK gene and the extent of the subclones generated from the four original recombinant M13 sequencing clones PC2.1, PC2.2, PC3.2, and PC3.4.

The origins of the DNA sequences are indicated:

- M13mp19 vector DNA
- multiple cloning site of M13 mp19.
- binding site of the universal primer
- P. chrysogenum DNA
- PGK coding region.

The direction of transcription of the PGK gene is shown by an arrow.

The symbols for restriction sites are as follows:
H = HindIII, Xa = XbaI, A = AccI, N = NcoI, K = KpnI, Xo = XhoI.

Restriction enzyme sites in the multiple cloning site are:

- AccI-
- 5’-HindIII-SphI-PstI-SalI-XbaI-BamHI-SmaI-KpnI-SacI-EcoRI-3’
4.1.1 Construction of subclones for sequence analysis by the use of restriction sites

Terminator clone PC2.1xs. A XhoI restriction site at the 3' end of the coding region of PGK and a SalI site in the multiple cloning site (MCS) of PC2.1 at the 5' end of the PGK coding region were used to construct PC2.1xs. Since the DNA ends generated by the enzymes SalI and XhoI are cohesive, 1321nt of the PGK coding region (Figure 4-1) between the two restriction sites may be removed by digesting the PC2.1 RF DNA with both enzymes and recircularising the fragments generated.

Approximately 1μg of PC2.1 DNA was digested with SalI and XhoI simultaneously (Chapter 2, section 6.1), after which proteins were removed by extraction with phenol/chloroform and the DNA recovered by ethanol precipitation. In order to promote the ligation of fragments to themselves rather than to each other a dilute (100μl) ligation reaction was set up with approximately 100ng of the linearised DNA (Chapter 2, section 6.5). One fifth of the ligation reaction mixture was used to transform the E.coli strain JM109 (Chapter 2, section 2.7) in which only the larger religated fragment containing the M13 vector sequence could propagate and form plaques. Several independently isolated plaques were used to inoculate separate cultures to prepare ssDNA for sequence analysis (Chapter 2, section 11). The clone PC2.1xs obtained in this way enabled sequence analysis of approximately 200nt of the terminator region downstream of the stop codon. Sequence in the opposite orientation was obtained directly from PC2.2 (Figure 4-2).

Promoter clones PC3.2k and PC3.4k. Restriction enzyme Kpnl sites 5' to the PGK coding region and in each of the MCS of PC3.2 and PC3.4 were used in the same way to construct clones PC3.2k and PC3.4k (Figure 4-1). Separate digestions of approximately 300ng of PC3.2 and PC3.4 RF DNA were carried out with Kpnl and the DNA purified, recovered, religated and transformed into the strain JM109 as described. The two plasmids PC3.2k and PC3.4k were constructed by this procedure (Figure 4-1).
Figure 4.2: Extent of sequence obtained for the promoter and terminator regions of the \textit{P. chrysogenum} PGK gene.

The Figure indicates the extent of sequence obtained from each of the sequencing subclones shown in Figure 4-1 and from the synthetic oligonucleotide \textit{5'-TGGTTTCCATGTACATG-3'} 646nt 5' to the transcriptional start point.

The numbering represents the distance from the transcriptional start point +1.

The arrows indicate the direction of sequencing and the portion of the promoter or terminator sequence obtained from that clone.

The box indicates the coding region of the PGK gene.

The symbols for the restriction enzymes are as follows: - \textit{H= HindIII, A= AccI, N= NcoI, K=KpnI, X= XhoI}. 
Promoter clones PC3.2n and PC3.4n. The NcoI site found in the promoter by restriction analysis, and the EcoR1 sites in each of the MCS of PC3.2 and PC3.4 were used to construct a second pair of plasmids PC3.2n and PC3.4n (Figure 4-1). The ends generated by the digestion of DNA with these enzymes are not cohesive and so a "fill in" reaction using the Klenow fragment of *E. coli* DNA polymerase I and dNTPs to convert the single stranded ends of the digested DNA into double stranded DNA was done before ligating the fragments in a blunt end reaction.

Approximately 600 ng of the RF DNA of PC3.4 and of PC3.2 were digested with NcoI and EcoRI together. After removal of proteins and recovery of the DNA, the DNA was resuspended in 18 μl of Klenow fill-in buffer and a "fill in" reaction using the Klenow fragment of *E. coli* DNA polymerase I done as described in Chapter 2, section 6.4. The Klenow fragment was inactivated by heating the reaction to 70°C for 10 minutes. Proteins were removed by phenol/chloroform extraction, the DNA recovered and the ligation reaction, transformation, and amplification of the clones carried out as before. Use of clones PC3.2n and PC3.4n allowed sequence to be determined from the translational start codon as far as the NcoI site at -348 (Figure 4-1; Figure 4-2).

4.1.2 The deletion of further sequence by Bal31 exonuclease digestion

In order to complete sequence analysis of the promoter region Bal31 exonuclease digestions were carried out on the M13 clones PC3.4 and PC3.2 using the NcoI site as start points and on PC3.2 using an AccI site 1 kb 5' to the first codon of PGK as the start point (Figure 4-1). The general strategy was to digest the DNA with NcoI or AccI then to do the exonuclease reaction in which DNA was removed from both ends of the DNA. To remove the unwanted DNA between the site of the deletions and the primer binding site a second digestion was done using an enzyme which cleaved the clone within the MCS. The DNA molecules were then made blunt ended using Klenow fragment and a ligation reaction performed.
Bal31 deletion from the NcoI site. About 10μg of RF DNA from each of the clones PC3.2 and PC3.4 was digested with NcoI and the DNA purified and recovered. The DNA was resuspended in 50μl of 2xBal31 buffer and a calculated amount (1 unit) of Bal31 added. The reaction was incubated at 30°C for five minutes and samples removed at 1 minute intervals when EGTA was added to 20mM to stop the reaction and the sample stored on ice (Chapter 2, section 6.2). The DNA was recovered as before and resuspended in distilled water. Half of each sample was examined by agarose gel electrophoresis to verify that the digestion had proceeded as expected. The other half of the sample was digested with a second enzyme EcoRI, the DNA recovered and incubated with the Klenow fragment of E. coli DNA Polymerase I and dNTPs in a "fill in" reaction to make blunt ended DNA molecules. The ligation reaction, transformation of the products into JM109 and the amplification of the clones were done as before. Single stranded DNA was prepared from six independently isolated M13 clones from each sample transformed into JM109, and analysed by carrying out sequencing analysis for one base only (t-tracking). Useful clones identified by this method were then fully sequenced. The clones named PC3.21, PC3.22, and PC 3.43a, PC3.43c, PC3.44, PC3.45a yielded overlapping sequence data extending to the AccI site 986nt 5′ to the first PGK codon (Figures 4-1 and 4-2).

Bal31 deletions from the AccI site. The AccI site 986nt from the PGK translational start codon in clone PC3.2 was used to produce clones yielding sequence data extending towards the NcoI site (Figure 4-2). Since there were three AccI sites in PC3.2 it was necessary to first linearise the molecule by partial digestion with AccI. The Bal31 reaction was then carried out on the mixture of linearised molecules followed by digestion with a second restriction enzyme and religation. Useful clones were identified after their recovery by doing single base sequence reactions (T-tracking). This strategy may appear unlikely to succeed but the yield of useful clones was two out of six tested.

About 18μg of clone PC3.2 RF DNA was digested with AccI until the majority of the DNA molecules appeared to be linear upon examination of a small sample (50-100ng) of the digested DNA by agarose gel electrophoresis. The linearised
DNA was recovered and incubated with 1μl Bal31 in 100μl of buffer. Samples were removed at 1 minute intervals up to 10 minutes and the DNA recovered. The DNA was then digested with SphI, which has a cleavage site in the MCS, and incubated with Klenow fragment of *E. coli* DNA polymerase I to make blunt ended DNA. A ligation reaction was set up and the products transformed into JM109. Single base sequence reactions were done with six clones and the sequence of two, PC0.4a and PC9.3a was fully determined, yielding data extending from the AccI site towards the NcoI site.

A synthetic primer of 17 bases which annealed to sequence between 646nt to 662nt 5' to the translational start codon was used to complete the sequence data to the NcoI site. Data was obtained on both strands of the DNA (Figure 4-2).
4.2.1 Promoter Sequence

The positions of sequence elements are given with reference to the major transcriptional start site which was located by experiments described in the next chapter (Chapter 5 section 2.1). This avoids the confusion of having two sets of reference numbers in use.

The promoter sequence from the AccI site (-947 bases) 5' to the transcriptional start (+1) to the first HindIII site (+108) in the coding region is shown in Figure 4.3. At the translational start the sequence found agrees with the consensus for filamentous fungi (Ballance 1986; Gurr et al. 1987) as shown in Table 4-1. No potential TATA homology or CAAT motif were observed close to the translational start. However a 37nt sequence which was composed entirely of pyrimidines with only three exceptions was observed at +10bp to -27bp. Pyrimidine rich sequences are sometimes observed near to the transcriptional start point in filamentous fungal genes, often those lacking TATA or CAAT motifs and in some highly expressed yeast genes (Gurr et al. 1987). They are thought to have a role in positioning the transcriptional start points. Further upstream there is a 9bp direct repeat at -324 and -333 and a 7bp repeat from -357 and -377. There are also four repeats of the pentamer TTCCC in this region. No significant inverted repeat sequences were found.

The promoter sequence was searched for the several motifs shown in Table 4-1. There was no match to the octamer sequence found in the A. nidulans PGK promoter with one mismatch (Streatfield 1990). A search of the promoter for sequences matching the glycolytic box (Punt et al. 1988) yielded a match to the core consensus proposed by Streatfield (1990) at -212 to -219. Searches were also made for motifs found in the yeast PGK promoter and at -307 to -319 a 11 of 13nt match found to the consensus yeast RAPI binding site (Chambers et al. 1990) with the two mismatches probably not being significant. However this match is close to the region of the promoter containing the repeat sequences described above. At -473 to -486 a 7 of 8nt match to the heat shock consensus, which also occurs in the yeast PGK promoter, was found (Pelham 1985).
Searches were made for similarities between the *P. chrysogenum* PGK promoter and the *A. nidulans* PGK promoter and two found are shown in Table 4-1. The first similarity found was a perfect match at -334 to -344 in the *P. chrysogenum* promoter and -270 to -280 in the *A. nidulans* promoter. The second was of the region -79 to -99 in the *P. chrysogenum* PGK promoter to -140 to -159 in the *A. nidulans* promoter with a gap of one base.
Table 4-1 A: Sequence motifs found in the *P. chrysogenum* PGK promoter

The positions in the *P. chrysogenum* PGK promoter are relative to the major transcriptional start point (+1) obtained by using data from Chapter 5, section 2.1. Similarly the positions of elements in other gene promoters are relative to the transcriptional start point.

<table>
<thead>
<tr>
<th>Name of element</th>
<th>Consensus sequence</th>
<th>Sequence found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock</td>
<td>C—GAA—TTC—G</td>
<td>C—GAA—TTC—C</td>
</tr>
<tr>
<td>(Pelham 1985)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAPI recognition site</td>
<td>AAATCCGTGCACCC</td>
<td>ACCTCCATGGCCC</td>
</tr>
<tr>
<td>(Chambers et al. 1990)</td>
<td>CC C A A C T T</td>
<td></td>
</tr>
<tr>
<td>Glycolytic box</td>
<td>T-TGAGGTG-A-TG</td>
<td></td>
</tr>
<tr>
<td>(Punt et al. 1988)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Streatfield 1990)</td>
<td>TGAGGTGT</td>
<td>TGAGGTAC</td>
</tr>
<tr>
<td></td>
<td>T AC</td>
<td></td>
</tr>
<tr>
<td>Octamer motif</td>
<td>ATGCAAAT</td>
<td>not found</td>
</tr>
<tr>
<td>(Rosales et al. 1987)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Translational start (DNA)</td>
<td>TCACAATGGC</td>
<td>CCACAATGTCTC</td>
</tr>
<tr>
<td>(Ballance 1986)</td>
<td>AC T</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-1B: Regions of similarity between the PGK promoters of *A. nidulans* and *P. chrysogenum*.

<table>
<thead>
<tr>
<th>PGK Promoter</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. nidulans</em></td>
<td>CTTCACCAGCA.GCCCCACCA -140</td>
</tr>
<tr>
<td><em>P. chrysogenum</em></td>
<td>CTTCACCAGGCCTGCCCCACCA -79</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>TCCGAGGCTAA -270</td>
</tr>
<tr>
<td><em>P. chrysogenum</em></td>
<td>TCCGAGGCTAA -334</td>
</tr>
</tbody>
</table>
Figure 4.3: Promoter sequence of *P. chrysogenum* PGK
The promoter sequence was obtained from the family of clones generated by PC3.4 and PC 3.2 up to 992nt 5' to the translational start.

Non-coding DNA is shown in lower case letters
Coding DNA in upper case letters.
The start codon is shown in bold letters.

Direct repeats are overlined and the letter below indicates which set of repeats the sequence belongs to
A- AATTCCC
B- TTCCC
C- GAGGCTAAA

Sequence motifs are indicated by underscores and a number:-
1- heat shock element
2- 11bp match with *A. nidulans* PGK promoter
3- 11/13 match to the yeast factor RAP1 recognition site
4- match to the glycolytic box core consensus
5- 19/21 match with *A. nidulans* PGK promoter
6- pyrimidine rich sequence
1  cgtgatgtct acctgcccc  cctaaagccc  aacttcggtc  tctggccggc
51  agtgccagtc  ctaaaactcc  gtgctattcc  cattcagtta  cagattgtga
101  gttaccaggg  tagtccccgg  cgagattgga  tctgtactga  cttctaaagc
151  cattcgtctc  aacggtggtg  attgcgttga  ccgccatacc  tttccctgac
201  taactccgccc  gaggaggttt  aattgaacgc  tattcaacgc  gaacatttattg
251  ataacgattc  gggcgataaa  ggctataaat  tgcactctttt  cttctgccaa
301  attatttagg  cgttccgtt gttttccatg  tacatgggattt
351  atggtatggc  ccacgggcca  gagatgctga  tttcaaaagg  tcggatttttg
401  cacatttggga  ataccaacct  gaggttttgc  tgcctaccttt  tgttttcgat
451  ttgacttgga  ttctttcagg  aaaaattccgc  tttttagcgt  ttcgggtcttt
501  taaccctctct  gggctttcaca  atgatttctgt  tagacacgga  aacgctctaa
551  gcatgtagcggctttagaag  ttcccatcag  tagaggccaa  tttccctagaat
601  ttccggttaaccgactaa  gagggtaaaag  aggacctcca  tggcccggcc
651  ttccctgtat  caacggeggcg  agctctgaca  tcaatccggc  gtcaagccga
701  tcagttccctc  ggggtactc  gggatcggggc  gtctgaggtat  ctgcttttat
751  agatatacct  aggaagagag  ggatttgctc  atactccgag  caagtacctt
801  attccgtcga  atacatcact  gggaggtacc  tgggttgcta  cgcgttgcga
851  cctcttcacc  ggcctggccc  accaaaactg  accccctcacc  tctttactga
901  tcgagactca  cggggccgctg  attatgtcttt  tctctttcttt  ctatccctttt
951  ttcgtcctcc  ttccatattg  tcatatactt  aactacccac  aATG TCT CT
1000  C TCC AAC AAG CTC CCC GTT ACT GAT GTC GAC CTC AAG GGC A
1042  AG CGT GTC TTG ATC CGG gttaagcctt
Figure 4.4 Sequence of the terminator region of \textit{P. chrysogenum PGK}.

This is the sequence obtained from clones PC2.2 and PC 2.1XS. It extends from the XhoI site 46nt 5' to the stop codon to the HindIII site 192nt 3' to the stop codon.

The \textbf{coding region} is shown in upper case letters.

The \textbf{noncoding region} is shown in lower case letters.

The \textbf{stop codon} is shown in bold letters.

Possible stem loop structures are underlined with arrows.

The \textbf{putative polyadenylation signal} is overlined.
CTCGAGGGCA AGGAGCTGCC CGGTGTTGCT GCTCTGTCCA GTAAGTAAat
atgaaaaat aggcggcgaa aggagtgcte acatcgggctc ttcttcgutc
tctgcatgttt atgaaactgt aatgtacaca gccaatgaat ttccttttaat
tacaagcact ttacctcttt taaagaatac tggatatga tcttagatcg
tataagctaa gtgtgtgccc caggtacaaa aacggaagctt
4.2.2 Terminator sequence

Sequence data was obtained extending to 192bp 3' to the termination codon (Figure 4.4). Regions which could form a stem loop structure in transcribed RNA and therefore could be involved with transcription termination were found at 117-129, 134-153, 163-184bp 3' to the stop codon. A search of the terminator sequence was made for the termination signal -CCTGTTCC- which may be involved in general eukaryotic transcription termination but no matches found (McLaughlan 1985). No potential polyadenylation signal sites (AAUAAA) were found (Proudfoot and Brownlee 1978).

4.3 Summary

The sequence data show motifs expected for the flanking sequences of a filamentous fungal gene. In the 5' sequence there is a pyrimidine rich region and the expected consensus for the translational start codon. Several short repeated motifs were also found clustered between -298 to -390 and there is a heat shock motif at -473. There are similarities in the P. chrysogenum PGK promoter to the PGK promoter in Aspergillus nidulans and to the S. cerevisae PGK promoter although the latter may not be significant. It is necessary to determine the transcriptional start points to assess the significance of the pyrimidine rich region and the stop points to assess the significance of motifs in the terminator. The analysis of the promoter and terminator regions is further discussed in Chapter 9.
Chapter 5

Analysis of the transcription of the PGK gene

Studies of DNA transcription can provide information about the size and number of possible different transcripts made from a gene, where they begin and end, where introns occur and how mRNA levels change under different conditions of growth. In this project the aim of the transcription analysis was first to establish the number of different possible transcripts from the PGK gene in P. chrysogenum, their size and their start and finish points, and secondly to determine the relative abundance of the PGK mRNA under different conditions of growth. In these experiments I have assumed that the only mRNA species detected is the mature cytoplasmic mRNA.

The PGK gene in P. chrysogenum contains two small introns of 55 and 62 nucleotides respectively (van Solingen et al. 1988). The length of the coding region of the P. chrysogenum PGK gene without the introns is 1247 nucleotides. Therefore the size of PGK mRNA in P. chrysogenum is expected to be at least 1.25kb. In this chapter an estimate of the size of the PGK message was obtained by hybridisation of a DNA probe to total cellular RNA which had been size separated on an agarose gel and transferred to a nitrocellulose filter (Northern analysis). A more accurate estimate was obtained by determining the points at which the mature PGK mRNA transcripts begin and end using S1 analysis. A radioactive probe of known size which overlapped the 5' end of the PGK mRNA and a similar probe which overlapped the 3' end of the mRNA were hybridised to total cellular RNA in separate experiments. Treatment of the hybridised nucleic acids with S1 nuclease removed any single stranded unhybridised nucleic acids leaving RNA/DNA hybrid molecules, the length of which is the number of nucleotides the mRNA transcript and the DNA probe have in common. The position of the transcriptional start and stop site of the mature mRNA was deduced from the length of the fragments protected by hybridisation to the mRNA.

It was necessary to know the position of the transcriptional start before manipulation of the promoter
and the information also helped to establish which flanking DNA sequences may have a role in the start and finish of transcription.

The concentration of PGK mRNA in cells of Aspergillus nidulans (Clements 1986) and Saccharomyces cerevisiae (Holland and Holland 1978) is relatively high and this was expected to also be the case in P. chrysogenum. Usually the abundance of an mRNA species is compared to that of ribosomal RNA or a mRNA of known amount that is likely to be unaffected by different conditions of growth. Unfortunately there are few reports of isolation of genes in P. chrysogenum, no comparisons of mRNA levels and no available probe for ribosomal RNA. Therefore the abundance of PGK mRNA was not determined. However an experiment relevant to the aims of the project was done in which the level of PGK mRNA was compared to the mRNA of the gene encoding the enzyme Isopenicillin-N synthase (IPNS). The relative abundance of these two mRNAs would indicate whether a gene fusion constructed between the PGK promoter and the IPNS coding region would be likely to increase the concentration of IPNS message.

Studies of the PGK genes of A. nidulans and yeast also suggest that their expression may be regulated by the carbon source on which the organism is grown (Clements 1986; Stanway et al. 1987). The gene is more highly expressed in growth on carbon sources metabolised by the gluconeogenic pathway. In the case of A. nidulans the increase in expression is about 1.5 fold. To investigate the effect of carbon sources on the expression of P. chrysogenum PGK total cellular RNA was isolated from cultures of Penicillium chrysogenum strain N.R.R.L. 1951 grown on different carbon sources and the abundance of PGK mRNA in each sample compared.

The method chosen to study the abundance of PGK mRNA was Northern analysis because small amounts of contaminating DNA in the preparation would not interfere with the hybridisation and it was more straightforward than S1 mapping, especially when several samples were being compared.
5.1 Preparation of total cellular RNA

Total cellular RNA was used in all the following studies since the PGK message is abundant and therefore it was not necessary to obtain polyadenylated mRNA.

In order to prevent the degradation of RNA by contaminating RNases several precautions were taken. Gloves were worn at all times when handling the RNA sample to prevent RNases on the skin from contaminating the RNA. All solutions not containing EDTA or Tris buffer were treated with 0.1% diethylpyrocarbonate (DEPC). Solutions containing EDTA or Tris were made from new stocks of chemicals kept specially for that purpose. All glassware was either treated by soaking in DEPC and autoclaving at 15lb pressure for 15 minutes or by baking at 200°C for four hours. The RNA preparations were aliquoted and stored at -20°C as an ethanol precipitate.

All of the experiments described in this chapter were done with young actively growing mycelium, expected to yield high mRNA levels, processed immediately to avoid intracellular turnover of the RNA. The RNA was prepared as described in Chapter 2 section 8.

