FACTORS INFLUENCING HETEROLOGOUS GENE
EXPRESSION IN THE YEAST SACCHAROMYCES
CEREVISIAE

A thesis submitted to the University of Leicester for the degree of
Doctor of Philosophy

BERNADETTE JORDAN
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FOR MUM AND DAD
I would like to thank everyone who has supported me for the years it has taken me to complete this thesis.

Graham deserves special thanks for listening, supporting and "putting up with me", but most of all for never allowing me to give up. Thanks to him for always being there. Particular thanks also go to my Mum and Dad for their never-ending love and encouragement.

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The yeast *Saccharomyces cerevisiae* is widely employed as a host for the production of recombinant proteins. In the work presented here, expression of recombinant reporter proteins has been analysed in yeast, with the aim of determining factors that contribute to the expression performance of recombinant genes.

Initially, expression of bacterial aminoglycoside phosphotransferase was analysed in yeast. This heterologous gene showed very low expression levels, resulting from a small decrease in mRNA abundance, plus a large decrease in translation efficiency. This appeared to be due to an unfavourable codon usage confirmed with an alcohol dehydrogenase reporter gene having highly favourable codon usage.

Addition of a PGK1, ADH1, or CYCl promoter to a promoterless aminoglycoside phosphotransferase gene on a 2μm-based plasmid resulted in a reduced transformation efficiency and copy number, despite the product being non-toxic to yeast. This was not manifest in ARS plasmids. The decrease in copy number, represented a loss of potential product-encoding genes. Further investigation implicated a number of factors; regulatory functions, transcription and codon usage/translation. The promoter-inhibition did not correlate with promoter strength, nor did it result from transcriptional bypass of terminators. It was increased by a PGK promoter without a coupled coding sequence, but was reduced by promoter deletions in heterologous gene constructions, although not homologous PGK. The copy number reduction was also accompanied by reduced background transcription which was not apparent in deleted promoters that had increased copy number.

A high codon bias gene lacking a promoter, showed both lower plasmid copy number and lower background transcription than one with low codon bias. Plasmid-borne PGK promoters were found to cause transactivation of chromosomal ADH expression, which did not occur with the ADH1 promoter. This was not influenced by expression of ADH protein, nor other promoters that were tested, but could be mediated by single additional PGK promoters. The effect was influenced by some promoter deletions.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>SECTION</th>
<th>CONTENTS</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td>Introduction</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>Host systems for the expression of cloned genes</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>The expression of cloned gene products in yeast</td>
<td></td>
</tr>
<tr>
<td>1.1.1</td>
<td>Transformation of <em>S. cerevisiae</em></td>
<td>2</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Transformant selection</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>The expression of heterologous genes</td>
<td></td>
</tr>
<tr>
<td>1.2.1</td>
<td>Sequence elements required for expression</td>
<td></td>
</tr>
<tr>
<td>1.2.1.1</td>
<td>Upstream activator sequence</td>
<td>7</td>
</tr>
<tr>
<td>1.2.1.2</td>
<td>TATA elements</td>
<td>8</td>
</tr>
<tr>
<td>1.2.1.3</td>
<td>Initiator sites</td>
<td>8</td>
</tr>
<tr>
<td>1.2.1.4</td>
<td>Terminators</td>
<td>9</td>
</tr>
<tr>
<td>1.3</td>
<td>Factors influencing expression of heterologous genes in yeast</td>
<td>10</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Promoter strength</td>
<td>10</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Terminator choice</td>
<td>12</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Translation</td>
<td>13</td>
</tr>
<tr>
<td>1.3.3.1</td>
<td>Translation Initiation</td>
<td>13</td>
</tr>
<tr>
<td>1.3.3.2</td>
<td>mRNA structure</td>
<td>14</td>
</tr>
<tr>
<td>1.3.3.3</td>
<td>mRNA stability</td>
<td>14</td>
</tr>
<tr>
<td>1.3.3.4</td>
<td>Codon Usage</td>
<td>15</td>
</tr>
<tr>
<td>1.3.4</td>
<td>Protein stability</td>
<td>16</td>
</tr>
<tr>
<td>1.3.5</td>
<td>Product toxicity</td>
<td>17</td>
</tr>
<tr>
<td>1.3.6</td>
<td>Plasmid copy number</td>
<td>17</td>
</tr>
<tr>
<td>1.3.7</td>
<td>Plasmid stability</td>
<td>17</td>
</tr>
<tr>
<td>1.3.8</td>
<td>Effects of plasmids on the host cell</td>
<td>18</td>
</tr>
<tr>
<td>1.4</td>
<td>Vectors employed for expression in <em>S. cerevisiae</em></td>
<td>18</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Yeast integrating plasmids</td>
<td>19</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Independently replicating vectors</td>
<td>19</td>
</tr>
<tr>
<td>1.4.2.1</td>
<td>Yeast replicating plasmids</td>
<td>20</td>
</tr>
<tr>
<td>1.4.2.2</td>
<td>Yeast centromeric plasmids</td>
<td>21</td>
</tr>
<tr>
<td>1.4.2.3</td>
<td>Yeast linear plasmids</td>
<td>21</td>
</tr>
<tr>
<td>1.4.2.4</td>
<td>Yeast episomal plasmids</td>
<td>22</td>
</tr>
<tr>
<td>1.5</td>
<td>The 2μm plasmid</td>
<td>22</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Elements of the 2μm circle replication system</td>
<td>23</td>
</tr>
<tr>
<td>1.5.2</td>
<td>2μm plasmid partitioning</td>
<td>25</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Composition of 2μm circle-based vectors</td>
<td>25</td>
</tr>
<tr>
<td>SECTION</td>
<td>CONTENTS</td>
<td>PAGE No</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>1.6</td>
<td>The use of reporter genes to study gene expression</td>
<td>27</td>
</tr>
<tr>
<td>1.6.1</td>
<td>β-galactosidase</td>
<td>27</td>
</tr>
<tr>
<td>1.6.2</td>
<td>β-glucuronidase</td>
<td>28</td>
</tr>
<tr>
<td>1.6.3</td>
<td>Chloramphenicol acetyltransferase</td>
<td>28</td>
</tr>
<tr>
<td>1.6.4</td>
<td>Aminoglycoside phosphotransferase</td>
<td>29</td>
</tr>
<tr>
<td>1.7</td>
<td>Aims and objectives of the thesis</td>
<td>30</td>
</tr>
</tbody>
</table>

### Chapter 2: Materials and Methods

<p>| 2.1 | Bacterial and yeast strains | 33 |
| 2.2 | Bacterial growth media | 33 |
| 2.2.1 | Luria Broth | 33 |
| 2.2.2 | Ampicillin and kanamycin medium | 34 |
| 2.2.3 | Amp / Xgal/IPTG agar medium | 34 |
| 2.3 | Yeast growth media | 34 |
| 2.3.1 | Yeast peptone medium and agar | 34 |
| 2.3.2 | YPD medium | 34 |
| 2.3.3 | YPD G418 medium | 35 |
| 2.3.4 | Minimal (semi-defined) medium and agar | 35 |
| 2.4 | Media Supplements | 35 |
| 2.4.1 | Carbon sources | 35 |
| 2.4.2 | Amino-acids | 35 |
| 2.4.3 | Antibiotics | 36 |
| 2.5 | Standard Buffers | 36 |
| 2.5.1 | Phosphate buffers | 36 |
| 2.5.2 | Phenol | 37 |
| 2.5.3 | Chloroform / Isopropylalcohol | 37 |
| 2.6 | Manipulation of DNA | 37 |
| 2.6.1 | Plasmids employed | 37 |
| 2.6.2 | Ethanol precipitation of DNA | 37 |
| 2.6.3 | Determination of DNA concentration | 38 |
| 2.6.4 | Phenol / Chloroform extraction of DNA | 38 |
| 2.6.5 | Endonuclease restriction of DNA fragments | 38 |
| 2.6.6 | Buffering conditions of the restriction Enzymes used in this thesis | 39 |
| 2.6.6.1 | RIAct Buffer composition | 39 |
| 2.6.7 | The separation of DNA restriction fragments | 39 |</p>
<table>
<thead>
<tr>
<th>SECTION</th>
<th>CONTENTS</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6.8</td>
<td>The isolation of DNA fragments for probe use</td>
<td>40</td>
</tr>
<tr>
<td>2.6.8.1</td>
<td>Oligo-labelling of DNA fragments by random hexamer priming for probe use</td>
<td>40</td>
</tr>
<tr>
<td>2.6.9</td>
<td>Removal of unincorporated nucleotides</td>
<td>41</td>
</tr>
<tr>
<td>2.6.9</td>
<td>The isolation of DNA restriction fragments for further modification and ligation</td>
<td>42</td>
</tr>
<tr>
<td>2.6.10</td>
<td>The elution of DNA from polyacrylamide gel fragments</td>
<td>43</td>
</tr>
<tr>
<td>2.6.11</td>
<td>Ligation of DNA fragments</td>
<td>44</td>
</tr>
<tr>
<td>2.6.11.1</td>
<td>To another fragment</td>
<td>44</td>
</tr>
<tr>
<td>2.6.11.2</td>
<td>Into a vector</td>
<td>44</td>
</tr>
<tr>
<td>2.7</td>
<td>E. coli Methods</td>
<td>45</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Transformation of E. coli cells</td>
<td>45</td>
</tr>
<tr>
<td>2.7.1.1</td>
<td>Preparation of competent E. coli cells</td>
<td>45</td>
</tr>
<tr>
<td>2.7.1.2</td>
<td>Transformation of competent E. coli cells</td>
<td>45</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Analysis of E. coli transformants</td>
<td>46</td>
</tr>
<tr>
<td>2.7.2.1</td>
<td>Replica plating E. coli transformant colonies</td>
<td>46</td>
</tr>
<tr>
<td>2.7.2.2</td>
<td>E. coli colony hybridisation</td>
<td>47</td>
</tr>
<tr>
<td>2.7.3</td>
<td>E. coli plasmid DNA preparation</td>
<td>47</td>
</tr>
<tr>
<td>2.7.3.1</td>
<td>Small scale E. coli DNA preparation</td>
<td>47</td>
</tr>
<tr>
<td>2.7.3.2</td>
<td>Large scale E. coli DNA preparation</td>
<td>48</td>
</tr>
<tr>
<td>2.8</td>
<td>Yeast Methods</td>
<td>50</td>
</tr>
<tr>
<td>2.8.1</td>
<td>Transformation of yeast cells</td>
<td>50</td>
</tr>
<tr>
<td>2.8.1.1</td>
<td>Preparation of competent yeast cells</td>
<td>50</td>
</tr>
<tr>
<td>2.8.1.2</td>
<td>Transformation of competent yeast cells</td>
<td>51</td>
</tr>
<tr>
<td>2.8.1.2.1</td>
<td>Using an auxotrophic marker for selection</td>
<td>51</td>
</tr>
<tr>
<td>2.8.1.2.2</td>
<td>Using a dominant marker for selection</td>
<td>51</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Yeast total DNA preparation</td>
<td>52</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Plasmid copy number determination</td>
<td>53</td>
</tr>
<tr>
<td>2.8.4</td>
<td>Yeast plasmid stability determination</td>
<td>55</td>
</tr>
<tr>
<td>2.8.5</td>
<td>Southern blotting of agarose gels</td>
<td>55</td>
</tr>
<tr>
<td>2.8.6</td>
<td>Southern hybridisation</td>
<td>56</td>
</tr>
<tr>
<td>2.8.7</td>
<td>Probe removal from Southern or Northern hybridised Hybond filters</td>
<td>57</td>
</tr>
<tr>
<td>2.9</td>
<td>RNA Preparation and manipulation</td>
<td>58</td>
</tr>
<tr>
<td>2.9.1</td>
<td>Yeast total RNA preparation</td>
<td>58</td>
</tr>
<tr>
<td>2.9.2</td>
<td>RNA gel electrophoresis</td>
<td>59</td>
</tr>
<tr>
<td>2.9.3</td>
<td>Northern blotting</td>
<td>59</td>
</tr>
<tr>
<td>2.9.4</td>
<td>Northern hybridisation</td>
<td>60</td>
</tr>
<tr>
<td>2.10</td>
<td>Protein Preparation and manipulation</td>
<td>61</td>
</tr>
<tr>
<td>2.10.1</td>
<td>Crude protein extraction from whole yeast cells</td>
<td>61</td>
</tr>
<tr>
<td>2.10.2</td>
<td>Protein assay</td>
<td>62</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (contd)

<table>
<thead>
<tr>
<th>SECTION</th>
<th>CONTENTS</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.10.3</td>
<td>SDS-Polyacrylamide gel electrophoresis of proteins</td>
<td>63</td>
</tr>
<tr>
<td>2.10.4</td>
<td>Staining protein gels</td>
<td>64</td>
</tr>
<tr>
<td>2.10.5</td>
<td>Alcohol dehydrogenase assay</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 3</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Expression of Bacterial Aminoglycoside Phosphotransferase in yeast as a model heterologous gene and dominant marker</strong></td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>66</td>
</tr>
<tr>
<td>3.2</td>
<td>The isolation of the APT coding sequence</td>
<td>69</td>
</tr>
<tr>
<td>3.3</td>
<td>Construction of the multi-copy APT determinant</td>
<td>72</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Promoter and terminator isolation</td>
<td>72</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Incorporation of a yeast replication origin onto the heterologous APT cartridge plasmid</td>
<td>75</td>
</tr>
<tr>
<td>3.4</td>
<td>Performance of the PGK promoter-driven APT expression construct as a G418 resistance determinant in E. coli and S. cerevisiae</td>
<td>77</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Transformation of the vectors in yeast</td>
<td>77</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Transformation of the vectors into E. coli</td>
<td>82</td>
</tr>
<tr>
<td>3.4.3</td>
<td>Growth, stability and copy number of G418 resistant transformants</td>
<td>83</td>
</tr>
<tr>
<td>3.4.3.1</td>
<td>Growth and viability of cells containing the APT gene</td>
<td>83</td>
</tr>
<tr>
<td>3.4.3.2</td>
<td>Plasmid stability</td>
<td>83</td>
</tr>
<tr>
<td>3.4.3.3</td>
<td>Determination of the plasmid copy number of pBEJ16 and pBEJ17</td>
<td>84</td>
</tr>
<tr>
<td>3.5</td>
<td>Determination of APT enzyme protein levels</td>
<td>86</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Development of the APT enzyme assay</td>
<td>89</td>
</tr>
<tr>
<td>3.5.2</td>
<td>APT activity of the heterologous plasmids</td>
<td>92</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Determination of the relationship between APT units and the % of total cell protein equivalent to APT</td>
<td>94</td>
</tr>
<tr>
<td>3.5.3.1</td>
<td>Overexpression of the APT protein</td>
<td>94</td>
</tr>
<tr>
<td>3.5.3.2</td>
<td>APT assay to equate APT units to % of APT protein</td>
<td>96</td>
</tr>
<tr>
<td>3.6</td>
<td>Transcript analysis of plasmid pBEJ16</td>
<td>99</td>
</tr>
<tr>
<td>3.7</td>
<td>A comparison of different translation initiation codons</td>
<td>99</td>
</tr>
<tr>
<td>3.7.1</td>
<td>Effect of the translation initiation environment on translation</td>
<td>101</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (contd)

<table>
<thead>
<tr>
<th>SECTION</th>
<th>CONTENTS</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>An analysis of secondary structure</td>
<td>102</td>
</tr>
<tr>
<td>3.9</td>
<td>Discussion</td>
<td>102</td>
</tr>
</tbody>
</table>