5.2 Determination of the 5' and 3' ends of the PGK mRNA by S1 exonuclease protection studies

A fully labelled radioactive DNA probe was synthesised from M13 phage single stranded DNA using the Klenow fragment of E. coli DNA polymerase, α-32P-dCTP, dNTPs and the universal primer to initiate synthesis. The double stranded molecule was then digested with the appropriate restriction enzyme to release the required fragment and the probe isolated following denaturation and gel electrophoresis. The radioactively labelled probe was hybridised to total RNA which had been isolated from young mycelium grown in minimal glucose medium. The S1 reaction was carried out for periods of 1 to 2 hours before being stopped, and the products separated by electrophoresis on a 6% acrylamide denaturing gel.
5.2.1 Determination of the 5' end of the message

A KpnI restriction site is conveniently located 162bp 5' to the translational start point. The transcriptional start was mapped using a single stranded DNA probe extending from the HindIII site in the first intron of the PGK gene to the KpnI site and complementary to the mRNA. The probe was made from the M13 recombinant phage PC3.4 and provided a 66nt overlap with the first exon of PGK and extended 8nt into the first intron (Figure 5-1A).

The probe fragment was prepared, hybridised to total cellular RNA and subjected to S1 nuclease digestion as described in Chapter 2 section 10. The single stranded DNA from clone PC3.4 was prepared by the same method used for preparation of sequencing templates (Chapter 2, section 11.3). The probe fragment was released from the radioactively labelled clone PC3.4 by digestion with KpnI, isolated by agarose gel electrophoresis, recovered from the gel and resuspended in H2O. The radioactive probe solution was divided into three equal aliquots. Two aliquots of probe were separately hybridised to 30µg P. chrysogenum total cellular RNA and the third was hybridised to the same amount of yeast tRNA. The S1 nuclease digestion of the experimental samples were done with 400 units and 1000 units of S1 nuclease for an hour. The control sample was digested with 1000 units S1 nuclease for the same period. The nucleic acids were recovered after ethanol precipitation and resuspended in T.E. buffer and half of the sample separated by electrophoresis on a 6% acrylamide gel alongside a set of sequencing reactions as size markers.

Three fragments which had been protected by hybridisation to the mRNA were observed 105, 116 and 128nt long. The most abundant was the 105nt fragment. The HindIII site at the 5' end of the probe is in the first PGK intron identified by sequence analysis (van Solingen et al. 1988). It is assumed that this intron is spliced out in the mature mRNA and that the 8nt of the probe which is complementary to the intron will not therefore hybridise to the mRNA. The lengths of the fragments correspond to the distances from the end of the first PGK exon to the transcriptional start points. The distances corresponded to start sites for
transcription of 39, 50 and 62 nucleotides 5' to the start codon. Thus the transcription start sites coincide with the highly pyrimidine rich region in the PGK promoter (Figure 5-2).

5.2.2 Determination of the 3' end of the message

The 3' end of eukaryotic RNA transcripts is cleaved during post transcriptional processing in the nucleus and polyadenylation occurs at this site. The true end of transcription is therefore difficult to identify since it is quickly removed in the formation of the mature mRNA and therefore the proportion of mRNA molecules in the cell which have not been processed is very low. However S1 nuclease analysis can be used to determine where the cleavage of the primary mRNA transcript occurs since the poly-A tail added during processing does not hybridise to the DNA probe.

The DNA probe prepared from PC2.2 used to determine the 3' end of the PGK transcripts was synthesised from the HindIII site 187bp 3' to the PGK stop codon and terminated at the BglII site in the coding region of PGK. The extent of the overlap with the coding region was 91nt (Figure 5-1B). It was prepared from the M13 clone PC2.2 in a similar way to the 5' probe, except that it was released from the RF DNA generated by the synthesis of the radioactive DNA strand by digestion with BglII. The S1 nuclease digestion reactions were done for one hour with 400 units of S1 nuclease.

The three protected fragments observed were 185, 194 and 198nt long with respect to the BglII site 5' to the stop codon, the most abundant being the 198nt fragment. The polyadenylation sites were therefore 94, 103 and 107 bases 3' to the stop codon (Figure 5-3). Several potential stem loop structures are found 3' to these sites (Chapter 4, Figure 4-4) and further examination of the nearby DNA sequences also reveals a potential polyadenylation signal (AATGAA) at 85nt 3' to the stop codon.

The results of the S1 nuclease analysis experiments show that there is a maximum of 9 possible different transcripts. The most frequent start site designated (+1) is 39 nucleotides 5' to the translational start and the most frequent stop site is at 107nt 3' to the stop codon. The
range of transcript lengths is 1381-1417nt and discrete transcripts would not therefore be detectable by Northern analysis using agarose gels. The size of the predicted single mRNA band which would be observed in Northern blot analysis would be approximately 1.4kb.
Figure 5-1: The DNA probes used in the analysis of P. chrysogenum PGK mRNA

A: The restriction map of the M13 recombinant phage clone PC3.4 shows the extent of the DNA probe used to map the 5' end of the PGK message.

The origins of the DNA sequences are indicated:

- M13 vector DNA
- Multiple cloning site
- Primer binding site
- The P. chrysogenum DNA
- PGK coding region
- The extent of the probe DNA.
- TAA Stop codon

The direction in which probe synthesis takes place is indicated by the single line arrow below each map and the direction of transcription by the open arrow above each map.

The numbers show the positions of sites with respect to the major transcriptional start site +1.

<table>
<thead>
<tr>
<th>Restriction sites</th>
<th>HindIII</th>
<th>NcoI</th>
</tr>
</thead>
<tbody>
<tr>
<td>BglII</td>
<td>kpnI</td>
<td></td>
</tr>
<tr>
<td>AccI</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B: The restriction map of the M13 recombinant phage clone PC 2.2 shows the extent of the DNA probe used to map the 3' end of the PGK message. The symbols used are as above.

C: The restriction map of the M13 recombinant phage clone PC2.1 shows the region of the clone used as a probe for PGK mRNA in Northern analysis.
A 274nt radioactively labelled DNA probe (Figure 5-1 A) complementary to the 5' end of the PGK mRNA and likely to extend 5' to its start was hybridised to 30μg of total cellular RNA from strain N.R.R.L. 1951 or to 30μg of yeast tRNA which was the negative control. The products were digested with 400 to 1000 units of S1 exonuclease and the remaining nucleic acid recovered and separated by electrophoresis on a 6% acrylamide gel alongside a set of sequencing reactions (Chapter 2 section 10). The autoradiograph shows the DNA fragments protected from digestion with S1 nuclease by hybridisation to the PGK mRNA from which the positions of the 5' ends of PGK mRNA may be deduced.

Lane 1 contains the 274 nucleotide Kpnl fragment from PC3.4 which was the probe.

Lane 2 shows the protected fragments obtained when the probe hybridised to 30μg yeast tRNA is digested with 1000 units S1 nuclease (control).

Lanes 3 and 4 show the products of an S1 digestion with different amounts of S1 nuclease (lane 3, 1000 units; lane 4, 400 units) when the probe DNA was hybridised to P. chrysogenum total cellular RNA.

The protected fragments are indicated by a dot. The last four lanes are a sequencing reaction (order GATC) and this is used to estimate the size and locations of the protected fragments.
Figure 5-3: S1 nuclease analysis of the 3' end of the PGK message.

A radioactively labelled 283nt DNA probe overlapping the 3' end of the PGK mRNA (Figure 5-1B) was hybridised to 30μg of total cellular RNA from strain N.R.R.L. 1951 or to 30μg of yeast tRNA as a negative control. The hybridisation products were incubated with 400 units of S1 nuclease after which the nucleic acids were recovered and separated by electrophoresis on a 6% acrylamide gel alongside a set of sequence reactions (Chapter 2 section 10). The autoradiograph shows the protected fragments obtained, from which the position of the 3' ends of the PGK message may be deduced.

Lane 1 contains the DNA probe, the 283nt BglII fragment from PC2.2.

Lane 2 contains the control, the products of an S1 digestion of the probe hybridised to 30μg yeast tRNA.

Lane 3 contains the protected fragments obtained from digestion of the probe hybridised to 30μg P. chrysogenum total cellular RNA.

Protected fragments are indicated by a dot. The last four lanes contain a sequencing reaction (order CTAG) used to estimate the size and location of the protected fragment.
5.3.1 Preparation of the DNA probes used to identify different mRNAs in Northern hybridisations.

The DNA probe used to identify the PGK mRNA in Northern hybridisations was a 1.5kb HindIII fragment containing the P. chrysogenum PGK coding region and 3' untranslated region, obtained from the M13 recombinant phage PC2.1 (Figure 5-1C). Several micrograms of RF DNA of this clone were incubated with the restriction enzyme HindIII and the products of the digestion separated by agarose gel electrophoresis. A slice containing the fragment required was cut out of the gel, and the DNA recovered by the method described in Chapter 2, section 6.3B. Approximately 3μg of fragment was prepared. About 100ng of this fragment, fully labelled with radioactive α-32P-dCTP by the random oligonucleotide priming method (Chapter 2, section 7.3) was used in each hybridisation.

The DNA probe used to identify the mRNA encoding the enzyme isopenicillin-N-synthetase (IPNS) was isolated in a similar way from the plasmid pCYX4 given to me by Professor G. Turner's group (Figure 8-1) and was a 1kb NcoI fragment containing the coding region of the IPNS gene which has no introns and 10nt of the 3' region. This probe was used at the same concentration as the PGK probe.

The DNA probe for the ATP synthase subunit 9 gene oliC was isolated from pPOL20 which was also given to me by G. Turner's group. It was isolated in the same way and was a HindIII-XhoI fragment 1.4kb in size, and contained the entire gene (Figure 6-1B).

5.3.2 The identification and estimation of the size of the PGK mRNA of P. chrysogenum.

The size of the PGK mRNA was estimated by Northern analysis (Chapter 2, section 9). Total cellular RNA was isolated from mycelium of strain N.R.R.L. 1951 grown in glucose minimal medium for about 16 hours. A sample of 3μg of total cellular RNA was separated by electrophoresis on a 1% formaldehyde/agarose gel and transferred in 20xSSC to a nitrocellulose filter which was found to give better results than nylon membrane. The immobilised RNA was hybridised to
100ng of PGK probe in 50% deionised formamide, 6xSSC, 0.25%
marvel milk (the SSC and Marvel were treated with DEPC) and
the size of the band estimated by comparison to the size
markers (Figure 5-4).

The size of PGK mRNA was approximately 1.3kb. This was
smaller than expected since the PGK coding region (without
the introns) is 1.247 kb in size.
Figure 5-4: Identification of the PGK message by Northern analysis.

The autoradiograph shows a Northern analysis of about 3$\mu$g P. chrysogenum total cellular RNA. To identify and estimate the size of the PGK mRNA population total cellular RNA was separated by electrophoresis on a 1% formaldehyde/agarose gel, transferred to a nitrocellulose filter, and hybridised to a 1.5Kb $\alpha-^{32}$P-dCTP labelled HindIII fragment isolated from PC2.1 containing the coding and 3' regions of the PGK gene (Figure 5-1). The hybridisation was done in 50% deionised formamide, 0.25% Marvel milk, 6xSSC at 42°C. Post hybridisation washes were done at 50°C in 0.1% SDS, 0.1X SSC.

The size markers are denatured phage $\lambda$ DNA which has been digested with HindIII.
5.3.3 The relative abundance of PGK mRNA and IPNS mRNA

The estimation of the relative abundance of the PGK and IPNS mRNAs was done with total cellular RNA isolated from young mycelium harvested at about the same stage of growth either on minimal medium with a glucose carbon source or on a complex growth medium GM used in fermentations. The culture density was monitored by determining the the wet weight of harvested mycelium.

The size of the *P. chrysogenum* PGK mRNA was 1.3kb and the size of the IPNS mRNA was estimated to be 1.1kb (Smith et al. 1989). The two mRNAs could not be clearly separated on a 1% formaldehyde/agarose gel and so the comparison was done by loading equal amounts of 20µg of the RNA preparation onto separate lanes of a gel, separating the RNAs by electrophoresis, transferring them to nitrocellulose and then cutting the resulting filter in half. The RNA on one half of the filter was hybridised to 100ng of the probe for PGK mRNA and the RNA on the other half hybridised to 100ng of the probe for the IPNS mRNA. The incorporation of radioactivity into each of the probes was measured and taken into account in the calculation of the relative levels of the two mRNAs (Figure 5-5).

In order to determine the relative amounts of total RNA loaded in each lane the PGK and IPNS DNA probes were stripped from the filters and the RNA on both filters hybridised to a probe containing the oliC gene. If the same amount of RNA was present the signal given by this probe would be the same in each track.

The autoradiographs were all scanned with a laser densitometer and the values obtained for the areas under the peak generated by each signal were used to calculate the relative abundance of the two mRNAs. The values obtained for the oliC probe were used to adjust the values obtained for the other probes for the amount of RNA which was loaded.

The results suggested that the abundance of PGK mRNA was 3 fold greater than that of the IPNS mRNA on the GM medium. On the minimal medium PGK mRNA was 10 fold more abundant than IPNS mRNA. On both media the PGK message was of similar abundance and readily detectable.
Figure 5-5: The relative levels of the PGK and IPNS mRNAs

Autoradiograph A shows the hybridisation of a PGK probe (Figure 5-1) or an IPNS probe (Chapter 8, Figure 8-1) to equal amounts of total cellular RNA isolated from P. chrysogenum, N.R.R.L. 1951 grown on glucose minimal medium (Lane 1) and GM medium (Lane 2). About 20μg of the RNA was loaded in duplicate and separated on a 1% formaldehyde/agarose gel. The RNA was transferred to 2 nitrocellulose membranes. One pair of samples was then hybridised to the PGK probe and the other to the IPNS probe as described in Chapter 2 section 9. The incorporation of radioactivity into the probes was measured (Chapter 2 section 7.7) and was 59% for the PGK probe and 88% for the IPNS probe. The resulting autoradiograph was scanned using a laser densitometer (Chapter 2 section 9.3) and the values obtained are shown below each lane.

Autoradiograph B shows the hybridisation of the RNA on the filters described above (after removal of the PGK and IPNS probes) to the probe for the ATP synthase subunit 9 gene, oliC (Chapter 6 Figure 6-1B). The autoradiograph was scanned with a laser densitometer and the values obtained are shown below each lane. These values were used to normalise the values obtained in A for the loading of the RNA samples.

The table shows the normalised results obtained from the densitometry scans of the two autoradiographs. These results were further adjusted for the different incorporation of radioactivity into the PGK and IPNS probes.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Probe</th>
<th>Relative value</th>
<th>Value adjusted for radioactivity of probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>PGK</td>
<td>1.1 = 1.48 (100%)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>PGK</td>
<td>1.1 = 1.13 (76%)</td>
<td>76%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>IPNS</td>
<td>0.14 = 0.144 (10%)</td>
<td>7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>GM</td>
<td>IPNS</td>
<td>0.61 = 0.54 (36%)</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.13</td>
<td></td>
</tr>
</tbody>
</table>
5.3.4 The relative abundance of PGK message on different carbon sources

Four defined carbon sources were used in this study, two glycolytic carbon sources, glucose, and glycerol, and two gluconeogenic carbon sources, acetate which feeds directly into the TCA cycle and quinic acid which feeds into the TCA cycle by the protocatechuic acid pathway (Chapter 1, Figure 1-1). The complex fermentation medium for *P. chrysogenum*, GM was also included in the study.

The strain N.R.R.L. 1951 was grown in minimal medium with the appropriate carbon source or on GM for 16-24 hours depending on the carbon source and harvested when it reached approximately the same stage of growth indicated by the wet weight of the harvested mycelium. Typically 1-2 grams of mycelium were harvested. Total cellular RNA was prepared from the mycelium as described, 3μg separated by electrophoresis on a 1% formaldehyde/agarose gel, transferred to nitrocellulose and hybridised to 100ng of PGK probe. To check that the same amount of total RNA was present in each sample the RNA was also hybridised to approximately 100ng of a probe for the *P. chrysogenum* oliC gene, which was expected not to be affected by the carbon source on which the cultures were grown (Chapter 6, Figure 6-1B). The autoradiograph obtained was also scanned using the densitometer and the relative abundance of PGK mRNA from mycelium grown on the different carbon sources calculated as before (Figure 5-6).

The resulting autoradiograph indicated that PGK mRNA is more abundant upon the carbon sources quinic acid and acetate, than on glucose or glycerol. The increased levels on quinic acid and acetate which require gluconeogenesis suggest that the PGK gene is transcribed at an increased level under those conditions and the promoter is more active.
Figure 5-6: Relative levels of PGK mRNA

 Autoradiograph A shows the hybridisation of samples of 3μg of P. chrysogenum total cellular RNA to a radioactively labelled probe containing the PGK gene (Figure 5-1). The RNA was isolated from mycelium grown on minimal medium with different carbon sources indicated below, and from mycelium grown on the complex medium GM. The RNA was separated by electrophoresis on a 1% formaldehyde/agarose gel, transferred to a nitrocellulose filter and the hybridisation done under the conditions described in Chapter 2 section 9. The autoradiograph obtained was scanned using a laser densitometer and the values obtained are shown below each lane.

 Autoradiograph B shows the hybridisation of the above RNA, after removal of the PGK probe, to a probe for the oliC gene (Figure 6-1) under the conditions used above, after the removal of the PGK probe from the nitrocellulose filter. The autoradiograph was also scanned with a laser densitometer and the values obtained are shown below each lane. The values were used to normalise the results obtained from autoradiograph A.

<table>
<thead>
<tr>
<th>lane</th>
<th>carbon source</th>
<th>Standardised value</th>
<th>Relative value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose (0.02M)</td>
<td>1.97 =0.454</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.33</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>GM</td>
<td>1.46 =0.337</td>
<td>74%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.57</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>glycerol (0.04M)</td>
<td>0.31 =0.11</td>
<td>24%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.73</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>quinic acid (1% w/v)</td>
<td>3.13 =0.87</td>
<td>190%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.79</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>acetate (0.1M)</td>
<td>2.67 =0.69</td>
<td>151%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.38</td>
<td></td>
</tr>
</tbody>
</table>
5.4 Summary

The determination of the start and end points of the mature PGK mRNA showed that transcription of the *P. chrysogenum* PGK gene starts and ends at several sites. The transcriptional start points lie in the pyrimidine rich region at 39, 50, 62nt from the translational start point, the major transcriptional start site being at 39nt and this has been taken as the reference point +1 in describing the positions of promoter elements. The polyadenylation sites were 94, 103 and 107nt from the stop codon and fell just 3' to a potential polyadenylation signal and a region which could form a stemloop structure in the mRNA described in Chapter 4. The range of sizes of the potential PGK transcripts is 1381-1417nt and in a Northern analysis one band of 1.3 kb was observed, 0.1 kb smaller than expected. Such a difference probably reflects inaccuracies in size estimations from the denatured DNA markers. The results confirm that the whole PGK gene lies on the 1.5 and 1.6kb HindIII fragments previously described.

The results of Northern analysis show that the PGK transcript is abundant, since it is easily detectable. The studies of relative levels of mRNA showed that PGK mRNA levels are three times higher than the levels of the *IPNS* transcript in young mycelium growing on the complex fermentation medium GM and the difference is greater in young mycelium growing on minimal medium. This suggests that a fusion between the PGK promoter and the coding region of the *IPNS* gene may be transcribed more efficiently than the native *IPNS* gene.

Studies of the relative PGK mRNA levels in mycelium harvested at same stage of growth, as judged by the yield of mycelium, show that transcription of the PGK gene appears to be modulated by the carbon source on which the mycelium is grown in a similar way to the *A. nidulans* PGK gene. Thus in young mycelium growing on carbon sources which are metabolised by the gluconeogenic pathway, the *P. chrysogenum* PGK mRNA is at least twice as abundant as in young mycelium grown on carbon sources which are metabolised by the glycolytic pathway (Clements 1986; Streatfield 1990). This result can be further explored by constructing a reporter
fusion between the PGK promoter and the coding region of the lacZ gene of E. coli, transforming this into P. chrysogenum and determining β-galactosidase activity in the transformed strain growing on different carbon sources.
Chapter 6

Construction and use of a reporter gene fusion of the P. chrysogenum PGK promoter to the E. coli lacZ gene.

Fusion of the PGK gene promoter to a convenient reporter gene would allow physiological studies of the expression of the PGK gene to be done and would readily provide the opportunity for functional analysis of the promoter. The lacZ gene of Escherichia coli is a widely used reporter gene which is easy to monitor and has been employed in the investigation of the expression of several filamentous fungal genes (Punt et al. 1990, Gomez-Pardo et al. 1990, Streatfield 1990). A series of vectors has been constructed containing the lacZ gene with a multiple cloning site at the 5' end in each reading frame. The terminator region of the E. coli lacZ gene has been replaced by that of the A. nidulans gene trpC to allow it to be expressed in filamentous fungi (van Gorcom et al. 1986) and these vectors have been given to us by van den Hondel's group.

This chapter describes the construction of a fusion between the PGK promoter and the coding region of the E. coli lacZ gene, its subsequent transformation into P. chrysogenum and the identification, by Southern analysis, of a strain in which the transforming plasmid has integrated at a defined locus. The A. nidulans PGK promoter-lacZ fusion (Streatfield 1990) was introduced into P. chrysogenum to verify that the E. coli lacZ gene would be expressed in P. chrysogenum.

Transforming DNA on a circular plasmid can integrate into the chromosome in one of three ways described by Hinnen et al. (1978). First the circular plasmid can integrate by a single crossover event at the homologous locus, in which case the entire plasmid is incorporated into the DNA and the target locus is therefore duplicated. Second the plasmid can integrate at a non-homologous locus by the same mechanism. Finally the selectable marker may integrate at the homologous locus by a double crossover in which case only the selectable marker and no other sequence is incorporated into the genome. The first type of transformant is the one which is required for the present analysis.
6.1 Transformation of *P. chrysogenum* N.R.R.L. 1951 with the *A. nidulans* PGK promoter-lacZ gene fusion.

A cotransformation experiment was the simplest way to introduce the *A. nidulans* PGK promoter-lacZ fusion into *P. chrysogenum* in order to determine whether the lacZ gene would be expressed in *P. chrysogenum*. In a cotransformation experiment the transforming DNA is a mixture of two different plasmids, and it has been shown that sphaeroplasts which have taken up one have a high chance of taking up the second. Transformation with the plasmid pPOL20 (Figure 6-1) was used to cotransform the plasmid p5'PGK- lacZ which contains the *A. nidulans* PGK promoter-lacZ fusion (Streatfield 1990, Figure 6-1), into *P. chrysogenum* strain N.R.R.L. 1951.

The general method used for transformation of *P. chrysogenum* was that of Bull et al., 1988 (Chapter 2, section 1.9). Two transformation experiments were done with different ratios of the two plasmids pPOL20 and p5'PGK-lacZ2; one with 1µg of pPOL20, and 4µg of p5'PGK-lacZ2, one with 2µg pPOL20 and 3µg of p5'PGK-lacZ2. The DNA was added in a total volume of 5µl of T.E. to 50µl aliquots of 10^8 protoplasts. Following the transformation procedure aliquots of protoplasts were mixed with an MEA overlay containing 0.9M sodium chloride and poured onto MEA plates. After incubation for 20 hours at 30°C, to allow regeneration of the protoplasts, the plates were overlaid with 25ml of MEA containing 3µg/ml oligomycin. Colonies appeared after 6-8 days' incubation at 30°C and were purified by replating the conidia from a single colony onto MEA plates containing oligomycin, isolating a single colony and repeating the procedure.

Six transformants were isolated and tested for the presence of β-galactosidase activity using X-gal plates (Chapter 2, section 1.5; Streatfield 1990). Conidia from the transformants were patched onto agar plates containing minimal glucose medium, buffered to pH6 with citric acid and disodium hydrogen phosphate, containing X-gal at 40 µg/ml. The glucose in the medium supressed endogenous β-galactosidase activity, and non-transformed N. R. R. L. 1951 gave colourless colonies on the test plates. Only one
of the six transformed strains, Tl gave a positive signal on the X-gal plates.

To confirm that Tl contained E. coli lacZ DNA a Southern analysis of genomic DNA from the transformants Tl, T4, T6 and N.R.R.L. 1951, was done (Chapter 2, section 7) using a probe for the lacZ gene. Genomic DNA was incubated with BamHI, the digestion products separated on a 0.7% agarose gel, transferred to Hybond-N membrane and hybridised to 200ng of radioactively labelled lacZ probe. The lacZ probe was a 4kb XhoI restriction fragment containing the lacZ coding region and the 3' region of trpC and was isolated using the method described in Chapter 2 section 6.3B, from the plasmid pAN923-42A described below. Genomic DNA fragments of size 3kb and 4.6kb from transformant Tl hybridised to the probe but no hybridisation was seen for any of the other strains. Therefore only transformant Tl contained lacZ DNA (Figure 6-2). The site and mode of integration was not deduced. The experiment shows that the lacZ system can be used in P. chrysogenum but it has not proved that the lacZ gene was being driven by the A. nidulans PGK promoter in this particular transformant.
Figure 6-1

A: Restriction map of p5'PGK-lacZ2

The origins of the DNA sequences of plasmid p5'PGK-lacZ2 (Streatfield 1990) are indicated:-

<table>
<thead>
<tr>
<th>Restriction sites</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. chrysogenum DNA</td>
<td>pUC7 DNA</td>
</tr>
<tr>
<td>Extent of hybridisation to A. nidulans oliC</td>
<td></td>
</tr>
</tbody>
</table>

B: Restriction map of pPOL20

The origins of the sequences in the plasmid pPOL20 (Bull, unpublished) are indicated:-

<table>
<thead>
<tr>
<th>Restriction sites</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. nidulans DNA</td>
<td>Direction of transcription</td>
</tr>
<tr>
<td>pBR325</td>
<td>PGK promoter</td>
</tr>
<tr>
<td>trpC terminator region</td>
<td></td>
</tr>
</tbody>
</table>

Restriction sites:
- Bg=BglII
- B=BamHI
- E=EcoRI
- H=HindIII
- Xo=XhoI
Figure 6-2: Southern analysis of P. chrysogenum strains cotransformed with the vectors pPOL20 and p5'PGK-lacZ2

Samples of genomic DNA from the transformed strains T1, T4, T6, and the recipient strain N.R.R.L. 1951 were digested with BamHI, the products separated by electrophoresis on an 0.7% agarose gel, transferred to Hybond-N and hybridised to 200ng of a radioactively labelled 4kb XhoI fragment containing the E. coli lacZ coding region and 0.7kb of the A. nidulans trpC terminator region. The hybridisation was done as described in Chapter 2 section 7.4.

DNA size markers (M) are lambda DNA digested with HindIII

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N.R.R.L. 1951 genomic DNA</td>
</tr>
<tr>
<td>2</td>
<td>T1 genomic DNA</td>
</tr>
<tr>
<td>3</td>
<td>T4 &quot; &quot;</td>
</tr>
<tr>
<td>4</td>
<td>T6 &quot; &quot;</td>
</tr>
</tbody>
</table>

The fragments in lane 3 which hybridised to the lacZ probe are 3 and 4.6kb in size.
6.2 Construction of a vector containing a *P. chrysogenum* PGK promoter-lacZ gene fusion

Since β-galactosidase was expressed from the *E. coli* lacZ gene in *P. chrysogenum*, a gene fusion between the PGK promoter of *P. chrysogenum* and the coding region of the *E. coli* lacZ gene was constructed. The *P. chrysogenum* PGK promoter was available as a 1.6kb HindIII fragment in the M13 clone PC3.2 (Chapter 3 Figure 3-8), and all of the 5′ sequence available of this fragment was incorporated into the construct. The lacZ gene was available on the series of plasmids pAN923-41A, -42A, -43A, which were given to us by van den Hondel’s group (Figure 6-3; Van Gorcom et al. 1986). In this series of plasmids the first few codons of the lacZ gene have been replaced by a multiple cloning site in each reading frame at the 5′ end of the lacZ gene to enable translational fusions to be made to the coding region in all reading frames. The plasmid pAN923-42A was chosen for the construction because it had the required sites for an in-frame fusion to the PGK coding region.

For the purposes of the study a transformation system was required which will produce low copy number transformants with integration at a defined gene locus. The transformation and selection system chosen which fulfilled these requirements was the oligomycin resistance marker which is encoded in *P. chrysogenum* by a mutant in the ATP synthase subunit 9 gene, oliC13. This mutant gene was isolated from *P. chrysogenum* (J. Bull et al. 1988) and was given to me on the plasmid pPOL20 by Professor G. Turner’s group (Figure 6-1)

The strategy for the construction of the promoter fusion to lacZ was first to construct a plasmid in which the HindIII fragment containing the PGK promoter isolated from PC3.2 was inserted into the HindIII site of the plasmid pPOL20 which contains the oligomycin resistance gene. The second step was to isolate a XhoI fragment containing the *E. coli* lacZ gene with the trpC terminator from pAN923-42A and insert it into the first plasmid at the SalI site 34 bases 3′ to the PGK translational start. The reading frame of the lacZ coding region would be unaltered when the DNA ends generated by XhoI at the 5′ end of the lacZ coding region and
by Sali at the 5' end of the PGK coding region are joined (Figure 6-4).

The products of the digestion of PC3.2 with HindIII were separated by electrophoresis on a 0.7% agarose gel and about 300ng of the 1.6kb HindIII fragment containing the PGK promoter was isolated by the method described in Chapter 2, section 6.3B. The plasmid pPOL20 was linearised by incubation with HindIII, proteins removed by phenol/chloroform extraction, the DNA recovered by ethanol precipitation and dissolved in sterile distilled water. Two ligation reactions were set up, one with the vector pPOL20 alone (positive control), one with estimated amounts of 5ng linearised pPOL20 and 100ng of the 1.6kb HindIII fragment from PC3.2. Half of the products of each ligation reaction was transformed into the E. coli strain JM83 (Chapter 2, section 2.5). The positive control yielded many transformants as expected and nine transformants were isolated from the products of the other ligation reactions. Restriction analysis with HindIII of plasmid DNA from the transformants showed that 3 (pOP5, pOP11, pOP12) contained the 1.6kb HindIII fragment. The orientation of the 1.6kb insert in the three plasmids was determined by restriction analysis with EcoRI and XbaI. The plasmid chosen to continue the construction was pOP12 which has the structure shown in Figure 6-4.

Approximately 100ng of the plasmid pOP12 was incubated with Sali and the linearised DNA recovered. The plasmid pAN923-42A was incubated with XhoI which released the lacZ gene and trpC terminator on a 4kb fragment. After separation of the digestion products by electrophoresis on an agarose gel, the 4kb fragment was isolated, purified as described and resuspended at a concentration of 10-20ng/μl. Three ligation reactions were set up. The estimated amounts in the first reaction were 10ng of linearised pOP12 and 150ng of the 4kb XhoI fragment, in the second 5ng of pOP12 and 150ng of the 4kb fragment, and in the third, 5ng of pOP12 alone (the positive control). Aliquots of the ligation reaction products were transformed into the E. coli strain JM83 and the transformants selected on medium containing X-gal and ampicillin. X-gal was used since it was possible that the E. coli transformants with lacZ inserts in the correct
orientation would express β-galactosidase, and on this medium those colonies would stain blue. However only one of the transformants appeared blue and plasmid DNA was prepared from twelve separate transformants including this one. Electrophoresis of native plasmid DNA on a 0.7% agarose gel showed that four of the plasmids contained inserts and separate digestion of the plasmid DNAs with XbaI, XhoI or EcoRI, identified two plasmids pOPL7 and pOPL8 with the correct orientation of the 4kb insert. The plasmid from the X-gal positive transformant had a modified insert.

Bulk plasmid DNA was prepared for pOPL8 and restriction analysis using the enzymes EcoRI, XhoI, XbaI, and HindIII separately and in all possible pairs showed that the plasmid yielded restriction fragments of the size predicted for the construct (Figure 6-5).
Figure 6-3: Restriction map of the plasmid pAN923-42A

The origins of the sequences in plasmid pAN923-42A are indicated:

- A. nidulans DNA
- E. coli plasmid DNA
- Direction of transcription
- trpC trpC terminator region
- Xb XbaI
- H HindIII
- E EcoRI
- Xo XhoI
polylinker

41A

\[
\begin{align*}
\text{Xo} & \quad B & \quad S & \quad P & \quad H \\
\text{CT.CGA.GGA.TCC.GTC.GAC.CTG.CAG.CCA.AGC.TTG.GCA.CTG.GCC.GTC.}
\end{align*}
\]

42A

\[
\begin{align*}
\text{Xo} & \quad B & \quad E \\
\text{CTC.GAG.GAT.CCC.GTC.}
\end{align*}
\]

43A

\[
\begin{align*}
\text{Xo} & \quad B & \quad E \\
\text{C.TCG.AGG.ATC.CCC.GGG.AAT.TCA.CTG.GCC.GTC.}
\end{align*}
\]
Figure 6-4: Strategy for construction of the fusion of the P. chrysogenum PGK promoter to the lacZ gene of E. coli.

Stage 1 Construction of pOP12
step 1 - digest pPOL20 with HindIII and recover DNA
step 2 - digest PC3.4 with HindIII and recover the 1.5kb fragment
step 3 - ligate DNAs

Stage 2 Construction of pOPL8
step 1 - digest pOP12 with SalI
step 2 - digest pAN923-42-A with XhoI and isolate the 4kb fragment
step 3 - ligate DNAs

The origins of the DNA sequences are indicated:

- P. chrysogenum DNA
- E. coli plasmid pUC9 (pPoL20)
- M13 phage mp19 (PC3.4)
- A. nidulans DNA
- E. coli plasmid DNA

The arrows indicate the direction of transcription of genes.

Restriction enzyme sites
H=HindIII
P=PstI
E=EcoRI
S=SalI
Xa=XbaI
N=NcoI
Bg=BglII
B=BamHI
Figure 6-5: Restriction analysis of plasmid pOPL8

Samples of plasmid pOPL8 DNA were digested singly and in all possible pairwise combinations with EcoR1, XbaI, HindIII, XhoI. The fragments generated were separated on an agarose gel and stained with ethidium bromide to produce the fragments shown. The DNA size markers are as described in Figure 6-2. A map of pOPL8 is presented in Figure 6-6.

<table>
<thead>
<tr>
<th>lane</th>
<th>Enzyme</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EcoRI</td>
<td>6.2, 3.7</td>
<td>5.7, 3.7</td>
</tr>
<tr>
<td>2</td>
<td>XbaI</td>
<td>5.4, 4.5</td>
<td>5.2, 4.4</td>
</tr>
<tr>
<td>3</td>
<td>HindIII</td>
<td>5.6, 4.3</td>
<td>5.3, 4</td>
</tr>
<tr>
<td>4</td>
<td>XhoI</td>
<td>9.9</td>
<td>9.4</td>
</tr>
<tr>
<td>5</td>
<td>EcoRI, XbaI</td>
<td>4.4, 2.7,</td>
<td>4.5, 2.85,</td>
</tr>
<tr>
<td></td>
<td>XbaI</td>
<td>1.8, 1.0</td>
<td>1.7, 0.98</td>
</tr>
<tr>
<td>6</td>
<td>HindIII, EcoRI</td>
<td>4.6, 1.0,</td>
<td>4.75, 2.85,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.7, 1.6</td>
<td>1.45, 0.99,</td>
</tr>
<tr>
<td>7</td>
<td>EcoRI, XhoI</td>
<td>6.0, 3.7,</td>
<td>6.1, 3.8</td>
</tr>
<tr>
<td></td>
<td>XhoI</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>HindIII, XbaI</td>
<td>5.4, 4.3,</td>
<td>5.3, 4.35</td>
</tr>
<tr>
<td></td>
<td>XbaI</td>
<td>0.2, 0.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>XbaI, XhoI</td>
<td>5.4, 2.9, 1.6</td>
<td>5.25, 2.9, 1.6</td>
</tr>
<tr>
<td>10</td>
<td>HindIII, XhoI</td>
<td>5.6, 2.9, 1.4</td>
<td>5.5, 2.8, 1.3</td>
</tr>
</tbody>
</table>
6.3 Transformation of the gene fusion vector pOPL8 into P. chrysogenum

The general method described above was used to transform 10μg of the circular plasmid pOPL8 alone into P. chrysogenum N.R.R.L. 1951. The DNA was added to a 50μl aliquot of protoplasts containing 2x10^8 sphaeroplasts/ml. The transformation efficiency was 4x10^-6 transformants per microgram of DNA per protoplast which was much as expected (Bull et al. 1988). Transformants were purified as described above and tested on X-gal medium for β-galactosidase activity. Of 15 transformants tested 14 had β-galactosidase activity and genomic DNA was prepared from each of these for Southern analysis to identify where integration had occurred.

6.4 Analysis of the transformants

In order to study the expression of the PGK promoter a transformant in which the vector pOPL8 had integrated by homologous recombination at a defined locus was required. There was some information about the map of the oliC region from previous Southern analysis and in Bull et al. (1988) from which maps could be drawn of the outcome of the predicted integration (Figure 6-6).

There is no SalI restriction site within the plasmid pOPL8. Therefore if the genomic DNA of the transformants was digested with SalI the integrated plasmid would remain intact in a DNA fragment of at least 9kb. If the DNA was hybridised to a probe for the oliC gene in a Southern analysis then one 7.8kb band would be predicted in non-transformed P. chrysogenum. A single band of 17.6kb would replace this band in transformants which had aquired a single copy of the plasmid by homologous integration. Transformants in which the plasmid had integrated elsewhere would produce two hybridising bands, the band detected in the original strain and a second of at least 9kb depending on the location of the adjacent SalI sites.

Genomic DNA isolated from the transformants was digested with SalI, the products separated by electrophoresis, transferred to Hybond-N and hybridised to a 1.4kb HindIII-XhoI fragment from pPOL20 containing the oliC13
gene. Among the strains tested, thirteen had the plasmid pOPL8 integrated at undefined loci and one, 8(9) had pOPL8 integrated at the oliC locus. An autoradiograph of the pattern of hybridisation of some of the transformants including 8(9) is shown in Figure 6-7.

Since the size of the hybridising fragment in transformant 8(9) was greater than 20kb there was probably more than one copy of pOPL8 integrated at the oliC locus. The copy number of integrated molecules was determined by further Southern analysis using BamHI and EcoRI to cleave the genomic DNA and the same radioactive probe. There is one BamHI site within pOPL8 and bands of 8.4 and 5.5kb were expected on the autoradiograph if there was only one copy of the plasmid. If more than one copy was integrated a third 9.9 kb band corresponding to the linearised plasmid would be expected and this would become more intense compared to the other bands in proportion to the number of copies of pOPL8. There are two EcoRI sites in pOPL8 but only two bands of 5.6 and 13.5kb were expected to hybridise to the oliC probe and if more than one copy of the plasmid had integrated then an extra 6.1kb band would be expected. The analysis showed that there were probably two copies of the plasmid pPOL8 integrated at the oliC locus because the predicted third band was present but was not noticeably more intense than the other bands on the autoradiograph (Figure 6-8).
Figure 6-6: Integration of pOPL8 at the oliC locus

The Figure shows the model for the prediction of the pattern of restriction sites generated when a single copy of plasmid pOPL8 integrates into the chromosome at the oliC locus.

The origins of the sequences is shown:—

- P. chrysogenum DNA
- A. nidulans DNA
- E. coli pUC9 DNA

The direction of transcription of relevant genes is indicated by an arrow.

The symbols used for restriction sites are:—

H=HindIII
B=BamHI
E=EcoRI
Xo=XhoI
Xa=XbaI
Figure 6-7: Southern analysis of *P. chrysogenum* strains transformed with the plasmid pOPL8

Genomic DNA from the strains indicated below was prepared and digested with SalI and a Southern analysis done as described in Chapter 2 section 7. The radioactive DNA probe was the 1.4kb HindIII-XhoI fragment from pPOL20 containing the oliC gene (Figure 6-1) labelled by the random oligonucleotide priming method. The DNA markers are as described in Figure 6-2.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Strain</th>
<th>Observed bands</th>
<th>Site of integration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recipient strain:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>N.R.R.L. 1951</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transformed strains:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8(9)</td>
<td>&gt;23</td>
<td>oliC (more than one copy)</td>
</tr>
<tr>
<td>3</td>
<td>7(4)</td>
<td>7.8, &gt;23</td>
<td>elsewhere</td>
</tr>
<tr>
<td>4</td>
<td>8(6)</td>
<td>7.8 &gt;23</td>
<td>elsewhere</td>
</tr>
<tr>
<td>5</td>
<td>7(6)</td>
<td>7.8, &gt;23</td>
<td>elsewhere</td>
</tr>
<tr>
<td>6</td>
<td>8(22)</td>
<td>7.8, &gt;23</td>
<td>elsewhere</td>
</tr>
</tbody>
</table>

The size of the fragment expected to hybridise to the probe for the recipient strain is 7.8kb. For a strain with a single copy of the plasmid integrated at the oliC gene, a single band 17.6Kb in size is expected, and a strain with a copy of the plasmid integrated elsewhere in the genome is expected to produce two bands of 7.8 Kb, and at least 9.8Kb.
Figure 6-8: Further Southern analysis of the transformed strain 8(9)

Genomic DNA from P. chrysogenum transformed strain 8(9) and the recipient strain N.R.R.L. 1951 was digested with EcoRI or BamHI and a Southern analysis done using the 1.4Kb oliC probe described in Figure 6-7. The DNA size markers are as described in Figure 6-2.

The predicted bands from a single copy of the plasmid integrated at the oliC locus are shown in the table below. If there is more than one copy of pOPL8 at the oliC locus then an additional band of 6.1kb in the EcoRI digestion and 9.8 kb in the BamHI digestion are expected. The fragment in parentheses may be due to incomplete digestion.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Strain</th>
<th>Enzyme</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8(9)</td>
<td>BamHI</td>
<td>8.4, 5.5</td>
<td>9.6, 8.2, 5.6</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>EcoRI</td>
<td>13.5, 5.6</td>
<td>14, (6,) 5.9, 5.3</td>
</tr>
<tr>
<td>3</td>
<td>1951</td>
<td>EcoRI</td>
<td>12.4</td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>BamHI</td>
<td>4.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>
6.5 Identification of the PGK promoter-lacZ fusion mRNA and comparison to the native PGK mRNA.

In order to assess how efficiently the PGK promoter-lacZ gene fusion was expressed at the oliC locus a Northern analysis of total cellular RNA from strain 8(9) was done using a DNA probe which contained only sequence common to the PGK promoter-lacZ gene fusion and the native gene. Conidia from the strain 8(9) and the strain N.R.R.L. 1951 were used to inoculate two cultures of minimal medium to 10^6 conidia per ml. The cultures were grown overnight at 30°C on a gyratory shaker at 250rpm. Total cellular RNA was isolated from the young mycelium of each strain and 10μg samples were separated by electrophoresis on a formaldehyde/agarose gel. The RNA was transferred to nitrocellulose and hybridised to 100ng of a 256nt KpnI-AccI fragment isolated from the M13 clone PC3.4 (Chapter 3, Figure 3-8, Chapter 4, Figure 4-1). The probe was isolated from PC3.4, after digestion with KpnI and AccI and labelled by the random priming method (Chapter 2, section 7.3). The hybridisation was done in 6xSSC, 0.25% Marvel milk, 50% deionised formamide at 42°C and post hybridisation washes done at a final stringency of 50°C in 0.1xSSC, 0.1% SDS (Chapter 2, section 9.2).