Chapter 4 Discovery and initial characterisation of an inhibition effect on episomal plasmids caused by strong chromosomal gene promoters

4.1 Introduction                                                   109
4.2 Investigation of the effect of the promoters                   110
  4.2.1 Promoters investigated                                       110
  4.2.2 Effect of promoters on transformability, expression, plasmid copy number and stability 114
  4.2.3 A comparison of transformability                             114
  4.2.4 Transformed cell doubling-time                               117
  4.2.5 APT expression from the promoter containing gene constructions 117
  4.2.6 The copy number and stability of cells containing the APT gene with different promoters 121
    4.2.6.1 Determination of plasmid copy numbers                     121
    4.2.6.2 Determination of plasmid stability                        121
  4.3 Discussion                                                    125

Chapter 5 Further investigation of the promoter-mediated plasmid inhibition

5.1 Introduction                                                   132
5.2 The influence of alternative plasmid configurations on the plasmid inhibition effect 133
  5.2.1 Plasmid-borne constructions with alternative or additional terminators 133
  5.2.2 Constructions with the plasmid-borne gene in the opposite orientation 136
  5.2.3 Altered plasmid construction configuration                     141
5.3 Transcript analysis of the various constructions                142
5.4 The effect of promoter addition on ARG based plasmids           145
### TABLE OF CONTENTS (contd)

<table>
<thead>
<tr>
<th>SECTION</th>
<th>CONTENTS</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4.1</td>
<td>ARS plasmid constructions</td>
<td>146</td>
</tr>
<tr>
<td>5.4.2</td>
<td>Transformability, stability and APT expression of the APT/ARS containing plasmids</td>
<td>146</td>
</tr>
<tr>
<td>5.5</td>
<td>Discussion</td>
<td>152</td>
</tr>
</tbody>
</table>

**Chapter 6** Homologous genes and the effect of promoter deletions on plasmid inhibition

| 6.1     | Introduction | 156     |
| 6.2     | Transformability and copy number of plasmids containing homologous genes | 156     |
| 6.3     | The effect of promoter deletions on plasmids expressing homologous and heterologous genes | 157     |
| 6.3.1   | Effects of promoter deletions on plasmids containing homologous genes | 159     |
| 6.3.1.1 | Plasmid transformability, copy number and stability | 159     |
| 6.3.1.2 | Transcript and protein analysis | 163     |
| 6.3.2   | Effects of promoter deletions on plasmids containing heterologous genes | 167     |
| 6.3.2.1 | Construction of heterologous promoter deletions | 167     |
| 6.3.2.1.1 | ADH1 promoter deletion construction | 168     |
| 6.3.2.1.2 | Construction of a TRP1 promoter deletion | 168     |
| 6.3.2.2 | Performance of the promoter deletion constructions | 171     |
| 6.3.2.2.1 | Plasmid transformability and APT expression | 171     |
| 6.3.2.2.2 | Plasmid copy number | 173     |
| 6.3.2.2.3 | Transcript analysis of plasmids containing the promoter deletions | 177     |
| 6.5     | Discussion | 180     |

**Chapter 7** Studies with an ADH reporter gene and the discovery and initial characterisation of possible transacting effects between plasmid and chromosomal genes

| 7.1     | Introduction | 183     |
| 7.2     | Expression studies with an ADH1 reporter gene | 184     |
# TABLE OF CONTENTS (contd)

<table>
<thead>
<tr>
<th>SECTION</th>
<th>CONTENTS</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2.1</td>
<td>Selection of a high codon bias reporter gene</td>
<td>184</td>
</tr>
<tr>
<td>7.2.1.1</td>
<td>Construction of promoterless and promoter-driven reporter gene plasmids</td>
<td>184</td>
</tr>
<tr>
<td>7.2.2</td>
<td>Investigation of the constructions</td>
<td>185</td>
</tr>
<tr>
<td>7.2.2.1</td>
<td>Transformation efficiencies</td>
<td>185</td>
</tr>
<tr>
<td>7.2.2.2</td>
<td>Stability and copy number of the transformants</td>
<td>190</td>
</tr>
<tr>
<td>7.2.3</td>
<td>Transcript analysis</td>
<td>190</td>
</tr>
<tr>
<td>7.3</td>
<td>Determination of ADH activity in the constructions</td>
<td>193</td>
</tr>
<tr>
<td>7.3.1</td>
<td>Construction of a standard curve of ADH activity versus the amount of ADH</td>
<td>195</td>
</tr>
<tr>
<td>7.3.2</td>
<td>Confirmation that activity determined was enzymatic</td>
<td>197</td>
</tr>
<tr>
<td>7.3.3</td>
<td>ADH activity of yeast transformants</td>
<td>197</td>
</tr>
<tr>
<td>7.3.4</td>
<td>ADH activity of cells containing other PGK1 promoter and whole gene plasmids</td>
<td>200</td>
</tr>
<tr>
<td>7.3.5</td>
<td>The effect of plasmids containing PGK1 promoter deletions on ADH activity</td>
<td>201</td>
</tr>
<tr>
<td>7.3.6</td>
<td>The effect of an integrated copy and increased copies of the PGK1 promoter on ADH activity</td>
<td>203</td>
</tr>
<tr>
<td>7.4</td>
<td>Discussion</td>
<td>205</td>
</tr>
</tbody>
</table>

Chapter 8 General Discussion | 211     |

Chapter 9 References | 220     |

Appendix | 243     |
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>TITLE</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5.1</td>
<td>Structural organisation of the 2μm circle.</td>
<td>24</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Schematic diagram showing the process of plasmid copy number determination.</td>
<td>54</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Derivation of APT coding sequence cartridges</td>
<td>70</td>
</tr>
<tr>
<td>3.2.1b</td>
<td>Sequence characteristics of the APT-coding sequence cartridges</td>
<td>71</td>
</tr>
<tr>
<td>3.3.1a</td>
<td>Construction of the PGK1 promoter and a terminator containing fragment</td>
<td>73</td>
</tr>
<tr>
<td>3.3.1b</td>
<td>Construction of the heterologous APT cartridge</td>
<td>74</td>
</tr>
<tr>
<td>3.3.2.1</td>
<td>Multi-copy plasmids containing the APT cartridge</td>
<td>76</td>
</tr>
<tr>
<td>3.4.1.1</td>
<td>Structure of plasmid pMP81</td>
<td>80</td>
</tr>
<tr>
<td>3.4.1.2</td>
<td>Frequency of G418^* transformants at various post-transformation expression times</td>
<td>81</td>
</tr>
<tr>
<td>3.4.3.1</td>
<td>Growth of G418^* transformants in YPD medium</td>
<td>85</td>
</tr>
<tr>
<td>3.4.3.2</td>
<td>Copy number determination of cells transformed with plasmids pBEJ16 and pBEJ17</td>
<td>87</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Analysis of apt protein in yeast cells transformed with the APT coding sequence</td>
<td>90</td>
</tr>
<tr>
<td>3.5.3.1a</td>
<td>Plasmid pTTQ18 employed for the overexpression of APT protein</td>
<td>95</td>
</tr>
<tr>
<td>3.5.3.1b</td>
<td>SDS-polyacrylamide gel electrophoresis separation of APT protein in soluble cell protein of transformed E. coli cells:</td>
<td>95</td>
</tr>
<tr>
<td>3.5.3.2</td>
<td>Log graph showing APT units/mg protein against % APT</td>
<td>98</td>
</tr>
<tr>
<td>3.6</td>
<td>Transcript analysis of plasmid pBEJ16</td>
<td>100</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Structure of plasmids pCJ17 and pCJ18</td>
<td>111</td>
</tr>
<tr>
<td>4.2.1.1</td>
<td>Sequence and structure of the promoters analysed</td>
<td>112</td>
</tr>
<tr>
<td>4.2.1.2</td>
<td>Structure of the differing promoter constructions</td>
<td>113</td>
</tr>
<tr>
<td>4.2.3.1a</td>
<td>A comparison of the transformability of plasmids without a promoter and with varying promoters</td>
<td>115</td>
</tr>
<tr>
<td>4.2.3.1b</td>
<td>Frequency of G418^* transformants with varying post-transformation expression time</td>
<td>115</td>
</tr>
<tr>
<td>4.2.5</td>
<td>Growth of cells containing the promasterless APT gene</td>
<td>118</td>
</tr>
<tr>
<td>4.2.4.1</td>
<td>APT expression from plasmids containing different promoters</td>
<td>119</td>
</tr>
<tr>
<td>4.2.5.1</td>
<td>Copy number determination of the promoter containing constructions</td>
<td>123</td>
</tr>
<tr>
<td>FIGURE</td>
<td>TITLE</td>
<td>PAGE No</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>---------</td>
</tr>
<tr>
<td>5.2.1.1</td>
<td>Structure of plasmids of alternative configuration</td>
<td>134</td>
</tr>
<tr>
<td>5.2.1.1b</td>
<td>Structure of plasmids pBEJ16 and pBEJ17 based on the LEU2 marker</td>
<td>135</td>
</tr>
<tr>
<td>5.2.1.2a</td>
<td>Transformability of plasmids with differing promoters and construction</td>
<td>137</td>
</tr>
<tr>
<td>5.2.1.2b</td>
<td>APT expression obtained with plasmids having differing promoters and construction</td>
<td>137</td>
</tr>
<tr>
<td>5.2.1.3</td>
<td>Copy number determination of plasmids of alternative configuration</td>
<td>139</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Northern blot analysis of cells transformed with plasmids containing different promoters and construction</td>
<td>143</td>
</tr>
<tr>
<td>5.4.1</td>
<td>Structure of ARS based plasmids containing the APT1 coding sequence and differing promoters</td>
<td>147</td>
</tr>
<tr>
<td>5.4.2.1</td>
<td>Transformability of ARS based plasmids</td>
<td>148</td>
</tr>
<tr>
<td>5.4.2.2</td>
<td>APT expression from ARS based plasmids</td>
<td>149</td>
</tr>
<tr>
<td>5.4.3.3a/b</td>
<td>Stability of ARS based plasmids containing the APT1 gene</td>
<td>151</td>
</tr>
<tr>
<td>6.3a</td>
<td>Structure of plasmid pCH215</td>
<td>160</td>
</tr>
<tr>
<td>6.3b</td>
<td>Structure of PGK1 promoter deletions</td>
<td>160</td>
</tr>
<tr>
<td>6.3.1.1</td>
<td>Copy number determination of plasmids containing various PGK1 promoter deletions</td>
<td>162</td>
</tr>
<tr>
<td>6.3.1.2.1</td>
<td>Northern blot analysis of yeast cells transformed with PGK1 promoter deletion plasmids</td>
<td>164</td>
</tr>
<tr>
<td>6.3.1.2.2</td>
<td>Analysis of total cell protein isolated from cells transformed with PGK1 promoter deletion containing plasmids</td>
<td>166</td>
</tr>
<tr>
<td>6.3.2.1.1</td>
<td>Structure of the ADH1 and TRP1 promoter deletion fragments</td>
<td>169</td>
</tr>
<tr>
<td>6.3.2.1.2</td>
<td>Construction of plasmids containing an ADH1 or TRP1 promoter deletion</td>
<td>170</td>
</tr>
<tr>
<td>6.3.2.2.2</td>
<td>Copy number of cells containing the truncated ADH1 or TRP1 promoter plasmids</td>
<td>175</td>
</tr>
<tr>
<td>6.3.2.2.3a</td>
<td>Northern blot analysis of yeast cells transformed with plasmids pBEJ38 and pBEJ39 and probed with APT</td>
<td>177</td>
</tr>
<tr>
<td>6.3.2.2.3b</td>
<td>Northern blot analysis of yeast cells transformed with plasmids pBEJ38 and pBEJ39 and probed with TRP1</td>
<td>178</td>
</tr>
<tr>
<td>7.2.1.1</td>
<td>Structure of the ADH1 gene and the fragment employed as the reporter gene fragment in subsequent constructions.</td>
<td>186</td>
</tr>
<tr>
<td>7.2.1.2</td>
<td>Construction and structure of ADH1 reporter gene plasmids.</td>
<td>187</td>
</tr>
<tr>
<td>FIGURE</td>
<td>TITLE</td>
<td>PAGE No</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>7.2.1.3</td>
<td>Structure of the LEU2 ADH1 reporter gene plasmid</td>
<td>188</td>
</tr>
<tr>
<td>7.2.2.2</td>
<td>Copy number determination of cells containing ADH1 reporter gene</td>
<td>192</td>
</tr>
<tr>
<td>7.3.3</td>
<td>Northern blot analysis of cells transformed with the ADH1 reporter containing plasmids</td>
<td>194</td>
</tr>
<tr>
<td>7.3.1</td>
<td>ADH Standard Curve, showing µg of ADH protein against Units of ADH Activity</td>
<td>196</td>
</tr>
<tr>
<td>7.3.2</td>
<td>The effect of the addition of Pyrazole to ADH enzyme reactions</td>
<td>198</td>
</tr>
<tr>
<td>7.4.5</td>
<td>The Glycolytic pathway in <em>S. cerevisiae</em></td>
<td>210</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>TITLE</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Heterologous proteins expressed in <em>Saccharomyces cerevisiae</em></td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Promoters commonly employed in expression vectors</td>
<td>11</td>
</tr>
<tr>
<td><strong>Chapter 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Growth of <em>S. cerevisiae</em> haploid laboratory strains in the presence of differing G418 concentrations</td>
<td>78</td>
</tr>
<tr>
<td>2</td>
<td>The number of G418 resistant transformants obtained after a post-expression time of 2 hours and auxotrophic transformants</td>
<td>81</td>
</tr>
<tr>
<td>3</td>
<td>Stability of plasmids pBEJ16 and pBEJ17 in selective and non-selective media</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>A comparison of plasmid copy number data obtained by scintillation counting a probed filter and by scanning an exposed autoradiograph for plasmids pBEJ16 and pBEJ17 and other plasmids</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>APT expression of cells containing plasmids pBEJ16 and pBEJ17 in minimal and rich selective media</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>Results of an APT assay employing standard amounts of APT protein and varying types of protein extract for dilution</td>
<td>97</td>
</tr>
<tr>
<td>7</td>
<td>A comparison of APT expression from plasmids containing either the APT1 or the APT2 cartridge</td>
<td>103</td>
</tr>
<tr>
<td><strong>Chapter 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Plasmid copy number of cells containing APT plasmids with differing promoters in selective rich or minimal media</td>
<td>122</td>
</tr>
<tr>
<td>2</td>
<td>Stability of APT plasmids with differing promoters in selective or non-selective media</td>
<td>124</td>
</tr>
</tbody>
</table>
## LIST OF TABLES (contd)