The resulting autoradiograph showed two bands for RNA isolated from 8(9) corresponding to the PGK mRNA and the PGK promoter-lacZ fusion mRNA, and one corresponding to the PGK mRNA in strain N.R.R.L. 1951 (Figure 6-9A). The second band present in the transformant 8(9) was approximately the size expected for the PGK promoter-lacZ fusion mRNA. The signal for the transformed strain 8(9) was analysed by scanning with a laser densitometer as described in Chapter 5, section 3.2. The resulting peaks showed that the fusion mRNA was 1.7 fold more abundant than the native PGK mRNA and that the band was broader indicating that the mRNA for the fusion protein may be less stable (Figure 6-9B). Since there are two copies of the PGK promoter-lacZ fusion in strain 8(9) the efficiency of transcription from the PGK promoter-lacZ fusion at the oliC locus is at least 85% of the native PGK gene.
Figure 6-9: Northern analysis of total cellular RNA from strain 8(9)

A: The autoradiograph shows a Northern analysis of total cellular RNA isolated from strain 8(9) and from N.R.R.L. 1951. Approximately 10μg of total cellular RNA from each strain was separated on a 1% formaldehyde/agarose gel and transferred to a nitrocellulose filter. The probe was a 203nt KpnI-AccI fragment from PC3.4 (Figure 4-1) which contained the coding sequence and 5' sequence which was common to both the PGK promoter-lacZ fusion and the native PGK gene. It would therefore hybridise to both the native PGK mRNA and the fusion mRNA with equal efficiency. The analysis was done as described in Chapter 2 section 9. The size markers are lambda DNA digested with HindIII and then denatured. The PGK mRNA is expected to be 1.4kb in size and the fusion mRNA, between 3 and 3.5kb.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Strain</th>
<th>Size of hybridising band (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8(9)</td>
<td>1.5, 3.2</td>
</tr>
<tr>
<td>2</td>
<td>8(9)</td>
<td>1.5, 3.2</td>
</tr>
<tr>
<td>3</td>
<td>N.R.R.L. 1951</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>N.R.R.L. 1951</td>
<td>1.5</td>
</tr>
</tbody>
</table>

B: Lane 3 from the autoradiograph was scanned using a laser densitometer as described in Chapter 2 section 9.3 and the trace obtained is shown. The areas under the peaks were measured and the values were 0.2 units for the native PGK message and 0.34 units for the PGK promoter-lacZ fusion message.
Absorbance

Absorbance

PGK promoter

native PGK

lacZ fusion

Y-position [mm]

Y-axis: is normalized

Y-start = 80  Y-stop = 120  Y-step = 1
6.6 Summary

The initial experiment described in this chapter showed that it is possible to use the *E. coli* lacZ gene (with the *A. nidulans* trpC terminator) as a reporter gene in *P. chrysogenum*. A construct containing the *A. nidulans* PGK promoter fused to the lacZ reporter gene was transformed into *P. chrysogenum* and one of the transformed strains had β-galactosidase activity. The nontransformed strain N.R.R.L 1951 was shown to give no background β-galactosidase activity when grown on medium containing glucose.

The *P. chrysogenum* PGK promoter was fused to the *E. coli* lacZ coding region and the construct transformed into *P. chrysogenum* strain N.R.R.L. 1951. Plate tests showed that *P. chrysogenum* strains transformed with the PGK promoter-lacZ fusion vector expressed the lacZ gene and they were screened by Southern analysis to identify one containing the vector integrated in a single copy at the oliC locus. However, although one such transformed strain was found, this contained two copies of the vector. The transformation procedure and subsequent analysis are rather time consuming and since I had no opportunity to compare lacZ fusion vectors with altered PGK promoters, I therefore decided to use this transformed strain for study of the expression of the PGK gene promoter under different physiological conditions. It is reasonable to assume that a single copy transformant would produce activities one half of those I would find but this would not be important for comparative study.
Chapter 7

Investigation of the physiological control of the *P. chrysogenum* PGK promoter

The previous chapter describes the construction of a gene fusion between the *Penicillium chrysogenum* PGK promoter and the *Escherichia coli* lacZ coding region, the transformation of the vector into *P. chrysogenum* and the identification of a transformed strain 8(9) in which two copies of the construct had integrated at the oliC locus. The first part of this chapter describes the use of the PGK promoter-lacZ reporter function in that strain for preliminary investigations of the modulation of PGK expression. Three areas of interest have been studied.

First, since the project arose through the interest of SmithKlineBeecham (SB) in isolating potentially strong promoters for possible application in the genetic manipulation of *P. chrysogenum* strains used for penicillin production, it would be relevant to know how the PGK promoter behaves during batch culture over the periods used in penicillin fermentations. Thus the reporter fusion was used to monitor PGK promoter activity through a batch fermentation.

Second, since studies of message levels described in Chapter 5 section 3.3 showed that *P. chrysogenum* PGK gene expression was modulated when the mycelium was grown on different carbon sources, the response of the *P. chrysogenum* PGK promoter under these conditions was further investigated using the reporter function.

Finally, since sequence analysis of the *P. chrysogenum* PGK promoter described in Chapter 4 section 2.1 had revealed a possible heat shock element at -473 to the major transcriptional start point the reporter function was used to assess the response of mycelium to incubation at elevated temperatures.

In the experiments described in this chapter the transformed strain of *P. chrysogenum* selected by resistance to oligomycin was stored on selective medium and conidia for inoculation of liquid cultures were harvested from mycelium grown on the same medium. However the batch cultures were
done in the absence of oligomycin to ensure good growth. Similarly transformed strains of A. nidulans selected by growth on quinic acid medium were stored on selective medium but were grown on glucose complex medium to ensure a good yield of conidia for the inoculation of liquid cultures. Liquid cultures were grown without selection excepting the experiments in which quinic acid was a carbon source amongst others tested.

The activity of β-galactosidase was measured in cell free extracts and the specific activity determined from the soluble protein content of the extract. If the promoter activity was constant under the conditions of the experiment and β-galactosidase was in a fixed proportion of total protein synthesis, the specific activity of the enzyme would remain the same. Furthermore if promoter activity were modulated positively this would produce a rise in specific activity and if negatively, a fall in specific activity would result.
7.1.1 Activity of the PGK promoter during growth in extended batch culture.

Batch fermentations for penicillin production are done over a period of 4-6 days and to monitor how the PGK promoter was expressed under these conditions the transformed strain 8(9) was grown in a batch fermentation for 6 days on complex FM medium (Chapter 2, section 1.2) and the β-galactosidase activity in mycelial extracts measured.

Four batch fermentations were set up in 1.5l micro-fermenters; one pair of fermenters were inoculated with 100ml of a 24 hour culture of the transformed strain 8(9) grown on FM seed medium and the other pair inoculated with the same volume of a 24 hour culture of the untransformed strain N.R.R.L. 1951. The untransformed strain N.R.R.L. 1951 provided a control to detect endogenous β-galactosidase activity because the FM medium contains lactose. The fermentation conditions are described in Chapter 2, section 1.3. The day to day running of the fermenters was done by Paul Beasley, Gerry Bromley and Mark Richardson at SB. Samples of 10-20ml of culture were taken each morning, cell free extracts prepared and the β-galactosidase activity determined. The mycelial growth was estimated by measuring the volume of mycelium in a 10ml sample of culture each day. The β-galactosidase assay (assay 1; Chapter 2, section 1.6) was based on a stop assay method described in Maniatis (1990) and the protein concentration was determined by the method of Lowry (1951). The results are presented in Figure 7-1.

To verify the stability of the transformed strain genomic DNA was prepared from mycelium collected from each fermenter on the sixth day of the fermentation. The DNA was digested with the restriction enzyme SalI and a Southern analysis was done using the oliC probe as described in Chapter 6, Figure 6-7. The pattern of hybridisation for each strain was unchanged compared to the previous Southern analysis, therefore the strains had not undergone major rearrangements in the absence of selection (data not shown).

The increase in the packed volume of mycelium in the culture showed that active growth of P. chrysogenum occured in the first two days of the fermentation. In the production
phase the packed volume of mycelium remained relatively constant (Figure 7-1B). The transformed strain 8(9) showed a rapid increase in β-galactosidase specific activity and hence PGK promoter activity in the first two days followed by a gradual decrease thereafter (Figure 7-1A). No β-galactosidase activity was detected for the untransformed strain N.R.R.L. 1951 until the fourth day probably because by that time glucose present in the medium had been exhausted and the endogenous β-galactosidase gene was derepressed. Thus expression of the PGK promoter varies during fermentation.
Figure 7-1: Expression of the PGK promoter in long term batch fermentation

The PGK promoter-lacZ reporter fusion integrated in two copies at the oliC locus of P. chrysogenum was used to report the PGK promoter activity in a six day fermentation on complex FM medium. The original strain N.R.R.L. 1951 was included as a control to monitor endogenous β-galactosidase activity. A volume of 1.5l of fermentation medium was inoculated with 100ml of 24 hour culture of the appropriate strain. The fermentation conditions were 1.5l air/minute, stirring at 1600rpm, pH between 6 and 8.

A: the graph shows the mycelial volumes measured over the period of the fermentation. The units are in ml per 10ml of culture and are the average values for two fermenters.

- • - transformed strain 8(9)
- ---O-- original strain N.R.R.L. 1951

B: shows the average β-galactosidase specific activities measured during the six day fermentation for each pair of fermenters. A volume of 10-20 ml of culture was harvested by filtration, cell free extracts prepared and the β-galactosidase specific activity determined. The specific activity is measured in nmoles ONPG hydrolysed per mg soluble protein per minute. The symbols are as in A. The values for the untransformed strain have been subtracted from the transformed strain.
A sediment
ml/10ml culture

B β-galactosidase activity

time of fermentation (hrs)

24 48 72 96 120 144

2000 — 1500 — 1000 — 500

96 120 24 144 72 48

24 48 72 96 120 144

2200

1500

1000

500
7.1.2 Expression of the PGK promoter during growth and on different carbon sources.

The data presented in Chapter 5, Figure 5-6) indicated that P. chrysogenum PGK mRNA levels are affected by the carbon source on which the mycelium is grown and were more abundant in mycelium from cultures containing carbon sources metabolised by gluconeogenesis. Further estimates of the relative activity of the PGK promoter were obtained by growing the transformed strain 8(9) on minimal media containing different carbon sources and monitoring the β-galactosidase activity. The gene fusion is integrated at the oliC locus. This gene encodes the mitochondrial ATPase subunit 9 protein and is not affected by growth of the strain on different carbon sources (G. Turner pers comm).

Several experiments were done in which the transformed strain 8(9) was grown in liquid medium with glucose, glycerol, acetate or quinic acid as carbon sources (Chapter 2, section 1.2). Cultures were grown in 2L flasks with 200ml minimal medium inoculated with $10^6$ conidia per ml and incubated at 30°C on a gyratory shaker at 250 rpm. The yield of mycelium was monitored by harvesting small samples of the culture and weighing the mycelial pad. The mycelium was harvested at the equivalent of 0.15g fresh weight per 100ml, cell free extracts prepared and the β-galactosidase specific activity determined using a continuous spectrophotometric procedure (assay 2; Chapter 2, section 1.6) and the Lowry method (1951) to determine protein concentration.

The range of β-galactosidase specific activities, relative to glucose (1.0) grown mycelium were, glycerol 0.94-1.4, quinic acid 2.4-3.0 acetate 1.5-1.9. Thus the relative activity of the PGK promoter is elevated on carbon sources which are metabolised by gluconeogenesis (quinic acid and acetate) rather than by glycolysis (glucose and glycerol). The increased β-galactosidase activities observed agree broadly with the results obtained from the mRNA studies (Chapter 5 section 3.3).

The activity of the PGK promoter was found to increase during the early phase of growth of mycelium on complex medium and was therefore monitored during exponential growth

119
in minimal medium on the four carbon sources, described above. Cultures in 2L flasks containing 400ml minimal medium were set up as previously described and mycelial samples harvested at intervals between 12 to 27 hours, weighed and stored overnight at -20°C.

The growth curves were found to be different for cultures grown on different carbon sources (Figure 7-2B). The glycerol, quinic acid and acetate cultures entered exponential growth later than the glucose culture. Presumably the enzymes for the metabolism of the different carbon sources were induced during this lag period. Once the cultures had entered exponential phase the doubling times were very similar for mycelium growing on glucose (3 hours), glycerol (4 hours) and quinic acid (3.5 hours), and about half that of the acetate culture (7.5 hours). Three samples were chosen from the exponential growth phase of each of the cultures, taken over the period of at least one doubling in mycelial weight. Cell free extracts were prepared from the samples and the β-galactosidase specific activity determined.

In exponential growth of each of the four cultures the β-galactosidase specific activity and therefore the PGK promoter activity increased as the mycelial weight increased. However the rates of increase of β-galactosidase specific activity were different for each growth medium, being 2-5 times greater on carbon sources metabolised by gluconeogenesis (Figure 7-2A).

Therefore the activity of the constitutive P. chrysogenum PGK promoter during exponential growth on minimal medium is positively modulated. Growth on different carbon sources affects the extent of modulation, which is greatest on carbon sources metabolised by gluconeogenesis.

7.1.3 The effect of heat on PGK promoter activity

A 7 of 8 nucleotide match to the consensus for the eukaryotic heat shock element C--GAA--TTC--G (Welshman 1985) occurs at position -473 from the transcriptional start in the PGK gene (Chapter 4, Figure 4-3). Since the PGK promoters in Saccharomyces cerevisiae and Trichoderma reesi also have heat shock elements the effect of incubation of
mycelium at elevated temperatures on the PGK promoter of P. chrysogenum was investigated by monitoring the β-galactosidase specific activity of the transformed strain 8(9) during incubation at 40°C. The strain 8(9) was grown for 16-18 hours in glucose minimal medium at 30°C and then part of the culture transferred to 40°C. Samples of mycelium were harvested after intervals of 20, and 40 and 180 minutes at 40°C, cell free extracts prepared, and β-galactosidase specific activity determined. If the expression of the PGK gene was induced by growth under these conditions and the β-galactosidase enzyme was stable at 40°C an increase in the β-galactosidase specific activity would be observed.

Specific activity of β-galactosidase remained fairly constant up to 40 minutes after transfer of mycelium to 40°C. After 180 minutes the specific activity increased compared to the control culture held at 30°C, suggesting an induction had occurred. However the total soluble protein in the later samples had fallen dramatically (Table 7-1). Overall the data suggest that the rate at which the PGK promoter initiates transcription was not increased under heat shock conditions.

The stability of β-galactosidase in P. chrysogenum cell free extracts was measured at 40°C compared to 30°C and 0°C and after two hours the activity was still 90% of the starting activity (data not shown). Therefore the β-galactosidase is not inherently unstable at elevated temperatures.
Figure 7-2: The expression of the PGK promoter in growth on different carbon sources.

Four cultures of minimal medium containing as a carbon source either glucose (0.02M), glycerol (0.04M), quinic acid (1% w/v), acetate (0.1M) were inoculated with the transformed strains P. chrysogenum 8(9), A. nidulans AL8, A. nidulans PL25 and incubated on a gyratory shaker at 250 rpm at the appropriate temperature. Samples of 30-50 ml were taken during logarithmic growth and the fresh weight of the mycelium determined (A). Cell free extracts were prepared from selected samples and the β-galactosidase specific activity determined from them (B).

A: the graph shows the increase in mycelial fresh weight calculated for 10ml of culture during the experiment.

- × glucose grown mycelium
- • glycerol grown mycelium
- △ quinic acid grown mycelium
- ○ acetate grown mycelium

Panel 1: P. chrysogenum 8(9). The P. chrysogenum PGK promoter-lacZ fusion transformed into P. chrysogenum strain N.R.R.L 1951 and integrated in two copies at the oliC locus.

Panel 2: A. nidulans AL8. The A. nidulans PGK promoter-lacZ fusion transformed into A. nidulans strain Q716 and integrated in one copy at the qutE locus.

Panel 3 A. Nidulans PL25. The P. chrysogenum PGK promoter-lacZ fusion transformed into A. nidulans strain Q716 and integrated in one copy at the qutE locus.

B: the graph shows the β-galactosidase specific activity observed for the selected samples plotted against the fresh mycelial weight/100ml of culture of the sample. The symbols for different carbon sources are as above. The specific activity is calculated as OD units at 420nm change x10^3 per minute per mg of protein. The panels are as described above.
B

1. β-galactosidase activity

2. β-galactosidase activity

3. β-galactosidase activity

yield g/100ml
Table 7-1 The effect of heat shock at 40°C on PGK promoter activity

A 200ml minimal medium culture in a 2L flask was inoculated with 2x10^8 conidia of the transformed strain 8(9) and incubated for 16-18 hours on a gyratory shaker at 250rpm at 30°C. Part of the culture was then transferred to another flask at 40°C for the intervals shown. The mycelium from both cultures was then harvested and the fresh mycelial weight measured. The β-galactosidase activity was then determined for cell free extracts prepared from the mycelium. The table shows the results obtained. The specific activity is expressed as the OD change at 420nm x 10^{-3} per mg protein per minute.

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Protein concentration (mg/ml)</th>
<th>Specific activity (u/mg protein)</th>
<th>Protein concentration (mg/ml)</th>
<th>Specific activity (u/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>59</td>
<td>2080</td>
<td>87</td>
<td>1750</td>
</tr>
<tr>
<td>40</td>
<td>82</td>
<td>1600</td>
<td>78</td>
<td>2470</td>
</tr>
<tr>
<td>180</td>
<td>63</td>
<td>1804</td>
<td>15</td>
<td>2941</td>
</tr>
</tbody>
</table>
7.2 The expression of the PGK promoter of *P. chrysogenum* in *A. nidulans*.

Sequence analysis of the PGK promoter of *P. chrysogenum* revealed both differences to the *A. nidulans* PGK promoter as well as some similarities.

Transfer of the *P. chrysogenum* PGK promoter-lacZ fusion to *A. nidulans* would show whether the promoter from *P. chrysogenum* retained its pattern of expression in *A. nidulans*. If the pattern of expression is retained then the sequences present in the *P. chrysogenum* PGK promoter are sufficient to control the promoter in *A. nidulans* implying that the appropriate transcription factors are present in *A. nidulans* and recognise the *P. chrysogenum* promoter sequences. If the pattern is different, then this may be because the appropriate factors are not present, cannot bind to the promoter or bind to it with a different affinity. An investigation of expression of the *P. chrysogenum* PGK promoter in *A. nidulans* is described in the second part of this chapter.

The expression of the *P. chrysogenum* PGK promoter may be compared to the *A. nidulans* PGK promoter by constructing an *A. nidulans* strain in which the *P. chrysogenum* PGK promoter-lacZ fusion is integrated at a defined locus. The strain could then be compared to an *A. nidulans* strain in which the *A. nidulans* PGK promoter-lacZ fusion is integrated at the same locus. The *A. nidulans* strain L8, in which a fusion of 487nt of the promoter and 72nt of the coding region of the PGK gene of *A. nidulans* to the lacZ gene of *E. coli* had been integrated in a single copy at the qutE locus was available for the comparison (Streatfield 1990). Therefore an equivalent strain in which the *P. chrysogenum* PGK promoter-lacZ reporter fusion was integrated at the qutE locus was constructed.

The DNA fragment containing the gene fusion in the plasmid pOPL8 was isolated and subcloned into a vector bearing a portion of the QUT gene cluster (pAL3.3) to form pH16 (Figure 7-3). The construct pH16 was transformed into *A. nidulans* strain 7-16 and the transformed strains screened for single copy integrants at the qutE locus. Under my supervision a third year undergraduate student, Leonard Wu verified the structure of the vector pH16, analysed the A.
nidulans transformants and compared the activity of the two PGK promoters under different conditions of growth using β-galactosidase assays.

7.2.1 The construction of a *P. chrysogenum* PGK lacZ fusion vector for transformation into *A. nidulans*.

The plasmid pAL3.3 contains a 3.3kb *A. nidulans* DNA fragment encompassing the QUTE gene, with 820 nt of 3' sequence including part of the 3' end of QUTA gene and 2037 nt 5' sequence including the QUTG open reading frame (Figure 7-4). There is a HindIII site 247nt from the BamHI site 3' to the QUTE gene and this was the site for insertion of a 5.5kb HindIII fragment from pOPL8 bearing the PGK promoter-lacZ fusion.

The plasmid pOPL8 was incubated with HindIII which released the *P. chrysogenum* PGK promoter-lacZ reporter fusion on a 5.5kb DNA fragment. About 1μg of the fragment was recovered and purified (Chapter 2 section 6.3B) as described previously. About 1μg of the plasmid pAL3.3 was also incubated with HindIII to linearise it 3' to the QUTE gene. The 5' phosphate groups were removed from the linearised pAL3.3 DNA by incubation with one unit of calf intestinal phosphatase as described in Chapter 2, section 6.6. The DNA was purified by phenol extraction and recovered. A ligation reaction (Chapter 2 section 6.5) was set up with 200ng of pAL3.3 and 300ng of HindIII fragment, and a control ligation reaction with the phosphatased vector alone. The *E. coli* strain JM83 was transformed with the products of the ligation reactions (Chapter 2 section 2.5) and some transformants were observed for the control which showed that the phosphatase reaction was not complete. However 24 transformants were isolated from the experimental sample and plasmid DNA prepared from them. The plasmid DNA was analysed by restriction digestion with HindIII for the presence of the 5.5kb HindIII fragment bearing the PGK promoter-lacZ reporter fusion. Two plasmids had the insert and one (pPH16) was subjected to further restriction analysis with the enzymes EcoRI, BamHI, XbaI singly and in pairs. The numbers of bands and their sizes agreed with those predicted confirming the structure of the vector pPH16 (Figure 7-5).
The plasmid PAL3.3 (Whittington et al. 1987) contains a 3.3 kb BamHI fragment from the QUT gene cluster of A. nidulans encompassing the QUTE gene, the QUTG open reading frame and part of the QUTA gene.

The plasmid PAL1 was made by isolation of the 1kb EcoRI-XbaI fragment encompassing the QUTE gene and ligation of that fragment with pUC19 vector linearised with EcoRI and XbaI.

The origins of the DNA sequences are indicated:

- A. nidulans DNA
- E. coli plasmid pBR325 (A)
- E. coli plasmid pUC19 (B)
- Extent of probe for Southern analysis

The direction of transcription of the genes are indicated by arrows.

**Restriction enzyme sites**

P=PstI
E=EcoRI
H=HindIII
B=BamHI
Xa=XbaI
Figure 7-4: Strategy for construction of the vector pPH16 used to transform the *P. chrysogenum* PGK promoter-lacZ fusion into *A. nidulans*.

The construction of pPH16 shown in this Figure was accomplished in one step from the plasmid pAL3.3 (Figure 7-3) and the plasmid pOPL8 (Chapter 6, Figure 6-6).

The origins of the DNA sequences is indicated:

- A. nidulans DNA
- P. chrysogenum DNA
- E. coli vector DNA

The direction of transcription of the genes is indicated by an arrow.

**Restriction enzyme sites**

- P = PstI
- E = EcoRI
- H = HindIII
- Xa = XbaI
Figure 7-5: Restriction analysis of the plasmid pPH16

Samples of the plasmid DNA were digested with the restriction enzymes EcoRI, BamHI, and XbaI singly and in pairs. The digestion products were separated by electrophoresis in a 0.7% agarose gel and stained with ethidium bromide. The size markers in lane M are phage lambda DNA digested with HindIII.

The table below shows the lane order, the predicted and the observed digestion fragments and their sizes. Some of the digestions were not complete and bands believed to be the products of partial digestion are shown in parentheses. Other bands are doublets and indicated by [X2].

<table>
<thead>
<tr>
<th>Lane</th>
<th>Enzyme</th>
<th>Predicted fragment size (Kb)</th>
<th>Observed fragment size (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EcoRI</td>
<td>2.0, 5.8, 7.1</td>
<td>2.2, 5.7, 7.0</td>
</tr>
<tr>
<td>2</td>
<td>XbaI</td>
<td>0.2, 1.0, 1.8, 5.4, 6.3</td>
<td>1.3, 2.1, 5.2, 6.6</td>
</tr>
<tr>
<td>3</td>
<td>BamHI</td>
<td>3.3, 4.4, 7.2</td>
<td>3.4, 4.4, 7.4</td>
</tr>
<tr>
<td>4</td>
<td>EcoRI,</td>
<td>0.2, 0.9, 1.0, 1.0, 1.0</td>
<td>0.8, 0.9, 1[x2]</td>
</tr>
<tr>
<td></td>
<td>XbaI</td>
<td>1.0, 1.4, 4.4, 4.5</td>
<td>1.3, 4.4, 5.1</td>
</tr>
<tr>
<td>5</td>
<td>EcoRI,</td>
<td>0.6, 1.3, 3.0, 2.6, 2.8[x2]</td>
<td>0.6, 1.3, 2.8[x2], 3.2, 4.6</td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
<td>2.8, 4.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>BamHI,</td>
<td>0.2, 0.3, 0.5, 1.3, 1.4, 4, 5.8</td>
<td>0.42, 0.47, 1.2[x2], 1.5, 3.9, (4.8), 6.2</td>
</tr>
<tr>
<td></td>
<td>XbaI</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7.2.2 Transformation of pPH 16 into A. nidulans and analysis of the transformants

The method used for the transformation of pPH16 into A. nidulans was that of Ballance and Turner (1985) as modified by Streatfield (1990) and described in Chapter 2, section 1.8. The recipient strain was 7-16, which has the genotype pyrG89 pabaA1 yA; bgaA4; qutE208 (Streatfield 1990). Vector pPH16 DNA was linearised by digestion with BglII which cleaves in A. nidulans DNA 5' to the QUTE gene. The use of such linear vectors was found to increase the transformation frequency and the proportion of plasmids integrating at the qutE locus (Streatfield 1990). About 10μg of pPH16 in a volume of 20μl of T.E. was added to 200μl of a suspension containing 4x10⁷ sphaeroplasts. Aliquots of the transformed sphaeroplast suspension were overlaid in minimal agar growth medium including 0.6M KCL, with quinic acid as the carbon source onto minimal agar plates of the same medium. The regeneration of the sphaeroplasts indicated 1.9x10⁷ viable sphaeroplasts in each aliquot. The control, with no DNA produced no colonies and the experimental sample, 34 colonies, a transformation frequency of 1.7 transformants per μg DNA per 10⁷ sphaeroplasts.

The transformants were purified by replating on selective medium to isolate single colonies and then tested for the activity of the PGK promoter in the gene fusion on X-gal plates (Chapter 2, section 1.5). Of the 32 transformed strains tested 18 produced β-galactosidase activity and therefore contained the gene fusion.

The identification of transformants which had a single copy of the plasmid integrated at the qutE locus was done in two stages to reduce the requirement for Southern analysis. Assays for β-galactosidase identified transformants which probably had a single copy of pPH16 and several of these then taken for Southern analysis to discover the nature of the transformation events.
7.2.3 Identification of transformed strains with probable single copies of the vector

The 18 transformed strains which had β-galactosidase activity on glucose medium, one strain which did not and Streatfield's strain L8 (now called Aspergillus L8, AL8) were inoculated into flasks of 200ml of minimal medium at $5 \times 10^5$ conidia/ml. The cultures were incubated overnight, and the β-galactosidase specific activity in cell free extracts determined as described previously (data not shown). The strain which gave no reaction in the plate test contained no β-galactosidase activity. The specific activities obtained fell into groups which were integer multiples of the lowest value and these groups presumably correspond to increasing copy numbers of the integrating plasmid (Streatfield 1990). Six of the strains (PL7, PL9, PL22, PL24, PL32, PL34) in the group corresponding to a single copy of the integrating plasmid, which also included the positive control strain AL8, were chosen for further analysis.

7.2.4 Identification of type I integrants by Southern analysis of single copy transformants

There is a single PstI site but no Smal site within the plasmid pPH16. Southern analysis of genomic DNA digested with PstI and Smal together will produce one 4.6kb band which hybridises to the QUTE gene probe for the recipient strain 7-16, and two of 6.8 and 12. kb in a transformed strain in which a single copy of pPH16 has integrated at the gutE locus. A transformed strain in which integration of pPH16 had occurred elsewhere would produce a band of 4.6 kb and another of variable size depending on the adjacent PstI and Smal sites. A diagram of the integration of a single copy of the plasmid at the gutE locus is presented in Figure 7-6.

A Southern analysis of genomic DNA from the six selected strains, the recipient strain 7-16 and the strain AL8 containing the PGK promomter-lacZ fusion was done (Chapter 2, section 7). The DNA was digested with PstI and Smal and hybridised to a 1kb probe for the A. nidulans QUTE gene. The
probe was isolated from pAL1, which is pUC19 with a 1kb EcoRI-Xbal insert encompassing the QUTE gene (Figure 7-3B), by the method described in Chapter 2, section 6.3.

Four of the transformed strains contained pPH16 DNA integrated at the gutE locus (Figure 7-7). Transformant PL25 with a single copy of the P. chrysogenum PGK promoter-lacZ reporter fusion at this locus was chosen to compare to strain AL8 which contains the A. nidulans PGK promoter-lacZ reporter fusion in a single copy at the same locus.
Figure 7-6: Predicted integration of the vector pPH16 at the A. nidulans QUTE locus

The top diagram is of the plasmid pPH16. The centre diagram shows the organisation of the QUT gene cluster in A. nidulans strain 7-16 and the diagram below a model predicting the outcome of the integration by a single crossover with the circular molecule of plasmid pPH16 at qutE locus.

The origins of the DNA sequences are indicated:

- A. nidulans DNA
- P. chrysogenum DNA
- E. coli vector DNA

The direction of transcription of each gene is indicated by an arrow.

The restriction sites shown are:

- B - BamHI
- E - EcoRI
- H - HindIII
- P - PstI
- Sm - SmaI
- Xa - XbaI
Genomic DNA was prepared from six *A. nidulans* strains transformed with linearised plasmid pPH16 (see text), the recipient strain 7-16, and the *A. nidulans* strain AL8 which contains the *A. nidulans* PGK promoter-ααζ fusion integrated at the gutE locus (Streatfield 1990). The DNA was digested with PstI and SmaI and separated by electrophoresis on a 0.7% agarose gel. The DNA was transferred to a Hybond-N membrane and a Southern analysis was done to determine the nature of the integration event. The probe was 100ng of a 1kb SalI-EcoRI fragment from pAL1 which bears the entire QUTE gene (Figure 7-3) and the analysis was done under the conditions described in Chapter 2 section 7.4.

The order of samples on the autoradiograph is shown below. The fragments predicted for the transformed strains are those which would result from the integration of a single copy of the transforming DNA at the gutE locus. The DNA size markers are as described in Figure 7-5.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Strain</th>
<th>Predicted fragment size (Kb)</th>
<th>observed fragment size (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7-16</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>AL8</td>
<td>10.9, 4.8</td>
<td>10.5, 4.8</td>
</tr>
<tr>
<td>3</td>
<td>PL7</td>
<td>12.8, 6.9</td>
<td>13, 6.6, 4.6</td>
</tr>
<tr>
<td>4</td>
<td>PL9</td>
<td>12.8, 6.9</td>
<td>13, 4.6</td>
</tr>
<tr>
<td>5</td>
<td>PL22</td>
<td>12.8, 6.9</td>
<td>13, 6.7</td>
</tr>
<tr>
<td>6</td>
<td>PL25</td>
<td>12.8, 6.9</td>
<td>13, 6.7</td>
</tr>
<tr>
<td>7</td>
<td>PL32</td>
<td>12.8, 6.9</td>
<td>13, 6.7</td>
</tr>
<tr>
<td>8</td>
<td>PL34</td>
<td>12.8, 6.9</td>
<td>13, 6.7</td>
</tr>
</tbody>
</table>
7.2.5 Expression of the Aspergillus and Penicillium PGK promoters in A. nidulans.

The P. chrysogenum PGK promoter is modulated by growth on different carbon sources and during exponential growth. Earlier work by Streatfield (1990) has shown that the A. nidulans PGK promoter is modulated by growth on different carbon sources. The modulation in exponential growth on glucose of the PGK promoter was also investigated and no change found (Streatfield 1990). Therefore an experiment equivalent to that described in section 7.2 was done using the reporter functions in the A. nidulans strains AL8 containing the A. nidulans PGK promoter fusion and PL25 containing the P. chrysogenum PGK promoter fusion, to establish how the A. nidulans and P. chrysogenum PGK promoters responded under these conditions in A. nidulans. The two strains were grown at 37°C for periods of up to 40 hours.

In the exponential phase of growth the doubling times were for AL8 5 hours on glucose, 5 hours on quinic acid, 9 hours on glycerol and 9 hours on acetate. For PL25 the doubling times were, 3 hours on glucose, 4.5 hours on quinic acid, 8 hours on glycerol and 4 hours on acetate (Figure 7-2B).

The β-galactosidase specific activities for AL8, the strain carrying the A. nidulans PGK promoter fusion at the gutE locus, showed that the activities were elevated in mycelium grown on quinic acid and acetate as expected (Figure 7-2A). The β-galactosidase activity in mycelium grown on glucose increased little with growth, which agreed with the results of Streatfield (1990), but on the other carbon sources the activities increased more quickly with quinic acid grown mycelium producing the greatest rate of change. The PGK promoter in the gene fusion in AL8 does not have the A. nidulans PGK glycolytic motif and therefore the levels of β-galactosidase on all the carbon sources were depressed compared to a promoter with this sequence.

The β-galactosidase activities for PL25, the strain containing the P. chrysogenum promoter fusion at the gutE locus, in mycelium grown on quinic acid or acetate were most similar to those observed for the P. chrysogenum strain 8(9) containing the Penicillium PGK promoter-lacZ fusion. There
was no change in growth on glucose medium as also observed for the A. nidulans PGK promoter. On glycerol medium the results indicated no change in activity during growth which is in contrast to the observations of PGK promoter activity in the strains AL8 and 8(9) (Figure 7-2A). Thus the P. chrysogenum PGK promoter functions in A. nidulans though with a slightly different pattern of modulation. This will be discussed further in Chapter 9.

7-3 Summary

The lacZ reporter function integrated in two copies at the oliC (oligomycin resistance) locus in the P. chrysogenum strain 8(9) was used in preliminary investigations of the P. chrysogenum PGK promoter activity under different physiological conditions namely, during the growth cycle, during the growth cycle in mycelium grown on different carbon sources, and in mycelium grown at elevated temperatures (40°C). The β-galactosidase activities observed in the fermentation experiment indicate that the promoter activity increases in the first two days in which mycelium is actively growing and then decreases when growth ceases in the production phase. Experiments were done in defined liquid culture containing different carbon sources and harvested at a fixed yield and then later at different points during exponential growth of the culture. These showed that the promoter is positively modulated in exponential growth and that the carbon source on which the culture is grown affects the rate of this increase together with a general increase in promoter activity on carbon sources metabolised by the gluconeogenic pathway.

The result of incubation at 40°C was an apparent increase to about 160% of the original activity three hours after the transfer. However since the increase in activity was coincident with a sharp drop in total protein concentration in the mycelium the conclusion was that PGK promoter activity is unchanged by elevated temperatures.

A plasmid was constructed which contained the P. chrysogenum PGK promoter-lacZ fusion gene and part of the A. nidulans QUT cluster including the QUTE gene. The construct was transformed into A. nidulans and a strain in which a
single copy of the plasmid had integrated at the gutE locus was identified. The β-galactosidase activity in this strain was compared to the activity of an A. nidulans strain which contained the A. nidulans PGK promoter fused to the lacZ gene integrated in a single copy at the same locus. The two transformed strains were grown in liquid culture in media containing different carbon sources in an experiment equivalent to that done previously and the β-galactosidase activity determined from several samples taken during exponential growth. The P. chrysogenum PGK promoter functioned in A. nidulans at a level comparable to the A. nidulans PGK promoter and both were positively modulated during exponential growth on gluconeogenic carbon sources. On glucose no increase in either promoter activity was observed and on glycerol the P. chrysogenum promoter showed no change in activity.

A number of considerations arise in applying results obtained by use of the lacZ reporter gene to interpret how the PGK promoter itself may be behaving. In the experiments one is observing β-galactosidase enzyme activity and this protein may be subject to degradation in the cell at a different rate to the PGK enzyme. Similarly the respective mRNA species may have different stabilities and possibly rates of translation. However experiments in which relative values are of interest will not be affected by these factors. The fermentation experiment can only be taken as an indication that PGK promoter activity is modulated in a fermentation. The data described in this chapter will be further discussed in Chapter 9.
Chapter 8

Expression of the IPNS gene under the control of the PGK promoter in a P. chrysogenum fermentation.

The final aim of the project was to assess the expression of a fusion between the P. chrysogenum PGK promoter and the IPNS gene coding region in fermentation upon complex FM medium by monitoring IPNS mRNA levels, IPNS enzyme activity and penicillin titre. It has been reported that increasing the copy number of the IPNS gene has no effect on penicillin titre (Smith 1989) but this observation was not investigated further. Using a different promoter such as the PGK promoter, to control the gene may have a beneficial effect on penicillin titre because regulatory factors which would normally control IPNS transcription would not operate. Data presented in the previous Chapter shows that the PGK promoter exhibits an increase in activity during exponential growth which then decreases slowly once exponential growth has ceased.

In addition, study of the relative levels of the PGK and IPNS mRNAs after overnight growth of mycelium of the strain N.R.R.L 1951 on different growth media including a complex growth medium suggested that PGK mRNA was the more highly expressed (Chapter 5, Figure 5-5). Therefore it may be possible to increase the abundance of IPNS mRNA by using the PGK promoter to drive transcription of the IPNS coding region.

Since it was not known whether the IPNS enzyme could tolerate extra amino acids at its N-terminus, the strategy adopted was to make a transcriptional fusion using a convenient NcoI site spanning the translational start codon of the IPNS gene. The construct was transformed into the P. chrysogenum strain N.R.R.L 1951 because any changes in IPNS levels would be most easily detected in a strain with low penicillin titre. A transformed strain in which a single copy of the transforming plasmid had integrated at the oliC (oligomycin resistance) locus was isolated and compared to strain N.R.R.L 1951 in a 6 day fermentation, monitoring the IPNS enzyme activity, penicillin titre and IPNS mRNA levels.

130
8.1 Construction of the fusion of the PGK promoter to the IPNS coding region.

The strategy for constructing the PGK promoter-IPNS fusion was long and complicated and is outlined below.

The IPNS gene was available on pCYX4 (Figure 8-1) and the PGK promoter was available the M13 clone PC3.4 (Chapter 3, Figure 3.8). The marker chosen for the selection of transformants was the oliC13 gene, which has a mutation conferring oligomycin resistance. This marker was least likely to affect growth or penicillin production of the transformant under nonselective conditions. Moreover this system gives low copy number integration of the transforming plasmid.

In order to construct a transcriptional fusion of the PGK promoter to the IPNS gene an NcoI site had to be removed from the PGK promoter to give PC3.4-N and another NcoI site constructed by site directed mutagenesis (SDM) at the ATG translational start codon. (Figure 8-2; stage 1). In addition to the desired changes, SDM can introduce random point mutations into the DNA on which the reaction is done. Therefore the clones generated by SDM must be verified either by determining the full sequence of the cloned insert or that of a small fragment which can then be subcloned into another vector. Thus the sequence of the 272 bases of the chosen clone SDM2 between the Kpnl site in the polylinker and the Kpnl site 5' to the start codon was determined. The equivalent Kpnl fragment in PC3.4-N encompassing the unmutated start codon was then replaced by the Kpnl fragment isolated from the clone SDM2 with the NcoI site at the translational start codon giving PC3.6.

To facilitate cloning a fragment bearing two NcoI sites 3' to the IPNS gene in the plasmid pCYX4 was deleted from pCYX4 to give plasmid pCYX4-B (Figure 8-3; stage 2). The IPNS promoter and leader sequence were then removed from pCYX-B by cleaving the plasmid with NcoI and HindIII and were replaced by the PGK promoter and leader sequence, isolated on an NcoI-HindIII fragment from PC3.6 to give pPC3.7 (Figure 8-4; stage 3). The BamHI fragment bearing the
3' untranslated sequences of the IPNS gene, deleted in stage 2 of the procedure, was then reinserted into the plasmid pPC3.7, at the single BamHI site, to give pPC3.8 (Figure 8-5; stage 4). The fusion of the PGK promoter to the IPNS gene was then excised from pPC3.8 on a 3.4Kb HindIII fragment and inserted into the HindIII site of the plasmid pPOL20 to form the final construct (Figure 8-6; stage 5).
Figure 8-1: Map of the plasmid pCYX4 carrying the IPNS gene of *Penicillium chrysogenum*

The origins of the DNA sequences in pCYX4 are:

- E. coli pUC9 DNA
- *P. chrysogenum* OLI13 DNA
- Direction of transcription
- Extent of probe used in Southern analysis

**Restriction enzyme sites**

H=HindIII
P=PstI
E=EcoRI
S=SalI
Xa=XbaI
N=NcoI
Bg=BglII
B=BamHI
Figure 8-2: Stage 1 of the construction of the gene fusion between the PGK promoter and the IPNS coding region in the vector pPIO1

The first stage in the construction of pPIO1 was to remove the NcoI site 5' to the PGK coding region and then by site directed mutagenesis construct a NcoI site at the translational start codon of PGK to construct SDM2.

**Step 1**

digest PC3.4 with NcoI, religate.

**Step 2**

anneal oligonucleotide:

5'-GAGAGCCATGGTGGGTAG-3' to PC3.4-N and do site-directed mutagenesis reaction.

\[ \text{PGK promoter} \]

\[ \text{M13mp19 DNA} \]

\[ \text{P. chrysogenum DNA} \]

\[ \text{transcriptional start and direction} \]

**Restriction enzyme sites**

H=HindIII

P=PstI

S=SalI

Xa=XbaI

N=NcoI

Bg=BglII

B=BamHI

Xo=XhoI
Figure 8-3: Stage 2 of the construction of pPIOL

Stage 2 in the construction of pPIOL was to remove two Ncol sites 3' to the IPNS gene in pCYX4 to construct pCYX4-B. The symbols used are as in Figures 8-1 and 8-2.

Step 4: cut pCYX4 with BamHI and religate 4.5Kb fragment.
Figure 8-4: **Stage 3 of the construction of pPIO1**

In stage 3 the promoter of the IPNS gene is replaced by the promoter of the PGK gene to construct pPC3.7. The symbols used are as in figures 8-1 and 8-2.

**Step 5** digest with NcoI and HindIII and isolate 3.4kb fragment.

**Step 6** digest with NcoI and HindIII and isolate 1.5kb fragment.

**Step 7** ligate 1.5kb and 3.4kb fragments
pCYX4-B
4.53kb
IPNS
amp

PC3.6
8.85kb

PC3.7
4.95kb
IPNS
amp

ori

H
S

Xa
N

P

B

P

H

Xa

N

H

B

P

ori

P_{PGK}
In stage 4 the sequences 3' to IPNS which were removed in stage 2 were replaced to construct pPC3.8. The symbols used are as in Figures 8.1 and 8.2.

Step 8  digest with BamHI, isolate 1.4kb fragment
Step 9  linearise with BamHI
Step 10 ligate fragments
Figure 8-6: Stage 5 of the construction of pPIO1

In the final stage (5) the PGK promoter-IPNS fusion was transferred to the vector pPOL20 carrying the oliC gene to construct pPIO1. The symbols used are those used in Figures 8-1 and 8-2.

**Step 11** digest with HindIII, isolate the 3.6kb fragment.

**Step 12** linearise with HindIII

**Step 13** ligate fragments
pPOL20 4.3kb
ori oIIC13
amp
Xo E

pPC3.8 6.25kb
ori
amp
IPNS
H N

11

12

13

pPIO1 7.68kb
ori
amp
pPGK
ori
oIIC13
Xo E

H N Bg B H N

Xa H
8.1.1 Stage 1 Removal of the NcoI site in PC3.4 and construction of a second NcoI site by site directed mutagenesis

Step 1 Removal of NcoI sites in PC3.4. About 1μg of DNA of the M13 recombinant phage clone PC3.4 was incubated with NcoI and the DNA recovered. To destroy the NcoI site the DNA was then made blunt ended by incubation for 20 minutes at room temperature with 10 units of the Klenow fragment of E. coli. DNA polymerase in the presence of ATP and deoxynucleotides. The reaction was stopped by heating the sample at 70°C for 10 minutes, the DNA recovered and a ligation reaction done in a volume of 100μl. The products of the ligation reaction were transformed into the E. coli strain JM109. Several plaques were isolated and replicative form (RF) DNA prepared. Those clones in which the NcoI site had been destroyed were identified by digestion of the RF DNA with the restriction enzymes NcoI and NsiI. The enzyme NsiI will cut the sequence generated by "filling in" an NcoI site. Twenty two clones were tested and one clone PC3.4-N with the required structure was used for the next step in the construction (Figure 8-2A).