<table>
<thead>
<tr>
<th>TABLE</th>
<th>TITLE</th>
<th>PAGE No</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Stability of APT plasmids with differing promoters and construction in selective and non-selective media</td>
<td>138</td>
</tr>
<tr>
<td>2</td>
<td>Plasmid copy number of cells containing APT plasmids with differing configuration in selective rich and minimal media</td>
<td>140</td>
</tr>
<tr>
<td><strong>Chapter 6</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Transformability and copy number of plasmids containing homologous genes</td>
<td>158</td>
</tr>
<tr>
<td>2</td>
<td>Transformability, plasmid stability and plasmid copy number of plasmids containing various PGK promoter deletions</td>
<td>161</td>
</tr>
<tr>
<td>3</td>
<td>Transformability and APT expression of plasmids containing the truncated TRP1 and ADH1 promoters</td>
<td>172</td>
</tr>
<tr>
<td>6</td>
<td>Plasmid copy number of cells containing the truncated TRP1 and ADH1 promoters</td>
<td>174</td>
</tr>
<tr>
<td><strong>Chapter 7</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Number of transformants obtained with plasmids containing varying promoter and ADH reporter cartridges in minimal selective medium</td>
<td>189</td>
</tr>
<tr>
<td>2</td>
<td>Plasmid copy number and plasmid stability over 10 generations of plasmids containing the ADH reporter gene</td>
<td>191</td>
</tr>
<tr>
<td>3</td>
<td>ADH units obtained from cells containing plasmids with the ADH gene and control plasmids</td>
<td>199</td>
</tr>
<tr>
<td>4</td>
<td>ADH units obtained from cells containing plasmids with a PGK1 promoter and a different coding region</td>
<td>202</td>
</tr>
<tr>
<td>5</td>
<td>ADH units obtained from cells containing plasmids with different promoters promoter and a different coding region</td>
<td>202</td>
</tr>
</tbody>
</table>
ADH units obtained from plasmids containing various PGK1 promoter deletions

ADH units obtained from cells containing differing copy number plasmids
LIST OF ABBREVIATIONS

ADH Alcohol Dehydrogenase
APT Aminoglycoside phosphotransferase
ATP Adenosine triphosphate
bp Base pairs
BSA Bovine serum albumin
cpm Counts per minute
CTP Cytosine triphosphate
CYC Cytochrome c
DNA Deoxyribonucleic acid
DTT Dithiothreitol
EDTA Ethylene diamine tetra-acetic acid
EtBr Ethidium bromide
G418 Geneticin
GPD Glyceraldehyde-3-Phosphate dehydrogenase
GTP Guanosine triphosphate
IMS Industrial methylated spirits
IPTG Isopropyl-B-thiogalactosidase
kD Kilodalton
kb Kilobase
LB Luria broth
MOPS 3-(N-Morpholino)propane sulfonic acid
dNTP Any 2' deoxynucleoside 5' triphosphate
OD Optical density
ORF Open Reading Frame
p promoter
PEG Polyethylene glycol
PGK Pyruvate glycerate kinase
PMSF Phenylmethylsulfonylfluoride
RNA Ribonucleic acid (prefix m = messenger, t = transfer)
rpm revs per minute
SDS Sodium dodecyl sulphate
SSC Sodium saline citrate
t terminator
TAE Tris base, EDTA
TBE Tris base, boric acid, EDTA
TE Tris HCl, EDTA
TEMED N,N',N,N' Tetramethylenediamine
Tris Tris (hydroxymethyl) aminomethane
TTP Thymidine 5' triphosphate
UAS Upstream activating sequence
UTP Uridine 5' triphosphate
UV Ultra violet
XGal 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD Yeast peptone glucose medium
μg microgram
CHAPTER ONE

INTRODUCTION
1.0 Host systems for the expression of cloned genes

In the early to mid 1970's, molecular genetic studies were principally centred upon the bacterium *Escherichia coli* and its plasmids and bacteriophages. Transformation systems were developed using *E. coli*, and in addition, antibiotic resistance determinants were exploited for use as dominant selectable markers. As a consequence the first available cloning vectors were *E. coli* plasmids and *E. coli* became established as the first host for recombinant DNA technology when in 1978 the somatostatin gene was expressed (Itakura et al., 1978). A number of attributes also made *E. coli* an ideal microorganism for expression, in that;

i) it has a high cell growth rate and thus a rapid generation of biomass
ii) its culture conditions are of low cost and
iii) recombinant proteins can be expressed at levels of 10-20% of the total cell protein

One of the major disadvantages of the system however, which limited its general usefulness is that it has a limited capacity to secrete proteins. Recombinant proteins produced in *E. coli*, are normally loaded intracellularly, in an insoluble and inactive form in inclusion bodies. Cells must therefore be treated with a denaturing agent to extract the proteins, resulting in only a small percentage of correctly refolded protein. As a consequence, complicated controlled refolding needs to be performed. *E. coli* is additionally unable to perform certain post-translational modifications, such as disulphide bond formation, glycosylation and acetylation, which ultimately leads to incorrect protein folding. Although a number of proteins may be obtained in high quantities, the purification and renaturation required to make the proteins biologically active, may be difficult and expensive. Additionally, many small polypeptides produced in *E. coli* are unstable owing to proteolytic degradation. As a result many can only be produced as fusion proteins, which then need to be cleaved to release the peptides. Since *E. coli* also produces toxic and pyrogenic cell wall components, it is a less acceptable host for the production of human pharmaceuticals and food products.

1.1 The expression of cloned gene products in yeast

As a consequence of the problems outlined above, alternative expression hosts were investigated. Yeasts were exploited as an alternative host for expression as a result of a number of important attributes. It is a eukaryote and thus possesses the same basic cell structure as multicellular higher eukaryotic microorganisms. It has a highly compartmentalised
intracellular organisation and an elaborate secretory pathway which is similar to higher eukaryotic systems (Schelmann and Novick, 1982). In addition it has the ability to perform post-translational modifications such as disulphide bond formation and N and O-linked glycosylation.

The use of yeast for expression was slowed considerably, owing to the lack of transformation procedures, plasmids or markers available for yeast. The initial transformation of yeast with recombinant plasmids was performed by Beggs (1978) and Hinnen et al. (1978) using *Saccharomyces cerevisiae* (described in section 1.2) and from this point *S. cerevisiae* became the focus of extensive characterisation and established as another major host for the expression of cloned foreign genes.

*S. cerevisiae* has proved to be a useful experimental organism for the expression and study of a wide variety of eukaryotic gene products, both for basic research and pharmaceutical applications. It also has an established safety for humans and thus has wide acceptability in the food industry having been employed in industrial brewing and the baking process for centuries. Unlike *E. coli*, *S. cerevisiae* secretes proteins into the culture medium and since few proteins are normally secreted, this is a major advantage for recovery and purification. Saccharomyces fermentation technology is well established for the large scale production of the yeast and its products, which can be readily adapted for the production of heterologous proteins. A wide range of heterologous proteins have been expressed in *S. cerevisiae*, a number of which are listed in Table 1.

1.1.1 Transformation of *S. cerevisiae*.

As mentioned previously, the wider exploitation of *S. cerevisiae* for heterologous protein expression was due to two particular advances in yeast technology;

i) the advent of techniques to introduce exogenous DNA (on a plasmid) into yeast cells (Hinnen et al., 1978, Beggs, 1978), leading to the development of a large number of vector systems, and,

ii) the demonstration that recombination of transforming DNA with the chromosome occurs via homology (Hinnen 1975) and that recombination can be directed (Orr-Weaver et al., 1981).
<table>
<thead>
<tr>
<th>HETEROLOGOUS PROTEIN</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human interferon α 2</td>
<td>Tuite et al, 1982</td>
</tr>
<tr>
<td>Calf prochymosin</td>
<td>Mellor et al, 1983</td>
</tr>
<tr>
<td>Gamma-interferon</td>
<td>Derynck et al, 1983</td>
</tr>
<tr>
<td>Human α-1 antitrypsin</td>
<td>Rosenberg et al, 1984</td>
</tr>
<tr>
<td>Zn/Cd superoxide human dismutase</td>
<td>Hallwell et al, 1987</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>Brake et al, 1984</td>
</tr>
<tr>
<td>Hepatitis B surface antigen particles</td>
<td>Valenzuela et al, 1982</td>
</tr>
<tr>
<td>Herpes simplex thymidine kinase</td>
<td>Zhu et al, 1985</td>
</tr>
<tr>
<td>Human interleukin 1β, 1A</td>
<td>Baldari et al, 1987, Livi et al, 1991</td>
</tr>
<tr>
<td>Insulin precursors</td>
<td>Thim et al, 1986</td>
</tr>
<tr>
<td>Hybrid human chorionic gonadotropin</td>
<td>Beesley et al, 1990</td>
</tr>
<tr>
<td>Mammalian β-2-adrenergic receptor</td>
<td>King et al, 1990</td>
</tr>
<tr>
<td>Rat cytochrome c</td>
<td>Clements et al, 1989</td>
</tr>
<tr>
<td>Human lysozyme</td>
<td>Castanon et al, 1988</td>
</tr>
</tbody>
</table>
The technique developed by Hinnen et al and Beggs (1978), to introduce foreign DNA into yeast cells involved the production of sphaeroplasts by the enzymatic removal of the yeast cell wall with glucanase or zymolyase. In the presence of an isotonic solution containing polyethylene glycol and Calcium ions, the sphaeroplasts were able to take up the DNA and under appropriate conditions the cell walls can be regenerated to permit propagation and selection of transformants. Transformant selection will be discussed in further detail later in this section. Transformation frequencies using this method with autonomously replicating plasmid DNA vary from $10^5 - 10^7$ transformants per μg DNA.

The method is however a complex procedure and can only be applied to strains that are efficiently sphaeroplasted. Alternative methods were therefore developed which did not require the production of sphaeroplasts.

Ito et al, (1983), developed a lithium acetate method, by which S. cerevisiae cells exposed to lithium ions (or other alkali metals such as Cs⁺, Rb⁺, K⁺) were induced to uptake the DNA. PEG is also required to facilitate the uptake. Transformation efficiencies with this method are in the region of $10^5$ transformants per μg DNA, but again dependent upon the DNA and strain employed.

A more recent method developed was electroporation, by which an electric pulse stimulates DNA uptake (Hashimoto et al, 1985; Becker and Guarente 1991). Transformation efficiencies in the order of $10^6$ transformants per μg DNA were obtained when early phase exponential cells were treated with dithiothreitol (Meilhoc, et al. 1990).

### 1.1.3 Transformant selection

To enable the identification of cells which have successfully taken up the exogenous plasmid DNA, a genetic marker needs to be employed, which should be present on the plasmid DNA. The most commonly employed procedure for detection, is to use an auxotrophic host strain and incorporate the corresponding wild-type gene into the plasmid containing the exogenous DNA. The wild-type gene complements the defective gene and on selection for that gene, only those cells containing the functional gene can grow. The genes coding for enzymes in the amino-acid biosynthetic pathway, such as TRP1 (Tschumper and Carbon, 1980) and LEU2 (Beggs 1978), encoding 3-isopropylmalate dehydrogenase and N(5’-phosphoribosyl)
anthranilate isomerase, respectively are commonly used, since it is fairly easy to obtain haploid auxotrophs for leucine and tryptophan biosynthesis. This is not the case for industrial yeast strains which are usually polyploid in their genetic make-up or of unknown genetic make-up. As a result it is very difficult to obtain stable auxotrophs without causing alteration of their physiological and fermentative characteristics (Stewart, 1981). Transformation of such strains has necessitated the development of the dominant selectable marker for transformant identification. A large number of both homologous and heterologous dominant markers are currently available, with the \text{CUP1} gene being one of the most commonly employed homologous marker. In addition a number of antibiotic resistance genes have been employed, although some problems are still encountered in terms of the resistance of the host strain to the antibiotic. Those that have been employed with varying success include chloramphenicol acetyltransferase (Cohen \textit{et al}, 1980; Hadfield \textit{et al}, 1986, 1987), thymidine kinase (McNeil and Friesen, 1981), hygromycin B phosphotransferase (Gritz and Davies, 1983), dihydrofolate reductase (Miyajima \textit{et al}, 1984; Zhu \textit{et al}, 1985) and aminoglycoside phosphotransferase (Jimenez and Davies, 1980; Webster and Dickson, 1983; Hadfield \textit{et al}, 1990).

1.2 The expression of heterologous genes

In order to express a gene, the coding sequence of the gene must be transcribed and the resultant mRNA translated. Many heterologous genes are poorly expressed and secreted when their own control and signal sequences are used for their expression in yeast. This is a result of differences in gene expression and protein secretion between other organisms and \textit{S. cerevisiae}. Translation in bacteria, for example, is initiated by ribosomes binding to a ribosome binding site a few bases 5' of the translation start codon of the operon (Shine and Dalgarno, 1974). In yeast, the ribosomes apparently recognise the 5' of the mRNA and then migrate down it until they reach the first most 5' translation start codon to initiate translation (Sherman and Stewart, 1982). In addition, bacterial genes are organised into operons, in which one promoter may transcribe several genes, whilst yeast genes are individual units, each gene having its own promoter.

To ensure efficient transcription and ultimately expression in \textit{S. cerevisiae}, the foreign gene is normally placed downstream of a natural yeast promoter, producing a heterologous gene construction. There are two ways in which this can be performed, either as a transcriptional
fusion or as a translational fusion. The method chosen will be dependent upon the protein to be expressed and its function. In a translational fusion, the 5' end of the coding sequence of a yeast gene is fused to the coding sequence of the foreign gene. Both the transcription and translation initiation codon is provided by the yeast fragment, in addition to the untranslated leader in the mRNA preceding it. The fusion in the coding sequence normally results in the polypeptides containing some N-terminal amino-acids of the yeast gene and thus the resultant product is different to the native product at its N-terminus.

In transcriptional fusions, transcription initiation is provided by the yeast promoter in yeast, but the translation initiation codon is provided by the heterologous coding sequence. The fusion junction is normally in the untranslated leader, and thus the same polypeptide is produced as in the native host.

1.2.1 Sequence elements required for expression.

The most important elements for the accurate and efficient expression of a gene in *S. cerevisiae*, are a promoter and its regulatory sequences and transcription termination elements. In addition, any gene product destined for secretion will require signal sequences. Since the majority of this work did not involve secretion this is not discussed.

Any promoter isolated from a yeast gene can be employed for the expression of a foreign gene in yeast. Since the expression of genes in yeast is controlled by the promoter and thus is normally subject to some form of regulation, a heterologous gene will also be affected by the regulation. As a consequence, therefore, transcriptional activity may reflect the growth phase or cellular physiology. The choice of promoter will therefore be dependent on whether constitutive or inducible transcription is required.

A large number of promoter regions have been analysed and the elements essential for promoter activity which are important determinants of transcriptional and translational efficiency, have been identified. These are;

i) an upstream activating sequence (UAS),
ii) upstream TATA sequences and
iii) initiator elements (I), known as cis-acting elements.
In addition to these elements, trans-acting proteins, encoded by separate genes, are also required to activate expression by binding to a number of promoter regions.

A large number of proteins which have been successfully produced in yeast have been expressed using a number of well defined yeast promoters and secretion signals. The PGK1 promoter has been used widely to express a number of different proteins, such as human interferon (Tuite et al, 1982), immunoglobulins (Wood et al, 1985), wheat α-amylase (Rothstein, 1984) and HIV antigens (Adams et al, 1987). Other promoters which have been successfully employed include the GPD promoter (Bitter and Egan, 1984), the α-factor expression system for epidermal growth factor (Brake et al, 1984) and the triose phosphate isomerase promoter to express human insulin precursor (Thim et al, 1986).