Step 2 Construction of an NcoI site at the start codon. A 22-mer oligonucleotide was designed complementary to the region around the start codon of the PGK gene but differing from it in two bases so that an NcoI site would be formed (Figure 8-2B). It was tested for similarity to other sequences in PC3.4-N using a computer search programme (FIND) and shown not to have any significant homology other than at the start codon of the PGK gene. A quarter of the DNA sample was transformed by electroporation as described in Chapter 2 section 2.6 into the E. coli strain TG1, which tolerates the presence of thionucleotides. Ten plaques were isolated after growth overnight at 37°C and single stranded DNA prepared from them. The sequences of four of the recombinant phage were determined using the universal primer to identify the required clones (Chapter 2 section 11). Three clones designated SDM1-3 had the required sequence.
**Step 3 Verification of the sequence of mutant clones and introduction of the mutation into PC3.4-N.** The DNA sequence of clone SDM2 from the HindIII site in the coding region to the KpnI site 161 bases 5' to the translational start codon was determined in order to verify that no changes had occurred in the DNA other than the desired ones. The 273nt KpnI fragment encompassing the start codon (Chapter 4 Figure 4-1) was then released by digestion of SDM2 DNA with KpnI and recovered after separation of the digestion products by electrophoresis on a 0.7% agarose gel. The unmutated clone PC3.4-N was also digested with KpnI and the large 8.5kb fragment recovered after separation of the digestion products by electrophoresis on a 0.7% agarose gel. Two ligation reactions were set up with 80ng of the 8.5kb fragment, one with 2ng and the second with 10ng of the 0.27kb fragment. The ligation products were transformed by electroporation into the strain TG1. Twenty nine of the transformants were tested for the presence of the 0.27kb KpnI fragment in the correct orientation by restriction analysis of the RF DNA and by doing single base sequencing reactions. One clone was found which had two inserts of the 0.27kb fragment. However a sequence determination showed that they were both in the required orientation. Since the second insert would be lost at the next step, the clone, designated PC3.6, was used to continue the construction.

**8.1.2 Stage 2 Removal of the NcoI sites 3' to the IPNS coding region**

The NcoI sites to be removed from the plasmid pCYX4 lie between the two BamHI sites in the plasmid and were therefore deleted by digestion of the plasmid with BamHI followed by recircularisation of the fragments. About 1μg of pCYX4 was incubated with BamHI and the products recovered (step 4). About 200ng of the digested DNA was included in a 100μl ligation reaction and the products transformed into the E. coli strain JM83. Six transformants were isolated and plasmid DNA prepared from them. To determine which plasmids had lost the 1.4kb BamHI fragment, a restriction analysis was done using BamHI. All the plasmids had lost the 1.4kb BamHI fragment and to save time the separate preparations were pooled and named pCYX4-B (Figure 8-3).
8.1.3 Stage 3 Replacement of the IPNS gene promoter with the promoter from the PGK gene.

The next stage was to isolate the PGK promoter on a 1.45kb NcoI-HindIII fragment and to insert this fragment into the plasmid pCYX4-B which had been digested with the same enzymes (Figure 8-4). The plasmid pCYX4-B was incubated with NcoI followed by Hind III and 2-3ng/μl of the 3.4Kb fragment recovered after electrophoretic separation of the digestion products (step 5). The M13 phage clone PC 3.6 was digested with the same enzymes and the 1.45kb fragment isolated following electrophoretic separation (step 6). The DNA was dissolved at the same concentration as the fragment of pCYX4-B. Two ligation reactions were set up with 20-30ng of pCYX4-B in each and 60-90ng or 100-150ng of the DNA fragment containing the PGK promoter (step 7). The products of the ligation reactions were transformed into the E. coli strain JM83. Six of the transformed colonies were isolated and used to prepare plasmid DNA. The plasmids were incubated with NcoI and HindIII to check that they contained the 1.45Kb fragment encompassing the PGK promoter, and one designated pPC3.7 was chosen for further analysis and the next stage of the construction.

The clone pPC3.7 was examined by restriction analysis with SalI and PstI to verify that the IPNS promoter had been removed and with NcoI, HindIII, BamHI, and NsiI to determine the orientation of the insert and to establish that the second insert in pPC3.6 had not been cloned. The analysis used the enzymes singly and in all possible pairwise combinations and the results proved that pPC3.7 had the structure required (data not shown).

8.1.4 Stage 4 Replacement of the 3' sequences of the IPNS gene.

The 3' sequences of IPNS present on the 1.4kb BamHI fragment of pCYX4 which were removed earlier, because they contained two NcoI sites, were now replaced (Figure 8-5). The plasmid pCYX4 was digested with BamHI and the 1.4kb BamHI fragment isolated (step 8). The plasmid pPC3.7 was also incubated with BamHI and the linearised molecule
recovered (step 9). A ligation reaction was set up with 100ng of the 1.4kb BamHI fragment and 400ng of the linearised pPC3.7 and the products of the ligation reaction transformed into E. coli JM83 (step 10). Plasmid DNA from 36 transformants were prepared and analysed by restriction digestion with BamHI which identified four plasmids containing the BamHI insert. The four clones were then digested with NcoI to determine the orientation of the insert. The plasmid chosen with the required structure was designated pPC3.8.

8.1.5 Stage 5 The transfer of the PGK promoter–IPNS gene fusion to the plasmid pPOL20.

The fusion of the PGK promoter and the IPNS coding and 3' regions was complete and it only remained to insert the fusion into plasmid pPOL20, which bears the oliC13 selectable marker conferring oligomycin resistance, for transformation into P. chrysogenum (Figure 8-6). About a microgram of pPC3.8 DNA was digested with HindIII, the 3.4kb fragment isolated and resuspended at a concentration of 5ng/μl (step 11). The plasmid pPOL20 was also incubated with HindIII and the DNA products recovered (step 12). In order to prevent religation of linearised plasmid the DNA was incubated with 1 unit of calf intestinal alkaline phosphatase in the buffer React 9 at 37°C for 30 minutes after which a further unit of phosphatase was added and the incubation continued for another 30 minutes (Chapter 2 section 6.6). The DNA was recovered following removal of the proteins by phenol/chloroform extraction and resuspended at a concentration of 50ng/μl. A ligation reaction was set up with 100ng of the fragment containing the PGK promoter–IPNS fusion and 20ng of the phosphatase treated plasmid (step 13). The ligation reaction products were transformed into JM83 and 12 of the transformants were analysed. Plasmid DNA was prepared and those plasmids with inserts were detected by separating the uncut plasmids by electrophoresis on a 0.7% agarose gel. Four plasmids with inserts were analysed further by restriction analysis using the enzymes XbaI, BglII and EcoRI. Two plasmids had the required structure and one designated pPIO1 was analysed
fully using the restriction enzymes BglII, BamHI, XbaI, HindIII and EcoRI singly and in all pairwise combinations. The fragment sizes obtained agreed fully with the predicted restriction map (Figure 8-7).
Figure 8-7: Restriction analysis to verify the structure of the vector pPIOl containing the PGK promoter-IPNS gene fusion.

Plasmid DNA for pPIOl was digested singly and in all possible pairs with the enzymes, HindIII, EcoRI, BamHI, BglII, XbaI. The DNA fragments generated were separated on a 0.7% agarose gel and stained with ethidium bromide. The order of the tracks in the photograph and the sizes of the fragments are shown in the table. The predicted fragment sizes are derived from sequence data and previous analysis. The marker lane (M) contains phage lambda DNA digested with HindIII.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Enzyme</th>
<th>Predicted fragment sizes (kb)</th>
<th>Observed fragment sizes (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EcoRI</td>
<td>7.68</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td>BamHI</td>
<td>7.68</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
<td>BglII</td>
<td>7.68</td>
<td>7.4</td>
</tr>
<tr>
<td>4</td>
<td>HindIII</td>
<td>4.3, 3.4</td>
<td>3.8, 3.4</td>
</tr>
<tr>
<td>5</td>
<td>XbaI</td>
<td>7.68</td>
<td>7.3</td>
</tr>
<tr>
<td>6</td>
<td>EcoRI, BamHI</td>
<td>5.0, 2.7</td>
<td>5.3, 2.5</td>
</tr>
<tr>
<td>7</td>
<td>EcoRI, BglII</td>
<td>5.6, 2.1</td>
<td>5.6, 2.0</td>
</tr>
<tr>
<td>8</td>
<td>EcoRI, HindIII</td>
<td>3.4, 2.7, 1.6</td>
<td>3.4, 2.6, 1.4</td>
</tr>
<tr>
<td>9</td>
<td>EcoRI, XbaI</td>
<td>4.8, 2.9</td>
<td>4.55, 2.85</td>
</tr>
<tr>
<td>10</td>
<td>BglII, BamHI</td>
<td>7.1, 0.6</td>
<td>7.8, 0.51</td>
</tr>
<tr>
<td>11</td>
<td>BamHI, HindIII</td>
<td>4.3, 1.1, 2.3</td>
<td>3.9, 2.5, 1.05</td>
</tr>
<tr>
<td>12</td>
<td>BamHI, XbaI</td>
<td>5.6, 2.1</td>
<td>5.65, 2.15</td>
</tr>
<tr>
<td>13</td>
<td>BglII, HindIII</td>
<td>4.3, 2.9, 0.52</td>
<td>4.1, 3.1, 0.58</td>
</tr>
<tr>
<td>14</td>
<td>BglII, XbaI</td>
<td>5.0, 2.7</td>
<td>5.1, 2.7</td>
</tr>
<tr>
<td>15</td>
<td>HindIII, XbaI</td>
<td>4.3, 3.2, 0.2</td>
<td>3.9, 3.3, 0.4</td>
</tr>
</tbody>
</table>
8.2 Transformation of the PGK promoter–IPNS fusion vector pPIOl into P. chrysogenum strain N.R.R.L 1951

Two transformation procedures were done (Chapter 2, section 1.9) each with about 10μg of linear and 10μg of circular vector DNA. The vector was linearised next to the oliC gene by digestion with the restriction enzyme XhoI. In the first experiment transformation of 1.6x10⁷ protoplasts with linear DNA yielded 1 transformant (efficiency 0.15 transformants per 10⁷ protoplast per μg DNA) and transformation with circular DNA yielded 26 transformed strains, (1.5 transformants per 10⁷ protoplasts per μg of DNA). In the second experiment, transformation of 1.7x10⁷ protoplasts yielded 7 transformants with linear DNA (0.4 transformants per 10⁷ protoplasts per μg of DNA) and 10 with uncut DNA (0.6 transformants/10⁷ protoplasts/μg DNA). The transformants were replated three times on selective medium to isolate single colonies and genomic DNA prepared from the purified strains.

8.3 Analysis of the transformed strains

In order to identify transformed strains in which the vector pIO1 had integrated at the oliC locus the genomic DNA of transformed strains and the original recipient strain N.R.R.L. 1951 was digested with SalI and a Southern analysis done using the plasmid pPOL20 as a probe. Previous analysis had shown that the vector sequences in the plasmid did not hybridise to N.R.R.L. 1951 and in this particular analysis it would give the same signal as the isolated IPNS fragment. The restriction enzyme SalI was known not to cleave the plasmid pPIO1. A transformed strain in which pPIO1 had integrated at the oliC locus would give a single hybridising band of 15.4kb and the 7.8kb band observed in the untransformed strain N.R.R.L. 1951 would be absent (Chapter 6 section 6.4; Figure 8-8). The analysis revealed one transformed strain, IC38 in which the 7.8Kb band observed in the untransformed strain N.R.R.L. 1951 was absent. In all the other strains the plasmid pPIO1 had integrated at other undefined loci. A Southern analysis of IC38, NRRL 1951 and several other transformants is presented in Figure 8-9.
size of the hybridising band for IC38 was 16kb which suggested that the vector was present as a single copy. Further digestion and Southern analysis with EcoRI and BamHI confirmed the nature of the integration event (Figure 8-10).

8.4 Use of the transformed strain IC38 in fermentation experiments.

The performance of the PGK promoter-IPNS fusion was monitored by growing the transformed strain IC38 in a fermentation with the untransformed strain N.R.R.L. 1951 as a control. Microfermenters containing 1.51 of complex FM medium were inoculated in duplicate with 100ml of a 24 hour old culture of either the untransformed control strain N.R.R.L 1951 or the transformed strain IC38 and the fermentation continued without selection for 5 days under the conditions described in Chapter 2 section 1.2. On each day of the fermentation penicillin titre was determined for a sample of the culture (Chapter 2 section 1.8) and 10ml of culture was used to make cell free extracts for the determination of IPNS enzyme activity. A sample of 10-20ml of culture was also taken for preparation of total cellular RNA at a later date.

8.4.1 Penicillin Titre

Penicillin titre was determined for each day of the fermentations. The values are averaged for all the cultures each strain and corrected for the growth of the culture by dividing these values by the concentration of soluble protein in the cell free extracts. Overall a small increase in penicillin yield was observed at the early part of the fermentation (Figure 8-11A).
Figure 8-8: Proposed organisation of genomic DNA resulting from a single copy integration of pPIO1 into the oliC locus of P. chrysogenum.

The model is derived by integration of one copy of a circular molecule of pPIO1 by homologous recombination via a single crossover event at the oliC locus.

\[
\begin{align*}
\text{oliC gene} & \quad \text{PGK promoter} \\
E. \text{coli vector DNA (pUC9)} & \quad P. \text{chrysogenum DNA}
\end{align*}
\]

Restriction enzyme sites

- H = HindIII
- P = PstI
- E = EcoRI
- S = SalI
- Xa = XbaI
- N = NcoI
- Bg = BglII
- B = BamHI
Figure 8-9: Southern analysis of putative single copy pPIOL transformants of *P. chrysogenum* 1951.

Genomic DNA was prepared from strain N.R.R.L. 1951 and the selected transformants, listed and digested with SalI. The products of the digestion were separated on a 0.7% agarose gel and transferred to nylon membrane. The DNA was hybridised to radioactively labelled plasmid pPOL20 (Chapter 6 Figure 6-1) under the conditions described in Chapter 2 section 7. The order on the gel is shown below with the sizes of the hybridising bands on the autoradiograph. The marker DNA (M) is described in Figure 8-7.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Strain</th>
<th>Observed fragment size (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N.R.R.L. 1951</td>
<td>7.7</td>
</tr>
<tr>
<td>2</td>
<td>113</td>
<td>7.7, &gt;23</td>
</tr>
<tr>
<td>3</td>
<td>IC30</td>
<td>7.7, &gt;23</td>
</tr>
<tr>
<td>4</td>
<td>IC38</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>IL10</td>
<td>7.7, &gt;23</td>
</tr>
</tbody>
</table>

Fragment sizes predicted for an integration of a single copy of pPIOL at the *oliC* locus is 15.4, for any other locus 7.8, >23. The non-transformed strain lane 1 is expected to give a 7.8kb fragment.
Figure 8-10: Further Southern analysis of the transformed strain IC38

Genomic DNA was prepared from the untransformed strain N.R.R.L. 1951 and the transformed strain IC38. The DNA was digested with EcoRI or BamHI and the fragments separated by electrophoresis on a 0.7% agarose gel. The DNA was transferred to a Hybond-N membrane and hybridised to the radioactively labelled 1.1kb IPNS probe as described in Chapter 2 section 7. The markers are those described in Figure 8-7. The order of lanes is shown in the table.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Strain</th>
<th>Enzyme</th>
<th>Fragment size (Kb)</th>
<th>predicted</th>
<th>observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N.R.R.L. 1951</td>
<td>EcoRI</td>
<td>12.4</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>N.R.R.L. 1951</td>
<td>BamHI</td>
<td>4.5</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>IC38</td>
<td>EcoRI</td>
<td>15, 5.5</td>
<td>15, 5.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>IC38</td>
<td>BamHI</td>
<td>7, 5.1</td>
<td>6.6, 5.1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8-11

A: **Penicillin titre during fermentation of 1951 and IC38**

The yield of mycelium/ml may not be the same in each fermentation and therefore the penicillin titre/ml was normalised for the soluble protein concentration obtained for a fixed volume of culture used to prepare the cell free extracts for the determination of IPNS activity. The average results of four fermentations for each strain are shown here. The maximum and minimum values are indicated by a bar.

- IC38
- N.R.R.L. 1951

B: **Isopenicillin-N-Synthetase (IPNS) specific activities observed for the original strain N.R.R.L. 1951 and the transformed strain IC38 during a fermentation**

The transformed strain IC38 and the untransformed strain N.R.R.L 1951 were each cultured in duplicate fermentations for a period of five days and a sample of 10-20ml taken for each day. Cell free extracts were prepared from each sample and assayed for IPNS activity. The protein concentration of the extract was determined by the method of Lowry (1951). The graph shows the average specific activities obtained for each strain. The specific activity is measured in moles of IPN converted per 15 minutes per ml of extract.

- Strain 1951
- transformant IC38
A
penicillin titre
units/mg protein

B
IPNS specific activity
8.4.2 Determination of IPNS enzyme activity.

The IPNS activity was determined for a 10ml sample of mycelium each day by a method based on that of Ramos et al. (1985) as modified by Smith et al. (1989) (Chapter 2, section 1.7). Total protein was determined by the method of Lowry et al. (1951).

On each day of the fermentation experiment the IPNS enzyme activity was determined in four different volumes of extract, from 150μl to 10μl, and for a fixed time, 30 minutes on the first day and 15 minutes thereafter. The results obtained provided an estimate of IPNS enzyme activity each day of the fermentation. The activities were calculated using the volumes of extract for each sample which produced a similar amount of product for reasons explained below.

The resulting graph shows that the IPNS enzyme activity in the untransformed strain is fairly constant throughout the fermentation. The transformed strain IC38 produced levels of enzyme double those for N.R.R.L 1951 in the first 3 days of the fermentation and then these levels dropped dramatically to those observed for the untransformed strain for the last two days (Figure 8-11B). The results indicated that the PGK promoter-IPNS gene fusion may not be expressed in the latter part of the fermentation. The increased enzyme levels were not as high as expected from the comparison made of mRNA levels of PGK and IPNS in mycelium grown overnight on minimal and complex (GM) medium described in Chapter 5 section 3.3.
8.4.3 Investigation of the IPNS assay

In order to determine the time course of the IPNS assay reaction and the best incubation period, a further reaction was set up for each sample on the first day of the fermentation. The time course of the reaction was monitored by removing a volume from the reaction mixture and stopping the reaction at 15, 30, 45 and 60 minutes.

The results indicated that by the time the reaction mixture had been incubated for 15 minutes the reaction had ceased (data not shown). The turnover of the substrate which would give the amount of product observed was calculated to be about 10% of the substrate available. Therefore instead of the substrate being the limiting factor in the reaction system something else was limiting it. For these reasons all the subsequent determinations of enzyme activity were done with incubations of 15 minutes.

Several experiments were done to determine what was limiting in the reaction. One possibility was that the enzyme was not stable in the extraction buffer. The approximate half life of the enzyme in the cell free extract was estimated to be at least 40 minutes by incubating the extract at 25°C for periods of up to 30 minutes before adding it to the reaction mixture. Thus enzyme instability in the cell free extract in the absence of substrate was not limiting the reaction.

To confirm that the reaction mix was correct a time course experiment was done using a cell free extract prepared from a sample of mycelium from a production strain. The amount of product generated indicated that the substrate was exhausted in 15 minutes (data not shown). Therefore there was nothing wrong with the components of the reaction mixture.

The conclusion from these experiments were that the IPNS enzyme may not be stable in the presence of the substrate ACV and can only undergo a limited number of catalytic cycles before losing activity. The inactivation effect was not observed in the production strain because it contained much higher levels of enzyme and in the time periods involved the substrate was completely metabolised. It was for these reasons that when comparing the transformed strain
IC38 to the untransformed strain N.R.R.L. 1951 the specific activities were calculated for volumes of extract which generated a similar amount of IPN and therefore contained a similar amount of enzyme.

8.4.4 Comparison of the mRNA abundance of the fusion gene and the native IPNS gene

The samples for total RNA preparation were frozen and ground up under liquid nitrogen as soon as possible after collection. The fine powder was resuspended in 4M guanidine thiocyanate, 25mM sodium citrate 0.5% sarkosyl, 0.1M β-mercaptoethanol and stored at -80°C until all the samples had been collected. Total cellular RNA was prepared as described in Chapter 2 section 8. Samples of 10-40μg of total RNA isolated from mycelium harvested on the first and fifth days of the fermentation were separated by electrophoresis on a 1% formaldehyde/agarose gel and transferred to a nitrocellulose membrane. The RNA was hybridised overnight at 42°C in 50% deionised formamide, 6xSSC, 0.25% Marvel milk (Chapter 2, section 9) to 200ng of a radioactively labelled 1.1kb NcoI fragment of pCYX4 encompassing the entire coding region of the IPNS gene (Figure 8-1), prepared by the method described in Chapter 2, section 6.3B. Four 30 minute post-hybridisation washes were done in a solution containing 0.1xSSC, 0.1% SDS at 50°C. The relative amounts of total RNA loaded were determined by stripping the IPNS probe from the filter (Chapter 2, section 9.2) and hybridising the RNA to 100ng of a 0.6kb probe containing part of the PGK coding region (Figure 8-12). This probe was isolated from a XhoI digestion of pPCl. Hybridisation of the RNA to the PGK probe would allow the comparison of mRNA levels for the transformed and untransformed strains each day but not comparison between days. Figure 8-12 presents the results which show that the RNA levels reflect the enzyme levels on the two days for which samples were analysed. In addition the mRNA from the fusion gene appears less stable than the IPNS mRNA. Densitometric scans using a laser densitometer were done on the autoradiographs to give numerical results.

The signal observed for the PGK probe which was the
control for the amount of RNA loaded in each lane is detectable in mycelium harvested on the last day of the fermentation. This result is in contrast to the observation that the mRNA from the PGK promoter–IPNS gene fusion is not detectable at that stage. A possible explanation is that the fusion gene is transcribed as efficiently as the PGK gene in the latter part of the fermentation but there is some change in mRNA stability. An alternative possibility is that the changes made to the PGK promoter have in some way affected the transcription of the fusion gene in the latter part of the fermentation.

A different medium was used in the fermentation to the media on which the relative levels of PGK message and IPNS message in young mycelium was determined (Chapter 5 Figure 5.5). Therefore the relative levels of PGK mRNA and IPNS mRNA on the first day of the fermentation were determined by Northern analysis as described in Chapter 5. Equal amounts of total RNA from N.R.R.L. 1951 were separated on a 1% formaldehyde/agarose gel and transferred to a nitrocellulose filter which was then cut in half. The RNA was hybridised either to 100ng of the 1.1kb IPNS probe or 100ng of the 0.6kb PGK probe under the conditions described above and exposed to X-ray film for the same length of time. The incorporation of radioactivity into each probe was the same. The relative amount of RNA loaded onto each filter was then determined by stripping the probe from the filter and rehybridising the RNA to 100ng of the oliC probe, the 1.4kb HindIII-XhoI fragment encompassing the oliC gene from pPOL20 described in Chapter 6 Figure 6-1. The results showed that the levels of PGK and IPNS mRNAs are equal on the first day of the fermentation experiment (Figure 8-13). The mRNA levels of the fusion gene and the native IPNS gene therefore show that the gene fusion between the PGK promoter and IPNS coding region is expressed efficiently.

The increase in IPNS enzyme levels was not as high as expected because under the conditions of the fermentation (FM medium) the IPNS gene was more highly expressed than when it was cultured on GM medium at an earlier stage of growth as in previous experiments.
Figure 8-12: Relative levels of IPNS mRNA and PGK promoter-IPNS gene fusion mRNA in the fermentation.

Total cellular RNA was prepared from a sample of mycelium collected on the first or fifth day of the fermentation and 10-40μg separated by electrophoresis on a formaldehyde/agarose gel and transferred to a Hybond-N membrane. The RNA was hybridised to 200ng of IPNS probe. The IPNS probe was a 0.8Kb NcoI fragment containing the coding region of IPNS isolated by the method described in Chapter 2 section 6.3 from the plasmid pCYX4. The resulting autoradiograph (top panel) was scanned by a laser densitometer. The radioactivity was then stripped from the filter and the RNA hybridised to a 0.6Kb probe for PGK coding region. The autoradiograph (bottom panel) obtained was also scanned with a laser densitometer and the values used to normalise the data obtained for the IPNS probe. The order of tracks and the densitometry data are presented below.

<table>
<thead>
<tr>
<th>Area under peak</th>
<th>Lane</th>
<th>Strain</th>
<th>IPNS probe</th>
<th>PGK probe</th>
<th>Relative value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>IC38</td>
<td>6.53</td>
<td>2.84</td>
<td>2.3.</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1951</td>
<td>3.52</td>
<td>2.73</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>IC38</td>
<td>8.56</td>
<td>3.94</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1951</td>
<td>1.28</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Day 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>IC38</td>
<td>0.84</td>
<td>0.44</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>1951</td>
<td>0.83</td>
<td>0.56</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>IC38</td>
<td>0.49</td>
<td>0.31</td>
<td>1.6</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1951</td>
<td>0.33</td>
<td>0.38</td>
<td>0.86</td>
</tr>
</tbody>
</table>
Figure 8-13: Relative levels of native PGK and IPNS mRNAs on the first day of the fermentation

Duplicate samples of 10μg total cellular RNA which had been prepared from the day 1 sample of strain N.R.R.L 1951, was separated by electrophoresis on a formaldehyde/agarose gel and transferred to nitrocellulose. The filter was cut in half and one half hybridised to 200ng of the IPNS probe (Figure 8-1) and the other to 200ng of the PGK probe (Chapter 5 Figure 5-1) under the conditions described in Figure 8-11. The autoradiograph is shown in A. The incorporation of the radioactivity was equal in this case. The radioactive probes were then stripped from the filters and they were hybridised in the same experiment to 200ng of the 3liC probe to check the relative amounts of RNA present in each lane (autoradiograph B). The resulting autoradiographs are shown.

Lane 1    RNA hybridised to the PGK probe
lane 2    RNA hybridised to the IPNS probe
8.4.5 Stability of the transformed strain

To verify the stability of the transformed strain genomic DNA was prepared from mycelium collected from each fermenter on the sixth day of the fermentation. The DNA was digested with the restriction enzyme Sall and a Southern analysis was done using the oliC probe described above. The pattern of hybridisation for each strain was unchanged compared to the previous Southern analysis described above, therefore the strains had not undergone major rearrangements at the oliC locus in the absence of selection in the period of the fermentation.

8.5 Summary

A plasmid was constructed bearing 1.5kb of the P. chrysogenum PGK promoter and leader sequence was fused at the translational start codon to the coding and 3' regions of the P. chrysogenum IPNS gene. The plasmid was transformed into the strain N.R.R.L 1951 and a transformant in which one copy of the transforming plasmid had integrated at the oliC locus was selected. The transformed strain was then cultured in a five day fermentation on FM medium and the penicillin titre, IPNS enzyme levels, and IPNS mRNA levels monitored in comparison to the untransformed strain N.R.R.L. 1951. The penicillin titre of the transformed strain, after normalising for the growth of the culture, was found to be slightly increased in the early phase of the fermentation but the final penicillin titre was the same as that of the untransformed strain. The IPNS enzyme and mRNA levels were found to be double those of the original strain during the first half of the fermentation but they declined to the level observed for the original strain in the latter part of the fermentation. The levels of the PGK and IPNS mRNAs on the first day of the fermentation were found to be equal.

The data indicates that in the early part of the fermentation the PGK promoter-IPNS gene fusion is expressed at similar levels to the PGK gene but is not expressed at all in the later stages of growth. Since the PGK mRNA levels were similar to the IPNS mRNA levels on the first day of the fermentation the expression of the gene fusion only equalled
that of the IPNS gene, resulting in a doubling of IPNS enzyme levels. The increased IPNS enzyme levels may have had an effect on penicillin titre in the early stages of the fermentation but since the IPNS enzyme levels did not remain high there was no overall increase in titre. In the latter half of the fermentation the mRNA levels for the strain containing the gene fusion were the same as for the untransformed strain indicating that the gene fusion may not be expressed. However PGK mRNA was still detectable, and so there may be some form of post transcriptional control such as a change in mRNA stability exerted on the gene fusion mRNA. However further experiments would be required to confirm this suggestion.
The PGK gene encoding the enzyme 3-phosphoglycerate kinase, which acts in carbon metabolism in both glycolysis and gluconeogenesis, is highly expressed from strong promoters in the yeast *Saccharomyces cerevisiae* and in the filamentous ascomycete *Aspergillus nidulans* (Holland and Holland 1978; Clements and Roberts 1986). It was therefore reasonable to expect that the PGK gene of another ascomycete, *Penicillium chrysogenum* which produces penicillin might also be highly expressed and have a strong promoter. SmithKlineBeecham were interested in isolating potentially strong promoters from *P. chrysogenum* for use in the manipulation of *P. chrysogenum* genes involved in penicillin biosynthesis. Consequently the aim of this project was to isolate and study the *P. chrysogenum* PGK promoter in the context of using it to drive the expression of other *P. chrysogenum* genes.

The *P. chrysogenum* PGK gene was isolated and the sequence of the promoter and terminator regions determined, the sequence of the coding region having been published by another group (van Solingen et al. 1988). The size of the PGK mRNA together with the points at which the mature transcript begins and ends were established and the message shown to be abundant. Having characterised the PGK gene in this manner the project was developed in two directions, one largely academic and one practical.

In the academic part of the project the expression of the PGK gene was examined by constructing a *P. chrysogenum* strain containing a fusion between the PGK promoter and the *Escherichia coli* lacZ reporter gene. The changes in the promoter activity through the growth cycle and during growth on media containing different carbon sources were the principle areas of study. The expression of the *Penicillium chrysogenum* PGK promoter in *A. nidulans* was also examined and its effectiveness compared to that of the *A. nidulans* PGK promoter.

The practical part of the project was to compare the expression of the PGK gene and the IPNS gene which encodes
the penicillin biosynthetic enzyme isopenicillin-N synthase. The PGK gene was the more highly expressed under the conditions employed. Therefore a P. chrysogenum strain containing a fusion between the PGK promoter and the coding region of the IPNS gene was constructed and the effect of the gene fusion on penicillin titre and IPNS enzyme levels examined in small scale fermentations.

9.1 Analysis of filamentous fungal genes encoding glycolytic enzymes

The isolation and analysis of the sequence of the A. nidulans genes tpi (Mcknight et al. 1986), PGK (Clements and Roberts 1985), gpd (Punt et al. 1988), encoding three of the enzymes which function in both glycolysis and gluconeogenesis has been reported. The pyk gene encoding pyruvate kinase which only functions in glycolysis has also been isolated both from A. nidulans and from A. niger (van de Graff 1989). The sequence of the promoter of the A. nidulans pyk gene has been published but not that of the A. niger pyk promoter. The PGK gene has been isolated from P. chrysogenum (Koekman et al. 1986) and from Trichoderma reesi (Vanhanen et al. 1989) but in both cases only the coding region sequence was published. The only reported analysis of the function of the promoters of the above genes is of the A. nidulans genes, especially the PGK and gpd promoters and this data is discussed below in greater detail.

Some similarities in sequence motifs in the promoters of the A. nidulans glycolytic genes have been observed (Punt 1988; Streatfield 1990). An 8 bp consensus sequence, generally called the "glycolytic box" (TG^T/AGGT^T/G^C) has been identified at varied distances 5' to the transcriptional start sites (+1). This glycolytic box may be a binding site for transcription factor(s) which regulate gene expression in a carbon source dependent manner (Figure 9-1). The genes also all have a pyrimidine rich (CT) region 5' to the transcriptional start point, which may be important in filamentous fungi in positioning the transcriptional start point(s) and the strength of the promoter. This region is varied in length and pyrimidine content (Figure 9-1).

Functional analysis of the promoters of the gpd and PGK
Figure 9-1 The promoters of genes encoding enzymes catalysing reactions in glycolysis and gluconeogenesis

The sequence elements are indicated:

- CT rich region
- glycolytic box
- essential region in A. nidulans PGK
- gpd box

The distances are with reference to the major transcriptional start point. The arrows indicate the direction of transcription.
A. NIDULANS GENES

-600  -400  -200  +1

tpi

PGK

-499

gpd

-592

PYK

-542

P. CHRYSOGENUM GENE

PGK

-212
gene by deletion of segments of each promoter has been done by others during the course of the work presented in this thesis (Punt et al. 1990; Streatfield 1990).

The results of the study on the gpd promoter support the hypothesis that the pyrimidine rich region is required for the positioning of the transcriptional start point. The deletion of this region resulted in an alteration of the positioning of the transcriptional initiation sites. Its removal also affected transcriptional efficiency, reducing mRNA levels by 80% compared to the mRNA levels observed for the original promoter. However it remains possible that the decreased message levels may reflect decreased stability of the transcripts rather than rate of initiation. Further deletion studies with the gpd promoter also revealed a second important region at -234 to -284 to the transcript ional start point, named the gpd box, because it shares similarity with the promoter of the gpd gene of A. niger (van Gorcom unpublished results). The deletion of this region resulted in decrease in the level of mRNA to half that of the original promoter. Lastly, although deletion of the promoter region containing the gpd glycolytic box in gpd had no effect on transcription of the gene when the mycelium was grown on glucose, the potential effect on mRNA levels in mycelium grown on other carbon sources was not explored.

The A. nidulans PGK gene is subject to modulation by carbon source and the levels of enzyme activity and mRNA levels are increased approximately two fold in mycelium grown on carbon sources such as acetate and quinate metabolised by the gluconeogenic pathway (Clements 1986; Streatfield 1990).

Deletion studies of the A. nidulans PGK promoter revealed a region essential for expression of the gene at -120 to -160 from the transcriptional start point. In contrast to the results for the gpd promoter, the apparent core promoter, consisting of a CAAT-like motif, a TATA-like motif, and a pyrimidine rich region, was found to be insufficient alone to enable transcription to occur. These results point to a different role for the pyrimidine rich region in the two promoters.

The effect of increasing the amount of the PGK coding
region in the gene fusion was also examined and a region between +72 and +694 was found to increase mRNA levels when included in the fusion. Thus the A. nidulans PGK gene may have a sequence similar to the coding region activation sequence of the PGK gene of S. cerevisiae (Ogden et al. 1987). Sequences 5' to -600 in the A. nidulans PGK promoter, the essential region at -120 to -160, and the element in the coding region between +72 to +694 each contribute about one third of the total mRNA levels. Deletion of the glycolytic box did not affect mRNA levels when the mycelium was grown on glucose, but depressed the transcription levels observed for the other carbon sources tested, quinic acid, glycerol and acetate. This data indicates that the glycolytic box may have a role in carbon source regulation of the glycolytic promoters discussed above.
9.2 Isolation of the *P. chrysogenum* PGK gene

The PGK gene of *P. chrysogenum* was isolated by screening a genomic library with a PGK coding region probe from *A. nidulans*. Verification of the identity of the clones isolated was obtained by DNA sequence analysis of part of the putative PGK coding region. Comparison of the sequence obtained to the published DNA sequence of the *P. chrysogenum* PGK (van Solingen et al. 1988) showed 98% identity of the two sequences. Comparison of DNA sequence to the PGK gene of *A. nidulans* showed a 74% similarity. The clone containing the *P. chrysogenum* PGK gene was transformed into a pgk-/mutant strain of *A. nidulans* in which the PGK gene had been disrupted by a pyr4 marker (Streatfield 1990) and pgk+ transformants were obtained at a good frequency (Streatfield, Hoskins and Roberts unpublished results). Taken together these observations strongly support the conclusion that the gene isolated was the *P. chrysogenum* PGK gene.

9.3 Sequence analysis of the *P. chrysogenum* PGK promoter

The promoter and terminator sequence of the PGK gene were determined in preparation for manipulation of the promoter together with the start and stop sites of transcription and the size of the mRNA transcripts. The promoter sequence was determined 947nt 5' to the transcriptional start +1 (Chapter 4, Figure 4-3). The promoter elements discovered are presented in Figure 9-1 and in Chapter 4, Table 4-1.

A pyrimidine rich region 37nt in length was the most immediately striking feature of the promoter at -27 to +10nt. The three transcript initiation sites of *P. chrysogenum* PGK lie within this region with the major site (+1) near the 3' end (Chapter 5, section 2.1). The pyrimidine rich region is thought to control the positioning of transcriptional start points (Chapter 1, section 2 and above). This region is also found in highly expressed *S. cerevisiae* promoters 8-10 bp from the transcriptional start point and its deletion from the yeast CYC1 gene promoter also affects the positioning of the transcriptional start sites (McNeil and Smith 1985).

The flanking sequences of the *P. chrysogenum* PGK gene
were searched for motifs and similarities to the PGK gene of Saccharomyces cerevisiae. The searches done are outlined in Table 4-1.

A heat shock element is found in yeast PGK promoter between -323 and -336 (Piper et al. 1985) and in the Trichoderma reesi PGK promoter at -345 (Vanhanen et al. 1989). The heat shock element in the yeast PGK promoter induces PGK transcription about five fold at elevated temperatures, but only when yeast is incubated on carbon sources metabolised by the glycolytic pathway (Piper et al. 1988). In the P. chrysogenum PGK promoter a 7 of 8nt match to the heat shock element is located at -473. The effect of growth at 40 °C on the expression of a fusion of the P. chrysogenum PGK promoter to the lacZ reporter gene was examined but no major increase in PGK promoter activity was observed using the reporter fusion at elevated temperatures. Therefore the apparent heat shock element may be non-functional. Interestingly the PGK promoter of P. chrysogenum may be induced transiently by another stress situation, anerobiosis (Renno et al. 1990).

**Similarities between the P. chrysogenum PGK promoter and the A. nidulans PGK promoter.** Searches of the P. chrysogenum PGK promoter for sequences thought to be important for the transcription and control in the A. nidulans PGK promoter revealed that there was an extensive match of 19 of 21nt within the essential 40bp region at -160 to -120bp in the A. nidulans PGK promoter. In the P. chrysogenum PGK promoter this sequence was located closer to the transcriptional start at -79nt. This 21nt sequence does not include the octamer sequence at -121 in the A. nidulans PGK promoter which is the only recognised sequence element previously found in this essential region (Streatfield 1990).

The 8nt glycolytic box sequence is present in all the promoters of A. nidulans genes encoding glycolytic enzymes, and was also found in the P. chrysogenum PGK promoter. It was however very much closer at -212 to the transcriptional start than the sequence in A. nidulans PGK promoter which is at -499 (Figure 9-1).

An exact match of 11bp (5'-TCCGAGGCTAA-3') was found in the P. chrysogenum PGK promoter in comparison to the A.
nidulans PGK promoter. This element was found at -334 in the P. chrysogenum PGK promoter and at -270 in the A. nidulans promoter. It occurs in a region of the A. nidulans promoter which does not appear important for transcription. A search for this exact sequence in the other A. nidulans glycolytic promoters did not produce any matches.

In conclusion, the arrangement of the P. chrysogenum PGK promoter is similar to the glycolytic promoters of the A. nidulans glycolytic genes in that there is a pyrimidine rich region at the transcriptional start point and there is a 8 bp consensus sequence, the glycolytic box further upstream. Other common sequences between the A. nidulans PGK promoter and the P.chrysogenum PGK promoter were found but their significance remains to be established.

9.4 The sequence analysis of the PGK terminator region

Sequence analysis of the terminator region revealed several regions which could form a stem loop structure in the mRNA between 117 and 184 nt 3' to the stop codon (Chapter 4 Figure 4-4). The P. chrysogenum PGK gene transcript exhibits three sites for cleavage and poly-adenylation 5' to this region at 94, 103, and 107nt from the stop codon (Chapter 5, section 2.2). There was a possible polyadenylation signal (AATGAA) five bases 5' to the first site although many filamentous fungal genes have no recognised polyadenylation signal (Gurr et al. 1987). There was no recognisable termination signal -CCTGTTCC- (Mclaughlan et al. 1985) in a 3' direction from the polyadenylation sites. The mean size of the PGK mRNA population, predicted from the positions of transcript initiation and 3' cleavage was 1.4kb. The PGK mRNA identified by Northern analysis of total cellular RNA was 1.3kb in size which suggests that the potential sites for cleavage and polyadenylation of the transcript are the ones used in vivo.

The PGK mRNA was easily detectable in a Northern analysis of 3-4μg of total cellular RNA with 100ng of radioactively labelled PGK probe (Chapter 5, Figure 5-4) and was therefore abundant, which implies that the PGK promoter of P. chrysogenum is a relatively strong promoter.
9.5 Expression of the PGK promoter

The *P. chrysogenum* PGK promoter was fused to the coding region of the *E. coli* lacZ reporter gene and the construct was transformed into *P. chrysogenum* N.R.R.L 1951. A transformed strain 8(9) containing two copies of the gene fusion integrated at the olic (ATPase subunit 9 gene) locus was selected and used for preliminary study of the expression of the PGK promoter. These studies focussed on two areas, expression of the promoter during growth in defined medium in batch culture, and during growth of the mycelium on different carbon sources.

9.5.1 Modulation of the *P. chrysogenum* PGK promoter by growth on different carbon sources: comparison at a fixed point in growth.

Previous work has shown a 1.5 fold increase in the specific activity of *A. nidulans* PGK in mycelium grown on carbon sources metabolised by the gluconeogenic pathway compared to those metabolised by the glycolytic pathway (Clements 1986). Later experiments using a fusion of the *A. nidulans* PGK promoter to the *E. coli* lacZ gene indicated that the increase was due to increased PGK promoter activity (Streatfield 1990).

The possibility that similar variation in the expression of *P. chrysogenum* PGK may occur was initially studied by comparing PGK mRNA levels in mycelium grown in liquid culture in media containing different carbon sources (Chapter 5 section 3.3). The mycelium was harvested at the same stage of growth judged by the yield of mycelium, and total cellular RNA prepared. The steady state PGK mRNA levels measured by Northern analysis were, in comparison to glucose (1.0) greater on media containing the carbon sources quinic acid (1.9) or acetate (1.5), which are metabolised by the gluconeogenic pathway.

Further studies using the PGK promoter-lacZ fusion gene in the transformed strain 8(9) were done in which mycelium was cultured and harvested as described above. The β-galactosidase activities measured were, relative to growth on glucose (1.0), 1.2 on glycerol, 2.7 on quinic acid, and
1.7 on acetate (Chapter 7, section 2). The results agree with the observations of PGK mRNA levels although the changes are only 2-3 fold. Thus the strong P. chrysogenum PGK promoter is modulated 2 to 3 fold by the carbon source on which the mycelium is grown in a similar way to the A. nidulans PGK promoter.

9.5.2 Modulation of the P. chrysogenum PGK promoter in the growth cycle and by carbon source

In the fermentation experiment described in Chapter 7 section 1, the PGK promoter activity varied during growth, increasing in early part of a fermentation experiment. The effect of growth on the modulation of the PGK promoter by the carbon source on which the mycelium is grown was investigated in defined media. The PGK promoter-lacZ reporter function in transformed strain 8(9) was monitored in shake flask cultures during growth in minimal medium with either glucose, glycerol, quinic acid or acetate as a carbon source. The mycelium was harvested at intervals during exponential growth and β-galactosidase specific activity determined (Chapter 7 section 3).

An increase in β-galactosidase activity and thus in promoter activity was observed in exponential growth of each culture and was more rapid in mycelium grown on carbon sources metabolised by gluconeogenesis (Chapter 7 Figure 7-2; Table 9-1). The results are difficult to interpret in terms of carbon metabolism during exponential growth since the concentrations of nutrients, particularly carbon sources are continuously changing in batch culture. The results support the hypothesis that PGK gene expression is higher in growth on carbon sources metabolised by gluconeogenesis which suggests that the flux through that pathway is greater than the flux through glycolysis. It is unlikely that the changes observed are in some manner a property of the reporter function since the rates varied with different carbon sources in the growth medium. The changes are also unlikely to be due to the site of integration of the reporter fusion, oliC, since the expression of the oliC gene is not affected by growth on different carbon sources (G. Turner personal communication).
9.5.3 Modulation of the *A. nidulans* PGK promoter in the growth cycle and by growth on different carbon sources.