1.2.1.1 Upstream activator sequence

UAS's are cis-acting elements found in the 5' regions of a number of yeast genes (Guarente, 1983, 1984). They are normally situated approximately 1400 and 100 nucleotides upstream of the TATA box (Guarente, 1987) and are required for efficient transcription of all S. cerevisiae genes. The activity of the UAS is dependent upon a number trans-acting proteins, which bind to sites within the UAS and allow gene regulation. These confer specific regulation which may be a response to physiological conditions such as oxygen (Zitomer et al, 1979), nutrients (Hope and Struhl, 1985) and heat-shock (Piper, et al 1988). UAS’s have been found to have variable nucleotide sequences dependent upon the promoter, although a number of common binding sites have been identified in the UAS of a number of promoters. For example a RAP1 binding-site has been determined in the PYK, PGK, ENO1 and ADH1 promoters (Chambers et al, 1989). A number of promoter UAS regions are found to contain two subsites, for example the CYC1 gene (Guarente et al, 1984), which are homologous but are distinctly regulated at the molecular level. In other instances the UAS regions are more complex and appear to interact with multiple regulatory factors. Examples include the UAS of the CYC7 and TRP1 (Mellor et al, 1991) gene which contain sub-sites which act synergistically to activate transcription. These sites may allow a careful modulation of UAS activity or coordinate induction responses to different physical signals, for example the sub-sites of the CYC7 UAS which mediates induction by haem and carbon source respectively.
Efficient and accurate initiation of transcription depends upon the presence of the TATA box, which has the consensus sequence of TATAAA. It is located at 40-120 bp upstream of the mRNA initiation site, depending on the promoter (Struhl, 1987) and strongly influences the level of transcription and the start site. The TATA element is a specific binding-site for the transcription factor TFIIID (Davison, et al, 1983) required to promote transcription complex formation (Buratowski et al, 1988). Some yeast promoters contain more than one TATA sequence, although it has been shown that different sequences of TATA element may have distinct functions. In the HIS3 gene one TATA element behaves like a constitutive element, whilst the second is necessary for transcription induction under conditions of amino-acid starvation (Struhl 1989). The CYC1 promoter region contains as many as 5 different TATA like sequences, but only two are functional. It is rare, in normal circumstances to have more than one functional TATA element (Lisherman, 1991), since if a gene has two TATA elements, only the 5' TATA box is functional which may be due to the closer proximity of the UAS (Li et al, 1991). Work by Singer et al (1990) with eukaryotic promoters indicated that a wide variety of sequences, many with no similarity to known TATA elements, can function efficiently as TATA sites in yeast. This is a consequence of the ability of the TATA binding protein (TFIID) to functionally interact with sequences other than the canonical TATA sequence, with sequences flanking the 6bp core of the TATA element influencing this interaction (Singer et al, 1990). In the absence of the TATA element, however, basal transcription may occur since the Initiator region directs the assembly of the activators with the general transcription factors, indicating that accurate use of the initiator can occur in the absence of TATA. At higher transcription, TATA may be required for the efficient assembly at the initiator. Without the TATA element, strong activators (e.g GCN4) may scan downstream recognising weak TATA elements, although disruption of a functional TATA element, results in a decrease in the expression level of a particular product or failure of initiation at the correct site.

Initiator sites

This sequence element is located very close to the mRNA start site and is responsible for determining the position of transcription initiation in yeast and can also signal the start of transcription when located 49, 77 or 106bp from the TATA sequence, but not at 30bp
A number of consensus sequences have been determined that can function as initiation sites, the predominant sequence being PyAAPu (Dobson et al., 1982), with initiation occurring at the first A.

In higher eukaryotes, the distance between the TATA site and the initiation site is fixed at about 30bp, but in yeast the spacing between the two sites is larger and more variable, ranging over 40 to 120bp downstream of the TATA element (Dobson et al., 1982; Hahn et al., 1985). Furter-Graves et al. (1991), analysed transcription initiation in six genes and found that sites were selected within windows spanning similar distance ranges from the TATA element. It was found that the SHI gene was involved in the establishment of the initiation window, but that the SH1 protein could have some role in the transcription initiation complex. It was speculated that the SH1 protein may be RNA Polymerase II or a transcription factor.

### Terminators

Precise transcription termination is essential for efficient and accurate gene expression (Zaret and Sherman, 1982) and a number of consensus sequences have been proposed. In eukaryotic cells, the mature mRNA is formed by posttranslational processing of a longer precursor transcript. The cleavage site is specified by a highly conserved sequence element AAUAAA, located 10-30bp upstream of the polyadenylation site. Although this site is not found in S. cerevisiae, however, it suggests that no single mechanism for 3' end formation exists. Observations that all mRNA in S. cerevisiae are polyadenylated indicated that transcription termination and polyadenylation may be directly coupled events (Iringer et al., 1991), by some component of RNA Polymerase II (Zaret and Sherman, 1982). This was indicated with the CYCl gene, since transcription termination was found to occur within a 38bp region, 8 nucleotides downstream from the CYCl poly (A) site (Russo and Sherman 1989). Two classes of different sequence polyadenylation site have been identified in yeast by Iringer et al. (1991), and their findings suggest that different mechanisms exist for mRNA 3' formation in S. cerevisiae and of different sequence elements directing this process. Unlike eukaryotes, transcription termination far downstream of the polyadenylation site is unlikely in S. cerevisiae.

All efficient polyadenylation sites contain the sequence element TTTTAT and in addition, have sequences with a high A+T content (about 80%) and A+T stretches of longer than
10bp at or near the mRNA 3'end, functioning in a strict orientation dependent manner. This could therefore pose a problem with foreign DNA sequences containing AT rich sites. This was illustrated by problems of fortuitous termination when expressing a *Clostridium tetani* toxin fragment C (Romanos et al., 1991), the DNA of which contained at least 6 AT rich sites. This was overcome by resynthesising the coding sequence to increase the GC content. Weaker polyadenylation sites are bidirectional and have the tripartite sequence TAG...TA(T)GT...TTT, which on deletion eliminates 3' end formation, although is unable to specify the 3' end alone.

1.3 Factors influencing expression of heterologous genes in yeast

In producing a heterologous construction to express a foreign gene, a number of factors are considered to influence expression. In most instances, the natural homologous gene is substituted by heterologous gene and in doing so a large sequence change can result. This in itself will have affects in translation, transcription as well as mRNA turnover and additionally in the loss of control regions and the interaction with regulatory elements (Chen et al., 1984). An example of the effect was shown by Mellor et al. (1985, 1987), when substitution of the *PGK1* coding sequence with interferon cDNA, resulted in a 6-fold reduction per gene copy in the amount of transcript. In determining the factors which might affect the yields of heterologous product all stages in gene expression must be considered. These factors could be the strength of the promoter directing expression, the stability of the final gene product, the efficiency of translation of the heterologous RNA and the stability of the heterologous transcript. In addition, the copy number and stability of the expression vector, will also have some effect. Each factor will be discussed in more detail below.

1.3.1 Promoter strength

The promoter signals from a large number of different *S. cerevisiae* genes have been employed to direct the expression of heterologous genes, shown in Table 2. The choice of promoter is dependent on whether constitutive or inducible expression is required. The most tightly regulated and inducible promoters are those of the galactose regulated genes, *GAL1*, *GAL10* and *GAL7*, whilst the glycolytic genes are the ones of choice if high level continuous
Table 2. Promoters commonly employed in expression vectors

<table>
<thead>
<tr>
<th>GENE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGK1</td>
<td>Dobson et al., 1982; Tuite et al., 1982; Wood et al, 1985; Rothstein, 1987; Adams et al, 1987</td>
</tr>
<tr>
<td>ADH1</td>
<td>Bennetzen and Hall, 1982b; Hitzeman et al., 1981</td>
</tr>
<tr>
<td>GAP3</td>
<td>Holland and Holland, 1980; Edens et al., 1984</td>
</tr>
<tr>
<td>ENO</td>
<td>Holland et al., 1981</td>
</tr>
<tr>
<td>PYK1</td>
<td>Burke et al., 1983</td>
</tr>
<tr>
<td>GPD</td>
<td>Bitter and Egan, 1984</td>
</tr>
<tr>
<td>Triose Phosphate Isomerase</td>
<td>Thim et al, 1986</td>
</tr>
<tr>
<td>MF α1 (α-factor)</td>
<td>Brake et al, 1984</td>
</tr>
<tr>
<td>CUP1</td>
<td>Etchevery, 1990</td>
</tr>
<tr>
<td>PHO5</td>
<td>Hinnen et al, 1989</td>
</tr>
<tr>
<td>PRB1</td>
<td>Sleep et al, 1991</td>
</tr>
</tbody>
</table>
expression is required. These promoters are not truly constitutive, since they are repressed to a certain extent in non-fermentable carbon sources. This can be overcome, however, by maintaining high glucose concentrations during growth. Elements important for promoter function have been discussed in a previous section (1.2).

The yields of heterologous proteins will be initially determined by the choice of promoter, since the strength of the promoter will determine, to some extent the amount of protein expressed. The fusion of a strong promoter to a gene will, however, only ensure frequent transcription of the gene and thus resultant protein levels will require the efficient translation of the resulting mRNA. As a result the efficiency of a promoter in expression cannot be predicted from the level of mRNA produced (Ernst, 1986). In general, the highest intracellular yields of heterologous proteins have been obtained by employing the promoters from genes that encode the glycolytic enzymes, since these each constitute 1-5% of the total cell protein and have correspondingly abundant mRNA. Conversely, the amino-acid biosynthetic genes are generally expressed at less than 0.1% of the total cell protein and their mRNA’s are rare. In some instances however, a strong promoter will result in the production of excess amounts of protein which could slow cell growth and the proteins will be degraded (Porro et al., 1991). This is also an important variable in optimal secretion of a protein encoded on a multi-copy plasmid, with optimal secretion obtained with a weaker promoter (Ernst, 1986).

1.3.2 Terminator choice

As mentioned previously, terminators are essential for accurate and efficient transcription. Zaret et al., (1984), observed that alterations in the end-point of yeast CYC1 mRNA, had major effects on CYC1 mRNA turnover and severely affected its translational efficiency. Mellor et al., (1982), determined that removal of the termination signal results in a decrease in mRNA yields, which is thought to result from the inherent instability of the mRNA owing to their length. This could result in overlapping transcription from an adjacent gene, causing neither gene to be fully transcribed. There is also evidence to suggest that the use of different terminator fragments in expression vectors may influence RNA levels, although there has been no detailed analysis of the precise termination sequence requirements for maximising heterologous gene expression. Terminators of prokaryotes and higher eukaryotes are not normally active in yeast, with the exception of the ADE8 terminator of Drosophila (Henikoff
1.3.3 Translation

The amount of protein expressed from a gene can be affected by a number of stages between its transcription and the translation of the message. Some of the factors which can affect expression at these stages are mRNA structure and stability, translation initiation efficiency and codon usage. The presence of intervening sequences (introns) within genes, can also effect expression, since mRNA containing non-yeast introns cannot be expressed in yeast. Relatively few *S. cerevisiae* genes have introns, although exceptions include the tRNA genes (Valenzuela et al, 1978), mitochondrial genes (Boss et al, 1978; Church et al, 1979) and ribosomal protein genes (Leer et al, 1985).

1.3.3.1 Translation initiation

Kozak, (1978), determined that the 40S ribosomal sub-unit binds to the 5' end of the mRNA, then migrates 3' until it reaches the initiation site corresponding to the first AUG. Sherman et al (1982), concluded that only the AUG codon was able to initiate translation, although conflicting evidence from Zitomer et al (1984), indicated that initiation could occur at the AUA and UUG codons, although at much lower frequencies than at AUG. It was additionally determined that the nearer the AUG to the 5' end of the mRNA, the efficiency of translation is increased, since the probability of initiation occurring at other sites is decreased. The context of the AUG was also deemed important in determining the efficiency of translation initiation and that the sequence of ACCAUGU is almost the optimal context for mammalian cells. A comparison of two contexts around the AUG codon of a yeast heterologous gene, by Hadfield et al (1990), indicated no influence of the context on the expression of the gene. These results were also in agreement to those of Baim and Sherman (1988), who found that base changes around the AUG initiator codon of the *CYC1* gene had only slight effects on translation efficiency.

Several additional structural features of the leader also affect the rate of translation initiation. Van den Heuvel (1990), found that the presence of an 18 nucleotide polyA tract in the leader
abolished translation of PGK mRNA, but leaders containing 40% of G residues interdispersed with either A or U, still allowed highly efficient translation.

1.3.3.2 mRNA structure

The translation and stability of a message could be affected by its primary and secondary structure. Baim and Sherman, (1988), demonstrated that the introduction of hairpin structures at the 5' end of CYC1 mRNA, in the vicinity of the AUG initiator codon inhibited translation of the message, which was proposed to be due to an inhibition of the scanning of the 40S initiation complex towards the initiation codon (Kozak, 1980). The degree of inhibition was found to be related to the stability of the hairpin and its position with respect to the AUG. When a hairpin structure was immediately downstream of the AUG, translation efficiency was reduced two-fold (Baim and Sherman, 1988). Additionally, a very stable structure, with a thermal stability of -58 kcal/mol, inhibited translation completely since it halts migration of the 40S ribosomal subunit, whereas a weaker one of -7.6 kcal/mol was only partially inhibitory. Bettany et al, (1989), found that a secondary structure of -18 kcal/mol was sufficient to inhibit translation of PYK1 message, indicating that a large amount of variability exists. It was proposed by Pelletier and Sonenberg, (1985), that a secondary structure close to the 5' end of a message, may affect the efficiency with which specific initiation factors bind to the mRNA (Roy et al, 1990). In addition, Kozak (1989b), determined that translation was not inhibited when a moderately stable hairpin of -30kcal/mol was present around the AUG (some distance from the CAP), but did cause inhibition when it is moved nearer to the 5' end.

Purvis et al (1987), found that translational efficiency of pyruvate kinase mRNA was drastically reduced by removal of about half of the mRNA trailer and by introduction of a stable hairpin immediately downstream of the translation stop codon. This effect is not observed in mammalian cells, but in yeast, the yeast 40S ribosomal subunits are less able to destabilise secondary structure and initiate translation than the mammalian 40S subunits.

1.3.3.3 mRNA stability

The half-life of yeast mRNA has a constitutive effect upon its translation and varies greatly from approximately 1 to greater than 100 minutes (Santiago et al, 1986). The fastest decaying
half-lives are in the region of 4-6 minutes, whilst the slowest are in the region of 40-60 minutes. The stability of most yeast mRNA is intermediate between both under steady-state conditions. It has been proposed, however, that stability is inversely related to its length, although this relationship is unclear since some relatively long mRNA are stable. An mRNA decapping enzyme has been purified from yeast and it was found that the longer mRNA's provided a better substrate for the enzyme than shorter mRNAs. The polyadenylation of mRNA in yeast may also affect its stability, since the presence of the poly(A) tail may provide some protection from exonuclease attack, which has been found in other eukaryotic systems (Bergmann and Brewerman, 1977; Huez et al., 1981). The 5'-cap, the length of the poly (A) tail (Santiago et al., 1987) and ribosome loading is not sufficient to determine the stability of an mRNA in yeast and it was postulated that destabilising elements may exist. Destabilising elements exist in eukaryotic systems, but as yet no well-defined examples in yeast exist. Brown et al., (1988) found that the fusion of URA3 sequences into the 3'-untranslated region of the relatively stable PYK1 mRNA caused this mRNA to be dramatically destabilised. This work implied the existence of a destabilising element within the URA3, but as yet the element has not been accurately mapped, nor have mechanisms by which the destabilising element operates. Herrick et al., (1990), noted that there was a direct correlation between the abundance of minor codons in transcripts and a reduced half-life.

1.3.3.4 Codon usage

A clear relationship has been determined between the level of expression of a number of genes and the degree of codon bias exhibited (Hoekema et al., 1987). The highly expressed genes were found to use almost exclusively, codons corresponding to the major tRNA species and completely avoid the use of minor tRNA's (Sharp et al., 1986). This tends to suggest that the tRNA may be the rate-limiting step in the translation process. In all cases the codon frequently used had a corresponding high tRNA level indicating that codon usage and tRNA availability were also adapted to each other. Kotula and Curtis (1991), found that when the immunoglobulin kappa chain gene from a mouse was optimised for expression in yeast, the presence of the preferred yeast codons resulted in a 50-fold increase in the amount of protein expressed, than in their absence. The same mRNA level was evident in both cases and thus this represented an increase in translation more than 50-fold.
A codon bias index system has been formulated which is a measure of the fraction of codon choices which is biased to a preferred set of 22 triplets (Bennetzen and Hall 1982). The degree of bias is allocated a value: an index of 1 suggests that for all the triplets in the mRNA only codons of a preferred variety are used whilst a value of 0 indicates totally random choice.

Codon usage varies between organisms, so that when a foreign gene is expressed in yeast, the codon usage has a different bias. This effect does not prevent expression, however.

1.3.4 **Protein stability**

In some instances, poor expression may not be a consequence of transcription or translation but could be due to the instability of the expressed heterologous protein in *S. cerevisiae*.

The rate of protein degradation is an important determinant of yield, which is balanced against the rate of protein synthesis. A protein with a long half-life has a greater chance of accumulation than a protein with a shorter half-life. The latter would only accumulate to a large extent if it was synthesised at a high rate. Regulatory type proteins tend to have short half-lives (less than 2.5h), whereas structural proteins are the converse (up to 160 hours) (Dice, 1987). Examples of highly stable proteins are Human Cu/Zn superoxide dismutase (Hallewell *et al.*, 1987) and Hepatitis B core Antigen (Kniskern *et al.*, 1986).

Proteolysis represents one of the greatest barriers to heterologous expression in any organism. The yeast vacuole contains several endoproteinas and exoproteases which are able to gain access to the heterologous gene products in the cytoplasm, when the cells are harvested and lysed. Proteins with a long half-life are normally degraded in the vacuole. In an attempt to overcome the proteolysis problem, many of the genes encoding the proteases have been cloned and null mutants constructed. Short half-life proteins, on the other hand are degraded in the cytosol by an ATP-dependent pathway involving ubiquitin. In this pathway, proteins are marked for rapid degradation by the covalent attachment to ubiquitin which then become the substrate for a cytosolic ATP-dependent proteasome. It is found that damaged or denatured proteins conjugate ubiquitin more effectively and are thus targeted for degradation. In some instances the proteases are present in the medium which can only be overcome by secretion into a medium in which they are lacking (Emr 1990).
1.3.5 Product toxicity

In most instances a cell in which foreign protein is expressed, is unaffected by its presence. In a number of cases, however, the protein being expressed is toxic to the cell and thus will adversely affect its growth or metabolism. The effect can vary in severity, with the protein merely reducing cell growth or cell viability, to the cases in which the expressed protein is lethal. Examples of the latter include the expression of Ricin, which is a toxin lectin from Soya bean and Diptheria toxin. Proteins having a lesser effect are insulin-like growth factor I (IGFI), which decreases growth rate reflected in the small colony size (Shuster et al., 1989) and human immune interferon, which results in the cells failing to grow to a high density and the plasmids unstable in selective medium (Fieschko et al., 1987). Both problems can be overcome by carefully controlling the expression of the foreign protein, to using inducible promoters and carefully monitoring growth.

1.3.6 Plasmid copy number

In addition to the factors outlined above, additional factors that can affect the mRNA abundance of cloned genes are vector copy number and stability (section 1.3.7).

In an attempt to increase the expression of a heterologous gene, high copy number vectors are often employed to increase the number of gene copies present in the cell. In doing so, the amount of product-encoding template will be increased and thus more product will be produced. Increasing the amount of product will however, have consequences on the metabolism of the yeast cell (discussed later). In addition, limiting factors, such as the relative abundance of required trans-acting factors may be a problem. If the copy number of a plasmid expressing protein which is inhibitory to the growth of the cell is increased, this may result ultimately in decreased stability and also copy number, the degree of which will be dependent upon the toxicity of the protein (Mason, 1991). Increasing copy number will not guarantee high yields since numerous other factors also play a role in expression.

1.3.7 Plasmid stability

The stability of the plasmid employed to express a foreign gene will also be important in determining the efficiency and level of expression of the heterologous proteins. To ensure
100% stability, the gene normally has to be integrated into the yeast genome, which limits the number of copies of the gene to one and thus limits the amount of product that will be expressed, unless multiple integration vectors are used (for example Lopes et al., 1989). Independently replicating vectors enable higher expression but are less stable, the stability being determined to some extent by the nature of the product being expressed. In addition, plasmid stability could be affected by physiological factors, since the presence of additional DNA might interfere directly or indirectly with the biosynthetic and secretory pathways and mechanisms that occur in the cell (Mason, 1991). Plasmid encoded functions are dependent on the host cell for factors for biosynthesis and thus the physiology of the cell will have an effect on plasmid stability and expression and conversely the expression of plasmid genes could result in competition or interference with various functions of the host cell.

1.3.8 Effects of plasmids on the host cell

The effect of the introduction of plasmids into the host \textit{S. cerevisiae} cell, is often overlooked when plasmids are employed to express heterologous proteins. Although the effects on the cell may not be immediately apparent, they could ultimately result in a number of changes to the metabolism of the cell which may result in the production of less heterologous protein (as described previously). This was illustrated by Birnbaum and Bailey (1991) using \textit{E. coli}. When a plasmid of an intermediate copy number (56 copies) was present in the cell, the level of several TCA cycle enzymes became elevated. If the copy number was increased to 240, an increase in the amount of heat-shock proteins was apparent, with a decrease in the growth rate and the ribosome content in the cell. Although an increased copy number favours greater cloned gene expression, the effects on metabolism of the cell are shown by decreased growth rates and less product formation. Although these effects were apparent in \textit{E. coli}, they may occur in \textit{S. cerevisiae}, but as yet no investigation has been performed.

1.4 Vectors employed for expression in \textit{S. cerevisiae}

The choice of vector used to express a foreign gene, also plays an important role in determining the level of expression of the gene, since copy number and stability of the vector alters gene dosage.
A large number of \textit{S. cerevisiae} vectors have been developed which contain a genetic marker and a number of unique restriction sites into which the foreign DNA fragment can be cloned. A large number of different types of vector are available, but many further developments in vectors normally reflect improvements in cloning technology.

Yeast vectors can broadly be divided into two main groups, those that are able to replicate and thus contain replication origins (independently replicating vectors) and those that cannot, which integrate into a chromosome and are inherited (integrating plasmids). The choice of vector employed, will ultimately be dependent on the requirement of the cloned foreign DNA.

1.4.1 Yeast Integrating Plasmids

Yeast integrating plasmids are circular DNA molecules which lack any replication sequences and are able to integrate into yeast genomic material by homologous recombination events. This was first demonstrated by Hinnen \textit{et al} (1978), when they employed a cloned yeast fragment containing the LEU2 gene to complement an auxotrophic (leu2) mutant to form a LEU2$^+$ transformant. The efficiency of transformation of these vectors occurs at much lower frequencies than with independently replicating vectors, (1-2 transformants/µg DNA) although this can be increased by linearisation of the vector within a region of homology with the host genomic DNA (Orr-Weaver \textit{et al}, 1981). This is a result of the highly recombinogenic nature of the ends of the DNA fragment which ultimately results in the incorporation of the entire vector within the genome and a duplication of the homologous sequences. Transformation with integrative vectors results in very stable transformants.

1.4.2 Independently Replicating Vectors

Beggs (1978), first demonstrated independently replicating multicopy vectors in yeast. These were found to contain a region of the 2µm plasmid (yeast endogenous plasmid) identified as being responsible for its replicative properties. Struhl (1979), discovered a different type of independently replicating vector which resulted in the identification of a yeast autonomously sequence or ARS. Both developments resulted in the design of a whole range of replicating plasmids.
The majority of replicating vectors which are designed are known as shuttle vectors, since they can replicate and be selected in both *E. coli* and *S. cerevisiae*. The incorporation of an *E. coli* replicon, most commonly derived from pBR322 sequences, provides an origin of replication and an antibiotic resistant gene, which enables the plasmids to replicate and be selected in *E. coli*. This permits all the recombinant DNA preparative procedures to be carried out using *E. coli* technology which offers speed and convenience of the ease of manipulation. In addition, most vectors contain unique restriction sites into which foreign genes can be cloned.

A number of different types of yeast replicating vector are available which yield quite different cellular copy levels and different stability values for the cloned gene. These include yeast replicating plasmids (YRp vectors), yeast episomal plasmids (YEp vectors), yeast centromeric plasmids (YCp vectors) and yeast linear plasmids (YLp vectors). A discussion of all the various vectors would not be feasible, but a brief overview of the different types can be made.

### 1.4.2.1 Yeast replicating plasmids

These plasmids contain an ARS element, which provides the plasmid with an origin of replication (Stinchcomb *et al*, 1979; Hsiao and Carbon 1979; Chan and Tye 1980), thus allowing them to replicate in the host cell. The sequences that confer autonomous replication are segments of chromosomal DNA, which are presumed to be origins of chromosomal replication. The plasmids transform yeast to relatively high frequencies (5000 - 20000 transformants/pg; Struhl *et al*, 1979), but are found to be highly unstable in the presence and absence of selection. In selectively grown cultures, typically only 5 - 25% of cells harbour the plasmid (Murray and Szostak 1983). This is thought to be a consequence of a strong maternal bias leading to unequal inheritance of the plasmids at cell division, which results in one daughter cell failing to receive plasmid in 30 to 60% of the cell divisions, which is dependent upon the plasmid (Murray and Szostak, 1983). This high rate of nondisjunction allows the copy number to increase in a minority of cells, and as a consequence the copy number of these plasmids can range from 20 -50 copies per plasmid-bearing cell (Hyman *et al*, 1982; Zakian and Kupfer, 1982). The plasmids can be stably inherited by integrating into the chromosome via homology with the auxotrophic marker. The plasmids have been
successfully used with non-*S. cerevisiae* yeast strains, e.g. *S. pombe* and *H. polymorpha*, where there are no naturally occurring endogenous plasmids to use as alternatives.

The following plasmids were adapted from those previously described and represent ways in which the plasmid stability can be increased.

1.4.2.2 Yeast centromeric plasmids

These plasmids are based on the YRP plasmids in that the replication origin is an **ARS**, but they contain an additional element that counteracts the mitotic instability of the **ARS** plasmids; a yeast centromere (**CEN**). The sequence of DNA required for a functional centromere resides within a segment of approximately 600 base pairs long (Bloom *et al.*, 1982). Plasmids carrying both an **ARS** and a **CEN** sequence are circular and are found to have a plasmid copy number of only one copy per cell, making them less desirable for use where high expression levels are required. The plasmids are more stably maintained during mitotic growth however, with about 90% of the cells containing plasmid in the absence of selection (Clarke and Carbon 1980). Stability can be further improved by increasing the size of the plasmid (Hieter, *et al.*, 1985). Short DNA segments from the centromeres of different chromosomes have been cloned in **ARS** plasmids and appear to function extrachromosomally.

The plasmids are useful alternatives to single-copy integrating vectors as the plasmid can be isolated. As a result they are particularly useful as vectors for genomic or cDNA libraries.

1.4.2.3 Yeast linear plasmids

Telomeres, which consist of tandem repeats of AC sequences and are essential to the replication and stability of chromosome ends, have been incorporated into the centromeric plasmids making them linear in form. Plasmids containing the telomeric sequences are usually present in reasonably high copy number, but have a poor mitotic and meiotic segregation. As a consequence they are lost at a fairly high frequency, with over 50% of cells from a selectively grown culture lacking the plasmid (Szostak and Blackburn, 1982). Addition of **CEN** sequences was found to improve stability leading to the development of the yeast artificial chromosome vector (YAC). These vectors are able to accommodate very large pieces
of cloned chromosomal DNA and thus could aid in the cloning of fragments from large genomes (Burke and Olsen, 1991). YAC's are constructed by initially propagating the vector as a circular plasmid in *E. coli* which contains a tandem double repeat. When this is then introduced into yeast it linearises to form two telomeric ends.

1.4.2.4 **Yeast episomal plasmids**

The most commonly employed plasmids for genetic transformation of yeast are those utilising the replication system of the naturally occurring yeast 2μm plasmid. This minimally comprises the origin of replication and the stability locus or *STB*. The problems of maternal bias found with YRp vectors are reduced by the *STB* region which facilitates stable partitioning of the vector at cell division.

Although 2μm based plasmids are more stable than the ARS based plasmids, they do not achieve the stability of the native 2μm, which is considered a consequence of the presence of exogenous yeast and bacterial sequences (Armstrong *et al.*, 1989). Thus the vectors are still lost at 1-2% per generation in the absence of selection, but are normally maintained at a fairly high copy number.

Throughout this work, expression of foreign genes which had been cloned into 2μm based plasmids were examined and as a consequence of the interesting observations that became apparent, greater description of the 2μm plasmid is warranted.

1.5 **The 2μm plasmid**

The 2μm plasmid is found in most laboratory strains of *S. cerevisiae* at approximately 30-100 copies/cell, as well as other Saccharomyces species (Clark-Walker and Miklos 1974; Gunge 1983) and is so-called since it has a monomeric contour length of approximately 2μm.

The wild-type 2μm plasmid is a covalently closed circular double-stranded DNA of 6318bp (Hartley and Donelson 1980), which contains an origin of replication and several genetic functions involved in plasmid propagation (Fig 1.5.1). The sequence of the entire plasmid has been determined (Hartley and Donaldson 1980) and reveals the presence of two identical 599 bp regions, which are precise inverted repeats of each other, separated by two single copy
domains of 2.3kb and 2.8kb. Intramolecular recombination readily occurs between the repeat sequences and generates two alternate forms of the plasmids (A and B) with different orientations of the two unique sequence regions. The two inversion forms of the plasmid co-exist in intracellular populations and thus 2μm plasmids isolated from yeast actually consist of a mixed population. This recombination event occurs by a specialised, site-specific mechanism which is catalysed by a plasmid-encoded function known as FLP (Broach and Hicks, 1980). A schematic diagram of the wild-type 2 μm plasmid is shown (Fig 1.5.1), in which the plasmid is represented as a dumbbell, aligning the homologous sequences of the inverted repeat.

### 1.5.1 Elements of the 2μm circle replication system

Four regions contain open translation reading frames of greater than 500bp. These were originally designated A, B, C and D and encoded predicted proteins of 423, 373, 296, and 181 amino-acids, respectively. They are now more commonly referred to as FLP, REP1, REP2 and ORFD (sometimes known as RAF) respectively. Each unique region contains a pair of ORF's the 5' ends of which lie within several hundred base pairs of each other near the centre of the region and which diverge on opposite DNA strands. The A,B and C coding regions extend into the inverted repeats.

The longest non-coding part of the unique regions, 1100bp, extends from the 3' end of the D ORF to the nearest inverted repeat. Within this area is found a series of inexact tandem direct repeats of a 62bp sequence. Five of these, centred on the stretch of DNA located between the unique Hpal site and the nearby Aval site of the 2μm circle are strikingly homologous. A sixth repeat lies distal to the Hpal site and part of the seventh lies distal to the Aval site, although neither is very homologous to the five core copies.

Recombination requires FLP and occurs within a site called FRT within each 599bp repeat. FRT consists of an 8bp core surrounded by a pair of 13 bp repeats. The repeats are 12 of 13 matches and are in inverted orientation. Recombination proceeds via a pair of staggered strand cuts at the ends of the core, which make an 8 bp 5’ overhang, with the recombining overhangs annealing.
Figure 1.5.1 Structural organisation of the $2\mu m$ circle.