Previous experiments described by Streatfield (1990) with an *A. nidulans* transformed strain AL8 containing the *A. nidulans* PGK promoter-lacZ fusion and in which no change in PGK promoter activity was observed in logarithmic growth on glucose minimal medium are in contrast with the results described above. Therefore an equivalent experiment was done in which the β-galactosidase activity of strain AL8 was monitored in growth on different carbon sources (Chapter 7 section 7.5).

The result obtained for mycelium of *A. nidulans* strain AL8 grown on glucose, showed little change in promoter activity through exponential growth as previously reported. However there was a increase in activity of the promoter in exponential growth of mycelium on glycerol, quinic acid or acetate (Chapter 7 Figure 7-2; Table 9-1) This increase in promoter activity was, as for the *P. chrysogenum* PGK promoter, most rapid on carbon sources such as quinic acid and acetate metabolised by the gluconeogenic pathway although the rates of increase differed.

Thus the overall response to growth of the mycelium on different carbon sources is similar for the *A. nidulans* and *P. chrysogenum* PGK promoters except in mycelium grown on glucose for which no increase in *A. nidulans* PGK promoter activity was observed.

9.5.4 Heterologous expression of the *P. chrysogenum* PGK promoter in *A. nidulans*

The expression of the *P. chrysogenum* PGK promoter in *A. nidulans* was examined by transforming a plasmid containing the *P. chrysogenum* PGK promoter-lacZ gene fusion into *A. nidulans*. The reporter fusion contained 1.5kb of the *P. chrysogenum* PGK promoter and PGK codons fused to the *E. coli* lacZ gene. The fusion was inserted into a plasmid containing 3.3kb of the QUT gene cluster including the QUTE gene with 820nt of 3' sequence and 2037 nt of 5' sequence which spans the QUTG open reading frame (Chapter 7 Figure 7-3 and 7-4). The plasmid was transformed into the *A. nidulans* strain 7-16 and a transformant PL25 with one copy of the gene fusion at
<table>
<thead>
<tr>
<th>Strain</th>
<th>Source of PGK promoter</th>
<th>Glucose</th>
<th>Glycerol</th>
<th>Quinic acid</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. chrysogenum 8(9)</td>
<td>P. chrysogenum</td>
<td>70</td>
<td>60</td>
<td>140</td>
<td>350</td>
</tr>
<tr>
<td>A. nidulans AL8</td>
<td>A. nidulans</td>
<td>20</td>
<td>150</td>
<td>1000</td>
<td>100</td>
</tr>
<tr>
<td>A. nidulans PL25</td>
<td>P. chrysogenum</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>200</td>
</tr>
</tbody>
</table>

Increase in β-gal specific activity per 0.1g increase in mycelial weight on:
the qutE locus was identified.

The \textit{P. chrysogenum PGK} promoter-lacZ reporter activity in the transformant PL25 was compared to the activity obtained in the experiments described above for the \textit{A. nidulans PGK} promoter-lacZ reporter gene fusion. The plasmid bearing the \textit{A. nidulans} reporter fusion in strain AL8 contained only 487bp of the \textit{A. nidulans PGK} promoter without the glycolytic motif and 13 N-terminal PGK codons. It also contained the \textit{QUTE} gene with 1.2kb of 5' sequence with an incomplete \textit{QUTG} ORF and 240bp of 3' sequence (Figure 6.1). The site of integration of the plasmids bearing the \textit{P. chrysogenum PGK} reporter function and the \textit{A. nidulans PGK} reporter function is slightly different, however the orientation of the reporter gene is the same with respect to the surrounding \textit{QUT} gene cluster.

An experiment equivalent to that discussed in section 9.5.2 was done with the transformed \textit{A. nidulans} strain PL25 containing the \textit{P. chrysogenum PGK} promoter fusion. The results indicated that the \textit{P. chrysogenum PGK} promoter was expressed at a similar level to the \textit{A. nidulans PGK} promoter (Chapter 7, Figure 7-2). It was modulated by growth on different carbon sources and during the growth cycle. The modulation on quinic acid and acetate was most similar to the promoter in \textit{P. chrysogenum} but the absence of modulation on glucose was like that of the \textit{A. nidulans PGK} promoter. There was no modulation on glycerol, in contradiction to previous results observed with the other strains (Table 9-1).

The data indicates that the transcription factors which modulate the \textit{A. nidulans PGK} promoter activity in growth on quinic acid, acetate and glucose are able to also control the \textit{P. chrysogenum PGK} promoter and therefore bind to their recognition sequences in the \textit{P. chrysogenum} promoter. However the binding efficiency of the factors may in some conditions be different resulting in changes in the pattern of modulation. The difference could equally be due to the absence of the glycolytic box in the \textit{A. nidulans PGK} promoter-lacZ fusion. The result for glycerol grown mycelium taken at face value indicates that the factors controlling \textit{A. nidulans PGK} activity when the mycelium is grown on glycerol cannot control the \textit{P. chrysogenum} promoter, perhaps
because they cannot recognise the appropriate sequence element. The sequence similarities noted between the two promoters may be regions to which some of these transcription factors bind. Their role in carbon source modulation could be investigated by further experiments with existing A. nidulans strains containing the appropriate A. nidulans PGK promoter deletions fused to the lacZ reporter gene and grown on the carbon sources described.

The integration of both the reporter fusions in A. nidulans at the qutE locus may have had an effect on the activity observed in mycelium grown on quinic acid since the QUT cluster genes are strongly induced in growth on quinic acid.
9.6 Use of the \textit{P. chrysogenum} PGK promoter to drive the expression of the IPNS gene of \textit{P. chrysogenum}.

When the work in this thesis was started the only genes encoding penicillin biosynthetic enzymes isolated were the genes for IPNS from \textit{C. acremonium} (Samson et al. 1985) \textit{P. chrysogenum} (Carr et al. 1986), \textit{Streptomyces} (Leskiw et al. 1988) and \textit{A. nidulans} (Ramon et al. 1987). Since that time the other two genes encoding the penicillin biosynthetic enzymes ACT (Barredo et al. 1989) and ACVS (Diez et al. 1990) have also been isolated. In addition the three genes have been found to be clustered in the genome in several fungi and bacteria including \textit{P. chrysogenum}. Some industrial \textit{P. chrysogenum} strains have been found to have 10-14 copies of the gene cluster (reviewed in Chapter 1 section 4).

SmithKlineBeecham were interested in the manipulation of genes involved in penicillin biosynthesis and therefore the second gene fusion constructed was of the PGK promoter to the gene encoding Isopenicillin-N synthetase (IPNS). The effect of placing the IPNS gene under a different control and increasing the levels of IPNS enzyme on penicillin titre was thus investigated. A transformed strain containing a single copy of the PGK promoter-IPNS fusion integrated at the oliC locus in addition to the native IPNS gene was grown in a small scale batch fermentation for five days and the penicillin titre, IPNS enzyme activity and IPNS mRNA levels were monitored. The penicillin titre was slightly increased in the early phase (2-4 days) of the fermentation compared to the untransformed strain, if normalised for different yields of mycelium. The final titre was lower than that of the untransformed strain (Chapter 8 Figure 8-11).

The IPNS specific activity in the strain containing the gene fusion was twice as great as in the untransformed strain for the first three days of fermentation indicating that the fusion was expressed at about the same level as the native IPNS gene. By the fourth day of fermentation the specific activity had declined to the levels observed for the untransformed strain (Chapter 8 Figure 8-11). This implies that the gene fusion is only being expressed early in the fermentation. This is in contrast to the experiment in which the \textit{lacZ} reporter function was monitored during a
fermentation and the promoter activity as indicated by the β-galactosidase activity was one third its maximum in the fifth day of the fermentation (Chapter 7 Figure 7-1).

The mRNA levels for IPNS in the transformed and original strains were also monitored on the first and fifth days of the fermentation and the difference between the mRNA levels for the fusion gene and the native IPNS gene were in the same proportion as the enzyme levels (Chapter 8, Figure 8-12). The control for sample loading was the PGK mRNA which allowed comparison between the strains on each day but not between days. In addition the control showed that on the fifth day of the fermentation there were still readily detectable levels of the PGK message at which stage the PGK promoter-IPNS gene fusion mRNA was not found. This difference between the mRNA levels of the gene fusion and the PGK gene may be due to a change in mRNA stability rather than a difference in transcription. An alternative though rather unlikely possibility is that the insertion of four bases at the NcoI site in the PGK promoter done in the construction of the gene fusion may affect promoter activity later in the fermentation.

The relative levels of PGK and IPNS mRNAs were monitored on the first day of the fermentation (Figure 8-13) since a different complex medium (FM) was used to that on which relative levels of PGK and IPNS mRNAs were originally determined (GM). Equal amounts of mRNA prepared from the untransformed strain N.R.R.L. 1951 on the first day of the fermentation were hybridised to DNA probes for PGK and IPNS. The results indicated that the two mRNAs had similar abundance. This result was in marked contrast to the previous experiments which compared the relative levels of the PGK and IPNS mRNAs in a similar manner but on a different complex medium (GM). The previous results showed that PGK was the more highly expressed gene (Figure 5-5).

The mycelium used to prepare RNA in the earlier experiment was from a 16 hour culture which had been inoculated with conidia. However on the first day of the fermentation the mycelium was harvested from a 24 hour old culture and which had been inoculated with a seed culture of mycelium. The mycelium was therefore older in the second experiment and this may account for the different result.
Equally the differences between the two complex media may cause the difference in the relative levels of PGK and IPNS mRNAs.
9.7 Summary and future work

The PGK gene from *P. chrysogenum* has been isolated and the sequence of the promoter and terminator regions determined. The promoter has a 37nt pyrimidine rich region which is a feature of about one third of filamentous fungal genes (Gurr et al. 1987). Transcription was initiated immediately 3' to this sequence in those genes and for *P. chrysogenum* PGK the major initiation point (+1) was near the 3' end of the motif and the other three initiation points were within the motif. There was also a 7 of 8nt match to the heat shock consensus sequence at -473 but the response of the promoter to heat shock was inconclusive. Similarities in the *P. chrysogenum* PGK promoter to regions of the *A. nidulans* PGK promoter have been noted but their functional significance remains to be tested. In the terminator region there were three sites 5' to a region which could form secondary structures at which the mature mRNA ended but no canonical polyadenylation signal or termination consensus sequence were observed.

The *P. chrysogenum* PGK promoter is strongly expressed and use of the PGK promoter-lacZ gene fusion indicates that it is modulated at least two to three fold by growth of the mycelium on different carbon sources, being greater on those metabolised by gluconeogenesis. The promoter activity was also modulated during growth of the culture. The increases in activity observed in logarithmic growth were again most rapid in mycelium grown on those carbon sources metabolised by gluconeogenesis. The *P. chrysogenum* promoter-lacZ fusion was active in *A. nidulans* at a similar level to the *A. nidulans* PGK promoter and it exhibited a modulation by carbon source similar to that observed for the *A. nidulans* promoter. Thus many of the appropriate transcription factors from *A. nidulans* recognise the *P. chrysogenum* PGK promoter.

A fusion of the PGK promoter to the IPNS gene did not produce a major change in the final penicillin titre in a fermentation. The gene fusion was only expressed early in the fermentation and at a level similar to the native IPNS gene. The expression level of the IPNS gene was the same as the PGK gene under the fermentation conditions used in contrast to earlier results.
The most obvious work which should be done with the PGK promoter of *P. chrysogenum* is to analyse the function of the promoter elements. This can be done either by progressive deletion of the promoter or by selective deletion of specific elements within it. Those elements identified as similar to elements in the *A. nidulans* PGK promoter are candidates for the latter approach. The plasmid pOPL8 containing the PGK promoter fused to the lacZ gene of *E. coli* would be the starting point for such a study. Transcriptional activity of the modified promoters could be assessed by monitoring β-galactosidase activity under different growth conditions. Another approach would be to isolate the DNA sequence elements which may be involved in transcriptional control and do DNA binding studies with cell free extracts prepared from mycelium grown under different conditions. Such experiments would reveal any proteins which bound to the specific sequences and would indicate their function. The identification of regions of the promoter involved in transcriptional control would enable manipulation of the promoter to improve its expression for practical applications.
Identification of clones from the cosmid library which may contain the catabolic dehydroquinase gene of P. chrysogenum

The genes encoding the enzymes of the pathway of quinic acid degradation of A. nidulans and N. crassa have been isolated and their regulation studied in detail at the molecular level (Hawkins et al. 1988; Geever et al. 1989). In both organisms the genes are clustered with genes which regulate their expression and all the proteins encoded by the genes in the cluster are inducible by quinate, some to a high level. The cosmid library described in Chapter 3 section was screened for clones which hybridised to a A. nidulans probe for the catabolic dehydroquinase gene QUTE. The isolation from P. chrysogenum of the equivalent gene to QUTE would enable the study of an inducible promoter which may be useful in expressing other genes and the investigation of the arrangement in the genome of the genes encoding the enzymes for quinic acid breakdown in P. chrysogenum. The isolation of a clone which may contain the P. chrysogenum gene encoding the enzyme catabolic dehydroquinase is described here.

A1.1 P. chrysogenum strains and construction of the cosmid library

The strains used are those described in Chapter 3 section 1 and the cosmid library screened is the one described in that section.

A1.2 Preparation of the catabolic dehydroquinase QUTE gene probe.

Approximately 30µg of the plasmid pAL3.3 (Figure 7-3) which bears the QUTE gene of A. nidulans was digested with XbaI and EcoRI and the digestion products separated by agarose gel electrophoresis. The 1kb fragment bearing the QUTE gene was excised and approximately 4µg purified from low melting point agarose by the method described in Chapter 2 section 6.3A. Samples of approximately 300-500ng of the
fragment were used in hybridisations to *P. chrysogenum* genomic DNA and labelled by the random oligonucleotide priming method (Chapter 2, section 7.3). The probe contains all the coding region of QUTE, 250bp 5' to the coding region and 238bp 3' to the coding region.

A1.3 Hybridisation of the *A. nidulans* QUTE probe to genomic DNA from *P. chrysogenum* N.R.R.L. 1951

A Southern analysis was done to establish the stringency of post-hybridisation washes needed to obtain a good signal when *P. chrysogenum* genomic DNA was hybridised to the QUTE probe. Samples of approximately 5μg of genomic DNA from N.R.R.L. 1951 were digested with EcoRI, BamHI or HindIII and 5μg of *A. nidulans* DNA digested with EcoRI as a positive control. The products of the digestion were separated by agarose gel electrophoresis. The DNA was transferred to Hybond-N and hybridised to 300ng of the *A. nidulans* QUTE probe at 60°C in 1.5X SSPE, 0.5% Marvel milk, 6% PEG 6000 (Chapter 2 section 7.4). Four 30 minute post hybridisation washes were done at 60°C in 0.1x SSC 0.1% SDS. Under these conditions the resulting autoradiograph yielded a good signal.

A single band hybridised to the *A. nidulans* QUTE probe for each of the digested DNAs. The sizes of these bands were, 6kb for the EcoRI digest of *A. nidulans* DNA and for *P. chrysogenum* DNA digested with EcoRI 6.6kb, digested with BamHI 4.0kb and digested with HindIII 20kb.

In a later experiment N.R.R.L. 1951 genomic DNA digested with HindIII or EcoRI was hybridised to the probe at 65°C. A range of post-hybridisation washes (2.5xSSC to 0.1xSSC) was done to determine at what concentration of SSC a signal could be obtained at 65°C. A good signal was obtained at 0.5xSSC, 0.1% SDS and these conditions were used later in the work.

A1.4 Screening of cosmid library

Approximately 12000 colonies were tested for hybridisation to the *A. nidulans* QUTE probe by the methods described above and in Chapter 3 section 1.4 and Chapter 2 section 4.2. The
initial screen produced 5 possible positively reacting clones. The clones were purified and re-tested. After two such cycles of colony hybridisation and purification further purification of positive clones was done by preparing samples of cosmid DNA. The DNA was denatured with 2M NaCl, 0.2M NaOH, for two minutes at 100°C and small samples (200ng-500ng) spotted onto a Hybond-N filter. The DNA was then hybridised to the QUTE probe fragment. Positive (the probe fragment for QUTE) and negative (the probe fragment for PGK described in Chapter 3) controls were also included. Two independent positive clones (Q1 and Q2) were isolated by this method.

The two clones Q1 and Q2 were purified by twice replating to isolate single colonies. Several of these single colonies were used to make small scale cultures and cosmid DNA prepared from these cultures. Samples of 10μl of the DNA were incubated with EcoRI, BamHI, HindIII, XhoI, and PstI. The DNA fragments produced were separated by electrophoresis on a 0.7% agarose gel, transferred to Hybond-N and hybridised to the A. nidulans QUTE probe as described. While the pattern obtained for Q2 was similar to that obtained for genomic DNA probed with the catabolic dehydroquinase gene some differences were found (Table 1). The pattern for Q1 was different to that obtained for genomic DNA and it was discarded.

A1.5 Summary

A cosmid clone Q2 containing P. chrysogenum genomic DNA which hybridises to the A. nidulans QUTE gene has been isolated. Its structure agrees in part with the structure of P. chrysogenum genomic DNA which hybridises to the QUTE probe. The differences noted between them which should be resolved by subcloning the region of the cosmid clone which hybridises to the probe and analysing it further. It would also be necessary to further locate the position of the QUTE gene equivalent in the cosmid clone in order to judge the likelihood that a gene cluster may have been isolated.
Table 1 The hybridisation of genomic and Q2 cosmid DNA to a probe for the A. nidulans QUTE gene

Genomic DNA prepared from P. chrysoenum N.R.R.L. 1951 and DNA prepared from the cosmids Q1 and Q2 was digested with the enzymes EcoRI, BamHI, or HindIII singly and in all possible pairs. The digested DNA was separated on a 0.7% agarose gel and transferred to Hybond N and hybridised to the 1kb QUTE probe at 65°C. The sizes of the fragments which hybridised to the probe are shown in the table.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Genomic DNA</th>
<th>Q2 cosmid DNA</th>
<th>Q1 cosmid DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>6.6</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>HindIII</td>
<td>23</td>
<td>6.2, 2.9</td>
<td>2.8, 2.1</td>
</tr>
<tr>
<td>BamHI</td>
<td>4.2</td>
<td>23, 4.2</td>
<td>23</td>
</tr>
<tr>
<td>EcoRI, BamHI</td>
<td>4.2, 5.2</td>
<td>3.9</td>
<td>6.1</td>
</tr>
<tr>
<td>EcoRI, HindIII</td>
<td>3.8</td>
<td>3.7</td>
<td>2</td>
</tr>
<tr>
<td>BamHI, HindIII</td>
<td>3.4</td>
<td>3.2</td>
<td>12, 6.2, 5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.5, 2.1</td>
</tr>
</tbody>
</table>
Appendix 2

Calculation of the number of colonies of the cosmid library to be screened in order to obtain a positive signal.

The calculation was done using the formula of Clarke and Carbon (1975). Since the genome size of P. chrysogenum was not known it was assumed to be twice that of the A. nidulans genome as a safe over estimate. The size of the PGK gene was taken to be 3kb since I wanted to isolate the upstream and downstream flanking sequence. The average size of the insert in the cosmid library was 30kb.

The formula is:

\[ N = \frac{\ln(1-P)}{\ln \left[1 - \frac{(L-x)}{M}\right]} \]

- \( N \) = number of colonies to be screened.
- \( P \) = the probability of obtaining one colony which is 0.99.
- \( L \) = average size of insert in cosmid library, 30kb
- \( X \) = size of gene to be found which is 3kb
- \( M \) = genome size, 5.4x10^4 kb

Using the above values the number of colonies to be screened was 9208.


Ball, C., (1973). The genetics of Penicillium chrysogenum. Progress in Industrial Microbiology, 12, 47-72.


characterisation of the acyl coenzyme A: 6-aminopenicillanic acid acyl transferase of Penicillium chrysogenum. 
Gene, 83, 291-300


Bernoist, C., O'Hare, K., Breathnach, R., and Chambon, P.,(1980). The ovalbumin gene: sequence of putative control regions. Nucleic Acids Research, 8, 127-142


Biggin, M.D., Gibson, T.J., and Hong, H.F., (1983). Buffer gradient gels and $^{35}$S label as an aid to rapid DNA sequencing. P.N.A.S. USA, 80, 3963-3965


expression of nuclear genes in *Saccharomyces cerevisiae*.  

Transformation of *Penicillium chrysogenum* with a dominant selectable marker. Current Genetics, 13, 377-382


Carey, M., Lin, Y.S., Green, M.R., Ptashne, M., (1990) A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives, Nature 345 361-


Cell 53, 3-4.


from Aspergillus nidulans. Gene, 44, 97-105


proteins. Cell, 50, 863-872

High efficiency transformation of E. coli by high voltage electroporation. Nucleic acids Research 16, 6127


Mutants of Aspergillus nidulans impaired in penicillin biosynthesis. Journal of General Microbiology 84, 420-422.


Grosschedl, R., Birnstiel, M.L.,(1980). Identification of regulatory sequences in the prelude sequence of an H2A
histone gene by the study of specific deletion mutants in vivo. P. N. A. S. U.S.A. 77, 1432-1436


three contiguous gene required for penicillin biosynthesis.
EMBO journal, 9, 279-287


Ogden J., Stanway C., Kim, S., Mellor J., Kingsman, A.J., and


Pontecorvo, G., Roper, J.A., Hemmons, J.M., Macdonald, K.D. and Bufton, A.W.J. (1953). The genetics of *Aspergillus*
nidulans. Advances in Genetics, 5, 141-238


The promoter of the glucoamylase encoding gene of Aspergillus niger functions in Ustilago maydis.
Gene 88 259-262.


Reversal of lysine inhibition of penicillin biosynthesis by α-amino adipic acid.
Archives of Biochemistry and Biophysics 93 238-241

Molecular Biology, 98, 503-517


Tyler, B., (1990). Two complex regions including a TATA sequence are required for transcription by RNA polymersase I in Neurospora crassa. Nucleic Acids Research 18, 1805-1811


Vieira, J. and Messing, J. (1982) The pUC plasmids, an Ml3mp7 derived system for the insertion
mutagenesis and sequencing with synthetic universal primers
Gene, 19 259-268


Further References