A diagram of the genomic organisation of the yeast plasmid

Form A

Form B
The mitotic stability of the wild-type 2µm plasmid approaches the stability of the chromosome, with a plasmid loss of 0.01% per cell per generation (Futcher 1986; Mead et al., 1982) with several components contributing to the stable plasmid maintenance. The major factor underlying the stable persistence of the plasmid is its ability to distribute plasmid copies more or less evenly between the mother and bud cells at mitosis. This is in contrast to artificial plasmids whose partitioning is strongly biased towards the mother cell. In addition to promoting equipartitioning, the plasmid is able to increase its copy number even though the replication only once in each generation. This is proposed to occur via the double-rolling circle model (Futcher, 1986), which assumes induced FLP recombinase activity. Semiconservative DNA replication proceeds bidirectionally from the plasmid origin. FLP-mediated recombination occurs at the FRT site and the forks are reorientated so that they no longer converge. Continuing replication in this mode yields a multimeric replication intermediate and recombination restores the converging orientation of the replication forks. Completion of replication yields a 2µm monomer and multimer and further FLP-mediated or general recombination resolves the multimers to monomers. Plasmid copy number is therefore amplified.

1.5.2 2µm Plasmid partitioning

Three parts of the 2µm circle have been identified as a requirement for efficient partitioning; two of these are the gene products of ORFB and ORFC, the REP1 and REP2 genes, the other is a region which encompasses the 62bp tandem repeat array, REP3 or STB (Kikuchi, 1983; Jayaram et al., 1983). The STB element is cis-acting and can endow YRp plasmids with unbiased mitotic partitioning and high stability in the presence of REP1 and REP2 gene products (Cashmore et al., 1986). Sequences flanking STB affect its activity in addition to transcription through its sequences. The way in which the STB system promotes plasmid stability, is as yet unexplained although a number of mechanisms have been postulated. It is possible that REP1 and REP2 bind to the STB sequence (Cashmore et al., 1986). The REP3 gene is involved in the regulation of FLP expression by antagonising REP1 and REP2 repression of the FLP promoter (Murray et al., 1987).

1.5.3 Composition of 2-µm circle-based vectors

The majority of plasmids employed for genetic transformation and gene expression, contain only parts of the previously described 2µm plasmid, to minimise plasmid size and the number
of restriction sites. The minimum extent of these sequences which must be retained have been defined by deletion analysis (Broach and Hicks, 1980; Jayaram et al, 1983). The replication origin which is located at the junction of the large unique region and one of the inverted repeats which encompasses a FLP recombination target site and REP3, the site near the origin necessary for the action of the REP system, must be retained on the recombinant plasmid because they act only in cis. The regions required are normally incorporated as a 2.2kb EcoRI fragment or the 2.1kb HindIII fragment form the B form, both fragments also span the plasmid origin of replication (Knowlton 1986). The REP1 and REP2 genes which are essential for maintaining high copy number, can be omitted from the expression vector if it is propagated in a cir+ strain so that these functions are provided by the REP genes of the endogenous plasmid. The FLP gene described is not essential in cis or trans and neither are the IR sequences, except for that associated with ori. Other requirements of the vector, as previously discussed, are a selectable marker, which is required both to obtain initial transformants and also to provide continual selection to maintain the vector in the culture and bacterial sequences to permit selection and propagation in E. coli, which are most commonly sequences from the pBR322 plasmid, although sequences from pUC plasmids are also employed, which permit higher plasmid yields from bacteria (Armstrong et al, 1989).

The singular feature of the 2μm circle based vectors is their ability to engage the 2μm partitioning system, which renders them more stable during mitotic growth than vectors based solely on ARS elements. Although the 2μm based plasmids are more stable than the YRp plasmids, they do not attain the stability of the wild-type 2μm plasmid, which is lost in the region of 0.01% per generation (Futcher and Cox, 1984). Under selective growth conditions, plasmid bearing cells usually comprise between 60% and 95% of the population depending upon the particular plasmid used and the selectable marker. It has been determined that plasmids based on the pUC plasmid tend to be somewhat more stable than the equivalent plasmid based on pBR322, which tends to indicate that the smaller the vector, the better the stability. The nature of the sequences cloned, will affect stability and the copy number. The addition of a gene with a product deleterious to the host cell will yield diminished stability and in general, plasmids with increased copy levels exhibit increased stability (Panchal, 1987). Generally 2 μm based plasmids normally have a copy number of 20 - 50 copies per cell (Ehrhart and Hollenberg, 1983; Hadfield et al, 1987, 1990), but as outlined this is variable.
1.6 The use of reporter genes to study gene expression

Reporter genes have been established as valuable tools to investigate the control of eukaryotic gene expression, since the product of the genes can be measured and equated to the expression of the gene. Sequences of the gene of interest to be analysed for regulatory function, or the promoter of a gene, are combined with the structural sequence of another gene whose product can be easily and quantitatively assayed (the reporter gene). After transfer of such a construct into eukaryotic cells the effect of the particular sequence on the level of gene expression can then be measured indirectly via the expression of the reporter gene. Sensitivity of reporter assays is determined both by the availability of enzyme assays and the absence of background or interfering activity in the system studied.

Two types of genes have been employed as reporter genes in *S. cerevisiae*, endogenous genes such as PGK (Rathjen et al., 1987) and exogenous coding sequences such as *lacZ* (Guarente and Ptashne, 1981; Ruby et al., 1983), which are the more commonly employed reporter genes or certain bacterial resistance genes which is a cumbersome procedure (Hollenberg, 1979). Bacterial β-lactamase, has been employed as a reporter gene in yeast, but its use appears to be restricted to specific applications (Kellermann et al., 1986). One difficulty arises from the fact that only 10% of the pre-protein is processed in an active form in yeast (Roggentinkamp et al., 1981). This can be circumvented by removal of the signal sequence, but in this case a translational start has to be supplied through an appropriate fusion. A number of reporter genes are commonly employed, those encoding β-Galactosidase (Guarente and Ptashne, 1981), chloramphenicol acetyltransferase (Herrera-Estrella et al., 1983) and more recently in yeast (although has been employed widely in plants and bacteria) β-Glucuronidase (Schmitz et al., 1990) and aminoglycoside phosphotransferase (Hadfield et al., 1990).

1.6.1 β-Galactosidase

This is a widely employed reporter gene and was one of the first to be employed in *S. cerevisiae* (Guarente and Ptashne, 1981). Genes are fused to sequences of the *lacZ* operon derived from *E. coli* and thus the promoter under study can be monitored by changes in the level of the β-Galactosidase enzyme. This can be assayed on a plate by inclusion of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase or quantitatively determined by measurement of the hydrolysis of ONPG (o-nitrophenyl-β-D-galactoside) (Miller, 1972).
Breeden and Nasmyth, (1987), noted that the measurement procedure of plasmid-borne activities might show variability in certain types of experiments.

### 1.6.2 β-Glucuronidase

This enzyme is encoded by the gusA locus of *E. coli* and is a hydrolase that catalyses the cleavage of a wide variety of beta-glucuronides (Stoeber, 1961). It has been widely developed for use in bacteria, plants and animals (Jefferson et al, 1986 and 1987; Kavanagh et al, 1988). It is very stable in yeast (with only 10% loss of activity observed following overnight incubation at 37°C), which in addition do not have measurable GUS activity, confirmed by Schmitz et al, (1990). Glucuronidase activity in yeast cell extracts may be monitored by a very sensitive fluorometric method by use of the histochemical reagent (X-Gluc), 5-bromo-4-chloro-3-indolylglucuronide (Jefferson, 1987). Spectrophotometric assays were found to be less sensitive and prone to inherent mistakes (Jefferson, 1987). Hirt (1991), showed that the gene can be used quantitative and qualitative as a reporter gene, with the development of a colony screening method, using a replica-plating technique, since yeast cells exclude the substrate (in contradiction to work by Schmitz et al, 1990). It was determined that a 100-fold difference in promoter strength could be observed on increased incubation (Hirt, 1991) enhancing the use of the gene. Expression of a bacterial glucuronidase permease (Jefferson et al, in prep.), would open new possibilities for the application of the GUS reporter gene in yeast, since the action of the permease would enhance the uptake of the glucuronidase. Large N-terminal additions of other sequences of varying lengths do not substantially affect the activity of the enzyme, (Jefferson, 1987), which is similar to the situation with β-galactosidase. The GUS gene has, however, been predominantly employed in plants, since the plant cells are amenable to in situ histochemical staining of GUS expressed in cells, both in tissue culture and tissue sections.

### 1.6.3 Chloramphenicol acetyl transferase (CAT)

The Chloramphenicol resistance gene from the bacterial transposable element Tn9, encodes the bacterial enzyme chloramphenicol-3-o-acetyltransferase (CAT), which inactivates the antibiotic by acetylation derived from acetyl-CoA. Enzyme activity can be easily and accurately quantified at high sensitivity by a number of assays (Shaw 1975; Kleanthos and Shaw 1984) and as a result has been widely employed as a reporter gene in several eukaryotic
systems, including *S. cerevisiae*, in which the gene is functionally expressed from its endogenous promoter (Hollenberg, 1979; Cohen *et al.*, 1980). The gene has also been successfully employed as a dominant marker in yeast (Hadfield *et al.*, 1987) and it was shown that the extent of CAT activity in the cells correlates with gene activity at the transcriptional level. The amount of enzyme produced should therefore reflect the activity of the transcriptional control elements present in a particular plasmid construct (Weiher *et al.*, 1983).

1.6.4 Aminoglycoside phosphotransferase (APT)

This reporter gene has been successfully employed in yeast by Hadfield *et al.* (1990). It can be detected at very low concentrations by a very sensitive radiolabelled assay, the APT enzyme can be expressed up to at least 2% of the total cell protein without having a detrimental effect on the host. The APT enzyme also provides a selectable phenotype in the form of antibiotic resistance in yeast to Geneticin G418 and bacteria to Kanamycin. Since very little needs to be expressed to provide the G418 resistance phenotype, the cells are not placed under selective pressure.

The aminoglycoside antibiotics, such as kanamycin, gentamycin, and neomycin, inhibit ribosome-protein synthesis, by interfering with drug transport. It is possible that aminoglycoside antibiotics are transported by sugar or aminosugar transport systems (Davies and Smith, 1978). The most common form of resistance to the aminoglycoside antibiotics depends on the presence of plasmid-coded modifying enzymes. The aminoglycoside antibiotics are classified according to their structure, and are divided into two classes, those containing streptidine or deoxystreptamine. The kanamycin, neomycin and gentamycin fit into the latter class. These are further subdivided into two groups dependent upon the nature of substitution on deoxystreptamine. Neomycin has substituents attached to the deoxystreptamine at the 4- and 5-hydroxyls, whereas kanamycin and gentamycin have substituents attached to the 4- and 6-hydroxyls. Aminoglycoside modifying enzymes are classified according to the mechanism of modification which can be N-acetylation, O-phosphorylation or O-nucleotidylation and their site of modification on the aminoglycoside. Aminoglycoside phosphotransferase (APT), phosphorylates the antibiotics containing the 2-deoxystreptamine moiety at the 3' hydroxyl position on amino-hexose I. The mechanism of resistance mediated by APT does not involve complete detoxification, since the culture medium contains unaltered drug. It is probable that
the enzymes are associated with the inner membrane of the cell where they would be accessible to substrates such as acetyl-CoA and ATP.

It has been established that transport of the aminoglycosides is active and requires the normal operation of bacterial membrane-energy function. The resistance mechanism for aminoglycoside antibiotics presumably involves interference with this transport. Since the modifying enzyme alone is necessary and sufficient to establish resistance, it can be assumed that modification of the antibiotic by O-phosphorylation, either interferes with or blocks transport directly by interacting with the transport mechanism/carrier. Alternatively, it was suggested that the rate of modification equals the slow rate of transport of the drug, thus preventing its entry into the cell.

1.7 Aims and Objectives of the thesis.

As previously illustrated, a number of factors have been identified which are known to influence gene expression in yeast, although continuing difficulties in the expression of heterologous genes indicate that not all factors have been identified. In some part, difficulties may arise from the diverse nature of the heterologous genes and the gene products themselves, although other factors must play a role.

The aim of this work was therefore to investigate factors that influence heterologous gene expression, using a model heterologous gene system. The heterologous gene system considered for this purpose was the *APT* gene, expressing the aminoglycoside phosphotransferase enzyme. This gene was considered since it had scope for use in *E. coli* and *S. cerevisiae*, which would facilitate construction work and in addition activity could be assayed. Although the *APT* gene had previously been employed as a dominant marker in yeast, expression was poor and thus to facilitate its use as a model system, improved expression would be required. The existing assay for APT was not optimal and thus the assay employed to detect APT activity could be improved to provide an easier and more reliable measurement procedure. Subsequent improvement of expression and the development of a more sensitive, quicker and reliable assay, indicated that in addition to the use as a reporter gene, the gene had scope as a dominant marker. The marker provided an antibiotic-resistance phenotype, which was expressed in *S. cerevisiae* and *E. coli*. Yeast transformants could be selected, directly or indirectly, dependent on the amount of expression whilst *E. coli*
transformants could be selected for one marker then selected for the APT gene, facilitating construction work and providing a distinct advantage over other markers. A number of factors also made the APT gene a useful reporter gene, an important factor being that the expression of the APT protein had no adverse effect on the cell (up to 2% of total cell protein). The development of the assay procedure enabled the determination of levels of APT protein in the region of 0.001% of total cell protein possible. Additionally a way of converting enzymatic activity units into percent amount of protein, provided a more useful measure of gene expression and enabled direct comparison between constructions. The apt gene was therefore a very useful reporter gene to measure gene expression.

The initial factor questioned, was whether the level of heterologous expression using different promoters reflected the relative level of expression of the natural "parental" gene. This study could only be performed by using the same coding region in each instance and thus the coding regions of the genes of the promoters investigated, namely the ADCl, PGK or CYC1 genes were substituted with the APT coding region to provide the heterologous constructions. The investigations revealed a number of surprising effects, one of which was that the addition of a strong promoter to a heterologous gene cartridge plasmid caused a phenomenon, which we termed replication inhibition, whereby the copy number and transformability of the plasmids containing the heterologous gene cartridge is reduced, but high stability is maintained. This discovery, required further investigation, in an attempt to determine why the effect was observed and importantly if the effect could be overcome. As a consequence, a large majority of the work was devoted to investigating the effect, although in all instances factors influencing the expression levels were considered. Differing constructions were examined to determine if the construction of the heterologous gene cartridge played a role and whether the effect was a consequence of transcriptional interference of the replication origin. This revealed that the effect was not a consequence of construction nor transcription. Analysis of a differing replication origin provided no further explanation for the effects observed. To determine if the effect was a consequence of promoter binding proteins, a number of deletions were considered, but no alleviation of the effect was observed. The investigations therefore revealed that not one factor alone was responsible for the observed effects. The last part of the study was devoted to determine if other genes were affected by the replication inhibition phenomenon and thus plasmids containing homologous gene cartridges were examined in addition to plasmids containing a high codon biased gene. Studies with the homologous gene revealed some similarities to the effect observed with the heterologous gene, but not to the
same order of magnitude. On consideration of the high codon biased gene, which was chosen as the ADH gene, a trans-effect on chromosomal ADH was revealed indicating an additional effect of heterologous gene expression which is not normally observed.
CHAPTER TWO

MATERIALS AND METHODS.
2.1 Bacterial and yeast strains

The strains of bacteria and yeast employed during this work are outlined.

2.1.1 E.coli

NM522 : Δ(lac-proAB), hsdA5(r-, m-, thi, supE, f'proAB, lacF7AΔM15
JM83 : r-, m-, supE, ara, Δ(lac-proAB) strA, thi, 88MlacZ, M15, recA-

2.1.1.2 S.cerevisiae

S150-2B  MATα, leu2-3, 12, his3-1, trp1-284, ura3-52, 2 μm-
DBY746  MATα, leu2-3, 12, his3-1, trp1-289, ura3-52
MDY40-4C MATα, leu2-3, 12, his3-1, trp1-289, ura2

Bacterial and yeast strains were stored at 4°C on M9 agar and YPD agar plates respectively and were restreaked at regular intervals onto fresh agar plates. Frozen strains were additionally stored as glycerol stocks at -70°C, which were obtained by mixing an aliquot of cells from a fresh overnight culture with an equal volume of 60% glycerol, in a small sterile glass universal bottle. This was mixed and then "flash" frozen at -70°C in a dry ice/IMS bath, after which the cells were stored.

2.2 Bacterial Growth Media

2.2.1 Luria broth (LB)

10g/l Bacto-tryptone, 5g/l Bacto-yeast extract, 5g/l sodium chloride, dissolved in Q water, which is distilled water that has been passed through a Millipore standard MilliQ system. The pH of the broth was adjusted to 7.2 with sodium hydroxide and the media autoclaved at 15psi/15 minutes.

Luria agar was made by the addition of 2% w/v Bacto agar to LB broth and the media was autoclaved at 15psi for 15 minutes. Supplements were added if selection for plasmids was required.
2.2.2  **Ampicillin and Kanamycin medium**

For the selection of Ampicillin or Kanamycin resistance, ampicillin or Kanamycin sulphate was added to sterile LB broth from stock solutions in Q to a concentration of 50μg/ml. For selection on agar plates the same concentration of antibiotic was added to sterile agar cooled to 55°C.

2.2.3  **Amp / XGal / IPTG agar medium**

This medium was employed as agar plates and provided a means of detecting bacterial transformants containing recombinant plasmids with a lacZ insert. Transformants lacking an insert yield blue colonies on this media, whilst those containing an insert are white. The plates were made by the addition, to a final concentration, of 50μg/ml Ampicillin, 40μg/ml XGal and 40μg/ml IPTG to molten Lagar cooled to 55°C. All solutions were added from stock solutions stored at -20°C.

2.3  **Yeast Growth Media**

2.3.1  **Yeast peptone media and agar**

10g/l Bactoyeast extract, 20g/l Bactopeptone, dissolved in Q water. The media was autoclaved at 15psi/15 minutes, without a Carbon source, after which sterile supplements could be added. Yeast peptone agar was made by the addition of 1.5g/l bactoagar to yeast peptone broth. The media was autoclaved at 15psi/15 minutes without a Carbon source, after which sterile supplements could be added.

2.3.2  **YPD media**

2% v/v sterile 40% glucose solution was added directly to the YP broth or to molten YP agar cooled to 55°C.
2.3.3 **YPD G418 media**

Geneticin G418 sulphate was added as a powder directly to the broth, to a final concentration dependent on the yeast strain employed. With *Saccharomyces cerevisiae* S150-2B, the concentration employed was 0.5 g/ml.

2.3.4 **Minimal (Semi-defined) Media and agar**

This medium comprised, 6.7g/l of Yeast Nitrogen base (without amino-acids) dissolved in Q water. Minimal agar was made by the addition of 2% w/v bactoagar. Both media were autoclaved at 10 psi/10 minutes after which the Carbon source (normally Glucose), was added to a concentration of 2% and the amino-acids required for selection.

2.4 **Media supplements**

2.4.1 **Carbon sources**

Glucose and Glycerol stocks were made to 40% w/v in Q, then autoclaved at 10 psi/10m and stored at room temperature. Ethanol, when employed, was added as Absolute Ethanol, which was sterilised by passing through a 20μm acrodisc filter prior to addition.

2.4.2 **Amino-acids**

All solutions were made in Q, then autoclaved at 10 psi/10 minutes, or alternatively passed through a 0.2 μm acrodisc filter.

<table>
<thead>
<tr>
<th>Amino-acid</th>
<th>Concentration</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>4mg/ml</td>
<td>4°C *</td>
</tr>
<tr>
<td>Uracil</td>
<td>2mg/ml</td>
<td>room temperature</td>
</tr>
<tr>
<td>Histidine</td>
<td>4mg/ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Leucine</td>
<td>4mg/ml</td>
<td>4°C</td>
</tr>
</tbody>
</table>

* stored in a light proof container
2.4.3 Antibiotics

**Amoxicillin and Kanamycin**: Stock solutions were prepared using the sodium salt and sulphate salt respectively, in Q to a concentration of 25mg/ml. Both were stored at -20°C and employed to a final concentration of 50 µg/ml.

**G418**: This antibiotic was always added directly to the media as a powder and employed at a concentration of 500 µg/ml. The powder was stored at room temperature.

**XGal**: This solution was prepared as a 20mg/ml stock in N,N Dimethylformamide and stored at -20°C.

**IPTG**: This solution was prepared as a 25 mg/ml stock in Q and stored at -20°C.

2.5 Standard Buffers

**TE** 10mM Tris-HCl, 1mM EDTA to pH 7.6 (unless stated) with conc HCl

10 x TAE 4.7g/l EDTA, 48.4g/l Tris base

10 x TBE 55g/l Boric acid, 9.3g/l EDTA, 108g/l Tris base.

20 x SSC 175.3g/l Sodium chloride, 88.2g/l Sodium citrate.

10 x MOPS 48g/l (3-[N-Morpholino]propane sulfonic acid), 6.8g/l Sodium acetate, 1.9g/l EDTA to pH 7.0 with NaOH.

2.5.1 Phosphate buffers

\[ x \text{ ml } 1M \text{ Na}_2\text{HPO}_4 + y \text{ ml } 1M \text{ NaH}_2\text{PO}_4 \]

<table>
<thead>
<tr>
<th>pH</th>
<th>x</th>
<th>y</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>7.4</td>
<td>81</td>
<td>19</td>
</tr>
<tr>
<td>7.6</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td>7.8</td>
<td>91.5</td>
<td>8.5</td>
</tr>
<tr>
<td>8.0</td>
<td>94.7</td>
<td>5.3</td>
</tr>
</tbody>
</table>
2.5.2 Phenol (Maniatis et al, 1982)

Frozen stocks which had been equilibrated and contained hydroxyquinoline were obtained from Fisons. Prior to use the stocks were defrosted slowly in a 55°C water-bath and thereafter stored at 4°C.

2.5.3 Chloroform/Isopropylalcohol

This was made by mixing 24 volumes of Chloroform to 1 volume of Isopropylalcohol.

2.6 MANIPULATION OF DNA

The methods employed for the manipulation of DNA for the construction of recombinant plasmids in *E. coli*, were essentially as those described in Maniatis et al (1982).

2.6.1 Plasmids employed

A number of plasmids were employed in order to introduce a number of polylinker sites when cloning DNA fragments and for generating DNA fragments for probe use.

- pIC19H Marsh *et al*, 1984
- pIC19R Marsh *et al*, 1984
- pMTL22 Chambers *et al*, 1988
- pUC19R Norrander *et al*, 1983
- pUC18R Norrander *et al*, 1983
- pAC21 Carter: unpublished
- pYRP7 Struhl *et al*, 1979, Stinchcomb et al, 1979

2.6.2 Ethanol precipitation of DNA

DNA was precipitated by the addition of 1/10 its volume of 3M sodium acetate pH 5.5 and 2 volumes of cold absolute ethanol. The sample was mixed, then placed into a dry ice/IMS
bath for 5 minutes. The DNA was pelleted by spinning in a microfuge at 13k for 15 minutes and the supernatant discarded. The DNA pellet was then washed with 0.5 ml of cold 70% ethanol and repelleted at 13k for 1 min. The resultant pellet was dried under vacuum and then resuspended in an appropriate volume of sterile TE buffer.

2.6.3 Determination of DNA concentration

DNA concentration was determined between an absorbance of 260-320nm by use of a Schimadzu spectrophotometer. The spectrophotometer was initially blanked using quartz cuvettes containing only TE. The sample in which the DNA concentration was to be determined was diluted either 10 or 100-fold and an aliquot mixed with TE to give a total volume of 1ml. This sample was added to a quartz glass cuvette and its absorbance scanned, using a quartz cuvette containing only TE as a reference. The concentration of the DNA was determined by using the relationship:

\[ \text{Absorbance} = 1 \text{ at } A_{250}\text{nm} \],

which corresponds to a DNA concentration of 60 \( \mu \)g/ml.

2.6.4 Phenol/chloroform extraction of DNA

An equal volume of phenol/chloroform (in a ratio of 1:1), was added to the DNA to be extracted. The mixture was carefully inverted several times to mix the solution, then the phases separated by spinning at 8k for 5 minutes. The upper aqueous phase was then transferred to a clean tube and the DNA precipitated as described in 2.2.2.

2.6.5 Endonuclease restriction of DNA fragments

DNA of the required amount (normally 10-100 \( \mu \)l), was restricted with the appropriate restriction endonuclease employing the buffer recommended by the supplier. The enzyme was normally added to 10x the digestion excess (1 Unit) and the buffer at 1/10 the total restriction volume. All endonuclease restrictions were performed for a period up to 60 minutes, at 37°C unless Smal or BglI were employed, in which case the restriction temperature was 30°C or 55°C respectively.
2.6.6 Buffering conditions of some commonly employed enzymes

All buffers outlined were supplied from BRL and those specified were the buffers in which the enzyme in question exhibits 100% unit activity.

<table>
<thead>
<tr>
<th>REact™ Buffer</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XbaI</td>
<td>XhoI</td>
<td>SalI</td>
<td>SmaI</td>
</tr>
<tr>
<td>XhoI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HindIII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bell</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6.6.1 REact™ Buffer composition

All were supplied as 10x concentrates.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>REact2</td>
<td>0.5M Tris-HCl (pH 8), 0.1M MgCl₂, 0.5M NaCl</td>
</tr>
<tr>
<td>REact3</td>
<td>0.5M Tris-HCl (pH 8), 0.1M MgCl₂, 1M NaCl</td>
</tr>
<tr>
<td>REact4</td>
<td>0.2M Tris-HCl (pH 7.4), 0.05M MgCl₂, 0.5M KCl</td>
</tr>
</tbody>
</table>

2.6.7 The separation of DNA restriction fragments

Horizontal agarose gels were employed to separate DNA fragments, essentially as described by Maniatis et al (1982).

The concentration of the agarose in the gel was dependent upon the fragment sizes generated from the restriction, but in most cases 0.8% - 1.5% w/v agarose was sufficient. HSB agarose was usually employed, made up in 1xTAE containing 0.5 µg/ml EtBr.

Prior to electrophoresis, 5 x loading buffer was added to the DNA sample, which was then loaded into the wells of the prepared gel, submerged in a tank containing 1xTAE running buffer which also contained 0.5 µg/ml EtBr. Electrophoresis was then performed at 20V if it was to be run overnight or up to 80V for several hours, until adequate separation had been obtained. This was noted by visualising the gel under long-wave U.V. When good separation
had been achieved the gel was photographed with long-wave U.V. using a Polaroid MP4 camera and type 55 film.

5 x loading buffer 10% PEG 6000,
0.05% Xylene cyanol,
0.05% Bromophenol Blue,
0.1% Orange G

2.6.8 The isolation of DNA fragments for probe use

The method employed, if restricted DNA fragments were required for probe use, was essentially as described by Dalgleish (1987). Low-melting point agarose without EtBr was employed in this instance and electrophoresis performed as previously described (section 2.2.6). The gel was then visualised under long-wave U.V and the required band fragment excised and placed into a pre-weighed eppendorf tube.

2.6.8.1 Oligo-labelling of DNA fragments by random hexamer priming, for probe use

Random hexamer priming was essentially as described by Feinberg and Vogelstein (1983). Sterile Q water was added at a ratio of 1.5 ml/g agarose, to the isolated DNA fragment contained in a pre-weighed eppendorf tube. The tube was then placed in a boiling water bath for 7 minutes to melt the agarose and denature the DNA, after which it was kept at 37°C for 10-60 minutes prior to initiating the labelling reaction. The DNA fragment was subsequently stored at -20°C and prior to use was reboiled for 3 minutes. The labelling reaction was carried out by the addition of the following reagents in the given order.

x µl Q water (to a total volume 25 µl)
5 µl OLB buffer
1 µl 10mg/ml BSA (Enzyme grade)
y µl DNA fragment (25ng)
5 µl [α-32P] dCTP (10 µCi/ml)
0.5 µl Klenow large fragment (PolII) (2 units)
This was then incubated at room temperature for at least 5h or O/N. The reaction was then stopped by the addition of 100 µl of stop buffer.

**Solution O** : 1.25M Tris-HCl (pH 8.0), 0.125M MgCl₂
**dNTP’s** : 100mM solutions in Q water
**Solution A** : 1ml solution O, 18µl β-mercaptoethanol, 5µl each dNTP, except dCTP
**Solution B** : 2M HEPES-NaOH (pH 6.6)
**Solution C** : Hexanucleotides (PL #2166) at 90 A₂₆₀/ml in TE buffer.
**OLB buffer** : A mixture of A:B:C in the ratios 10:25:15
**Stop buffer** : 20mM Tris-HCl (pH 7.5), 20mM NaCl, 2mM EDTA, 0.25% SDS, 1µM dCTP.

Labelled dATP could be used in which case solution A replaced dATP with dCTP and stop buffer contained dATP and not dCTP.

### 2.6.8.2 Removal of unincorporated nucleotides

Unincorporated nucleotides were removed by fractionation using a Sephadex G50 column. The end of a glass Pasteur pippete was removed using a diamond etch pencil and plugged with a small bung of sterile polyallomer wool. It was then carefully packed with medium grade sephadex G50 in TE, to within 5-10mm of the top of the pippete. The labelled DNA fragment was loaded onto the top of the column, followed by 200 µl aliquots of TE, which were subsequently collected in eppendorf tubes. Fractions containing the incorporated nucleotides, normally located in the fourth to seventh fraction were identified as the first hottest peak, when the tubes were cerenkow counted in a scintillation counter. A second peak was also obtained, which represented the unincorporated nucleotides, which were subsequently discarded. The hottest fractions were pooled and boiled for 7 minutes in a 100°C PEG bath, prior to addition to a prehybridised filter.
2.6.9 The Isolation of DNA restriction fragments for further modification and isolation

DNA restriction fragments required for further modification were isolated by the use of polyacrylamide gels as described in Maniatis et al (1982). The percentage gel employed was determined by the size of the restriction fragment required. In most instances a 3.5% gel was sufficient but the voltage and length of time for which the gel was run was altered.

The gel apparatus consisting of two glass plates with 2 mm spacers between them was assembled prior to making the gel solution. The glass plates were carefully cleaned using IMS and Acetone, then assembled with the spacers, using adhesive tape. Bulldog clips were then fastened onto the taped areas and the gel could now be made. A 3.5% polyacrylamide gel was made by mixing the following in a Buchner flask:

- 11.6 ml stock acrylamide,
- 2.1 ml 10% ammonium persulphate,
- 10 ml 10x TBE
- 76.3 ml Q water.

The solution was then degassed using a main vacuum line, 30 μl TEMED added and the solution carefully poured between the assembled plates. A spacer containing the required number of wells was then carefully placed at the top of the gel apparatus between the plates. The gel was then allowed to polymerise. Once the gel had polymerised (takes approximately 30 - 60 minutes) the plates were connected using the bulldog clips to a vertical gel tank. 1×TBE was then poured into the top and bottom reservoirs and air bubbles eliminated by flushing the regions with buffer in a syringe.

The DNA sample to be separated was mixed with 5 x loading buffer and loaded into the wells of the gel using a Gilson, alongside a size marker. The gel was then run at a constant current with a voltage determined by the size of fragment required. As a rough guide,

<table>
<thead>
<tr>
<th>Fragment size</th>
<th>Voltage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 - 8 kb</td>
<td>100V</td>
<td>24h</td>
</tr>
<tr>
<td>2 - 1.4 kb</td>
<td>50V</td>
<td>24h</td>
</tr>
<tr>
<td>0.6 kb</td>
<td>50V</td>
<td>16h</td>
</tr>
</tbody>
</table>
On completion of electrophoresis, the gel was disassembled and stained in 1xTBE containing 0.5µg/ml ethidium bromide for 15 minutes. The stained gel was then viewed under short-wave U.V to visualise the DNA and the required fragment excised using a scalpel. The gel was normally photographed under long-wave U.V using a Polaroid MP4 camera once the fragment was excised, to ensure the correct fragment had been isolated.

**Acrylamide solution:**
- 30% acrylamide
- 0.8% bisacrylamide

made up in Q water, which was stored at 4°C in a light proof container.

2.6.10  **The elution of DNA from polyacrylamide gel fragments**

The excised gel fragment isolated in 2.6.9, was squashed into smaller fragments using a scalpel and returned to an eppendorf tube. 400 µl of DNA elution buffer was then added and the tube incubated at 37°C for 2 hours. The tube was then spun in an eppendorf microfuge at 13k 5 minutes and the eluent carefully removed into a fresh tube stored at 4°C. Further elution buffer was then added and the process repeated several times.

Before the DNA sample could be employed for further processes it was important that any acrylamide which was present in the sample was removed as this could interfere with any subsequent reactions. The base of a 0.5 ml eppendorf tube was pierced with a hot syringe needle and a small amount of polyallomer wool placed over the hole. The tube was then suspended in the mouth of a 15 ml plastic centrifuge tube and the collected eluent carefully added into the eppendorf tube. The centrifuge tube was then spun for 10s at 1k in a Hareus centrifuge, which was sufficient to separate the DNA from the acrylamide. The DNA was then precipitated as in 2.2.2 and resuspended in 400 µl T.E. The DNA was then transferred into an eppendorf tube and stored at - 20°C until required.

**DNA Elution buffer:**
- 0.5 M Ammonium Acetate,
- 1mM EDTA pH 8.0
2.6.11 Ligation of DNA fragments

DNA fragments for ligation to another fragment or into a vector were prepared as in 2.6.9.

2.6.11.1 To another fragment

The relative molar concentration of the ends of the fragment were initially calculated, as outlined in Maniatis (1972) so that the ligation would favour the formation of intermolecular dimers. Ligations were preferentially performed in a total volume of 10μl and if the volume required for the ligation exceeded this, the two fragments were co-precipitated. The resultant DNA pellet was then resuspended in 8μl 5mM Tris-HCl pH 7.4 and 1μl 10x ligation buffer and 1μl T4 Ligase (1-2 Units) added. The ligation was then incubated at 12°C overnight. If the ligation was between ends that were cohesive, for example those generated from ECoRI restriction, the ligation was performed for 5 hours. Half the ligation mix was then employed for transformation into E. coli. The remaining volume was stored at 4°C.

Ligation buffer:
- 0.5M Tris HCl,
- 0.1M MgCl₂,
- 0.1M dithiotheritol,
- 10mM spermidine,
- 10 mM ATP,
- 50 μg/ml BSA

2.6.11.2 Into a vector

The vector into which the fragment was to be ligated, was normally restricted with the required enzymes then phenol/chloroform extracted to inactivate the enzyme(s). The DNA was subsequently precipitated as previously described, and resuspended in a small volume of TE. The ligation was then set up as in 2.6.11.1.
2.7 E. COLI METHODS

2.7.1 Transformation of E. Coli

2.7.1.1 Preparation of competent E. Coli cells.

The strain to be transformed was initially inoculated into 20ml Lbroth in a universal container and grown up overnight at 37°C. 1ml of this O/N culture was then employed to inoculate 50ml of L.Broth in a 250ml shake flask. This was subsequently incubated at 37°C with vigorous shaking until the absorbance at 650nm against an L.Broth blank was 0.45 - 0.5 absorbance units, corresponding to approximately 2x10^8 cells/ml.

20ml aliquots were then decanted into universals and pelleted in a Hareus Christ centrifuge at 5k for 5 minutes at 0°C. The supernatant was then poured off and the cells were gently resuspended in 10ml of ice-cold 0.1M MgCl₂. The cells were repelleted as before and resuspended in 10ml of ice-cold 0.1M CaCl₂. Following the final pelleting, the cells were resuspended in 1ml of 0.1M CaCl₂ and the cells kept on ice for a minimum of 60 minutes. Cells could now be used for transformation and could also be frozen.

The competent cells were frozen by the addition of 1 volume of 50% w/v glycerol containing 0.1M CaCl₂ to 3 volumes of competent cells. The mixture was then aliquoted into eppendorf tubes and immediately frozen using a dry ice/IMS bath, then stored at -80°C until required for a maximum of 3 months. Prior to use the frozen competent cells were slowly thawed on ice.

2.7.1.2 Transformation of competent E. coli cells.

1μg of vector DNA or 5 μl of ligation mix to be transformed was added to 200 μl of competent cells in an eppendorf tube. Two controls were also set up at the same time, one a no DNA control to check competent cell purity and another pUC19 DNA which should transform cells very well. The tubes were gently mixed and left on ice for 30 minutes after which the cells were heatshocked at 42°C for 2.5 minutes. The tubes were then returned to ice for a further 30 minutes. 1ml LB broth was then added to the mixture and incubated at 37°C for 20 minutes. Cells were then pelleted at 5k for 5 minutes in a benchtop microfuge.
and the supernatant removed. The cells were resuspended in 200μl of LBroth and were then plated onto the required selective plates and incubated overnight at 37°C. In all E.coli transformations,

2.7.2 Analysis of E.coli transformants.

E.coli transformants could be screened by one of two methods. If a large number of transformants were to be screened, a colony hybridisation could be performed to eliminate transformants not containing the fragment of interest. The positives identified in this way could then be analysed by the second method of small scale plasmid DNA preparation, which was employed when a smaller number of transformants were to be screened.

Colonies to be analysed by colony hybridisation were either replica-plated or picked, dependent upon the number of colonies to be investigated and the separation of the colonies on a plate.

2.7.2.1 Replica-plating E.coli transformant colonies (Lederberg and Lederberg 1952)

Well separated colonies on plates were replica-plated onto fresh plates or filters in the following manner. A platform slightly smaller than the petri-dish was employed which was marked for orientation and a piece of Whatman 3 MM filter paper placed onto it. The plates were also marked and after lining up the marks on the platform, were placed face downwards onto the filter paper. The plate was gently pressed down, then very carefully lifted from the platform leaving colonies on the filter paper. The plate onto which the colonies were to be transferred, was also marked then orientated onto the platform, then gently pressed down for several seconds. The plate was then carefully removed, ensuring that only a minimal of material was transferred. A duplicate plate was also made. If required a number of plates could be placed onto the platform following one transfer and the plates incubated at 37°C overnight. For every different plate to be replica plated, the filter was replaced.

If colonies on the plate to be screened were not well spaced, the colonies could be picked. A Hybond-N filter suitably marked with pencil for orientation was placed onto a selective LAgar plate. Using sterile toothpicks the colonies were picked onto the grid array marked on
the filter and a replica plate made without the filter. The plates were then incubated at 37°C overnight.

2.7.2  \textit{E. coli} colony hybridisation. (Maas 1983)

Following incubation of the replica-plated plates, Hybond-N filters marked for orientation, were placed onto the plates using forceps and then carefully removed. For those colonies picked onto the filters, the filters were also carefully removed from the plates. The filters were allowed to dry for a few minutes and the bacteria lysed by placing the filters colony uppermost onto sheets of 3MM paper soaked in 0.5M NaOH for 10 minutes. The filters were then transferred onto a pad of 3MM paper soaked in denaturing solution for 3-5 minutes, followed by 3-5 minutes on a pad soaked in neutralise solution. The filters were finally transferred onto a pad soaked in 0.5M Tris-HCl pH 8.0, 2xSSC for 3-5 minutes, after which they were compressed between two sheets of 3MM. The remaining colonies on the filters were in this way removed since they adhered to the 3MM paper. The filters were then cross-linked under long-wave UV for 1-2 minutes. The positive colonies could then be identified by Southern hybridisation of the filters described in section 2.8.6, with the fragment of interest labelled with $^3$P as described in section 2.6.8.1. Transformants containing the fragment could be identified after autoradiography and DNA prepared and analysed as previously described (2.7.3.1 and 2.7.2).

\textbf{Denaturing solution}: 0.5 M NaOH, 1.5 M NaCl

\textbf{Neutralise solution}: 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0

2.7.3  \textit{E. coli} plasmid DNA preparation.

2.7.3.1 Small-scale \textit{E. coli} DNA preparation

The small scale preparation of bacterial plasmid DNA was performed using the alkaline lysis method of Birnboim and Doly (1979).
Single bacterial colonies were picked from selective plates using sterile toothpicks and inoculated into 2.5 ml of LB broth in sterile capped tubes containing the appropriate selection. These were then incubated at 37°C overnight. 1.5 ml of culture was then transferred to a 1.5 ml eppendorf tube and the cells pelleted at 5k 5 minutes in a bench-top microfuge. The supernatant was then poured off and the cells resuspended using a Gilson, in 100μl of ice-cold lysis buffer containing 2mg/ml lysozyme which was added immediately prior to use. The tube was then left on ice for 30 minutes after which time 200μl alkaline SDS (freshly made) was added. The tube was mixed by inversion, retained on ice for 5 minutes and 150 μl 3M NaAc pH 5.5 added. The tube was mixed again by inversion and left on ice for 30-60 minutes during which time a heavy white precipitate formed. The tube was then spun at 13k for 5 minutes in a benchtop microfuge and the supernatant was transferred to a new eppendorf using a Gilson; the pellet was discarded. The supernatant was phenol/chloroform extracted, spun at 13k for 5 minutes and the upper aqueous phase transferred to a new eppendorf and the DNA precipitated as in 2.6.2. This was subsequently resuspended in 20μl T.E and 10μl employed for DNA restriction, to determine if the transformant was of the correct orientation. To destroy RNA present in the miniprep DNA, 2μl 10mg/ml RNaseA was added to the sample as it was restricted at 37°C. The DNA was then examined as previously described (2.6.7).

**Lysis buffer:** 25mM Tris-HCl pH 8.0,
10mM EDTA pH8.0,
50mM Glucose.

This solution was normally made for storage, but prior to use 2mg/ml lysozyme was added.

**Alkaline SDS:** 4.3 ml Q water,
0.2 ml 5M NaOH,
0.5 ml 10% SDS, freshly made each time.

2.7.3.2 **Large scale E. coli DNA preparation**

The method employed was based on that described by Clewell and Helsinki (1969).

400ml LB broth containing a selective antibiotic for the plasmid in a 2 litre flask was inoculated with either a colony known to be correct or 500μl of a miniprep culture remaining from 2.7.3.1
The flask was then incubated at 37°C with vigorous shaking overnight. The cells were then harvested at 8k for 10 minutes in a Sorvall GS3 rotor and the supernatant poured off. The pellet was then drained thoroughly and the cells resuspended in 10ml of ice-cold 25% sucrose, 50mM Tris-HCl pH 8.0 using a pipette. This was then transferred to a capped 50ml centrifuge tube and kept on ice. 1ml of freshly made 10mg/ml Lysozyme in 50mM Tris-HCl was then added, the tube thoroughly mixed and left on ice for 5 minutes. This was followed by the addition of 2ml 0.25M EDTA pH 8.0 and the tube mixed and retained on ice for a further 10 minutes. The cells were then lysed by the addition of 15 ml lysis buffer and the lysate cleared by centrifugation at 18k for 60 minutes at 4°C in a Sorvall SS34 rotor.

25ml of this supernatant was decanted into a measuring cylinder. 25g of Caesium chloride, ground to a fine powder using a pestle and mortar, was then added and the solution mixed to dissolve the Caesium. This was then poured into a 35ml polyyallomer ultracentrifuge crimp tube (Sorvall 03989) and 2.5ml 10mg/ml Ethidium bromide added and the tube mixed. The tubes to be spun were balanced in pairs to within 0.1g and then sealed using the Sorvall ultracrimp system. The tubes were then loaded into a TV850 ultra-vertical rotor and centrifuged at 40k, 20°C for 18h after which the tubes were carefully removed and viewed under long-wave U.V. Two bands are normally visible in the centre and lower half of the gradient, with the upper band corresponding to linear chromosomal and open-circular plasmid DNA and the lower band super-coiled covalently closed circular plasmid DNA. Since the tubes were sealed, they were punctured at the top using a syringe needle, to allow air to enter the tubes before the DNA could be removed. The lower band was then removed using a 5ml syringe with a 19 gauge needle. The needle was inserted through the side of the tube just below the band which was then carefully extracted in a 5ml volume. This was transferred to a 6ml polyyallomer ultracentrifuge tube and as before, the tubes were balanced and sealed, then centrifuged in a Sorvall TV865 ultra-centrifuge rotor at 60k, 20°C for 18h or 5h. Following the spin, the tubes were again viewed under long-wave U.V.

The lower band (normally only one band is apparent but two can be observed when the initial band extraction is contaminated) was removed and transferred to a clear capped plastic tube. 1 volume of water-saturated Caesium chloride saturated isopropanol was added and the tube mixed well. The two phases were then allowed to separate out and the upper isopropanol phase removed and discarded using a Pasteur pipette. Further extractions were then performed until the aqueous phase was clear, upon which it was placed into a dialysis bag knotted at the
base and the top clipped. This was dialysed against TE at 4°C, to remove the Caesium chloride. Dialysis was performed for at least two hours, after which the TE was replaced with fresh solution. This was done a further 2 times then dialysis done overnight. Once dialysis was complete the DNA was transferred to a storage eppendorf and the concentration determined.

Lysis buffer : 2% Triton X-100, 50mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0

2.8 YEAST METHODS

2.8.1 Transformation of yeast cells

2.8.1.1 Preparation of yeast competent cells

The method employed was derived from that originally described by Ito et al (1983). For all steps, it was important that detergent-free bottles and solutions were employed. The yeast strain to be transformed, was initially inoculated into 20 ml of YPD media in a 25 ml plastic universal container and grown up overnight at 30°C. This culture was then employed to inoculate another 20ml of YPD media, to give a culture containing approximately 2.5 x 10⁶ cells/ml, determined by use of a haemocytometer. The culture was then incubated at 30°C with shaking, until the cell count was 1x10⁷ cells/ml, after approximately 3 - 4h. The cells were then pelleted in a Hareus centrifuge at 4k for 5 minutes at 20°C and the supernatant discarded. The cells were then resuspended in 10ml of transformation TE and then pelleted at 4k for 5 minutes and the process repeated. The cells were then gently resuspended in 10 ml LA and incubated with shaking at 30°C for 60 minutes. The cells were again pelleted and resuspended in 2.5 ml LA providing sufficient cells for 6 transformations.

Transformation TE : 10 mM Tris-HCl pH 7.6, 0.1 mM EDTA.

LA : 0.1 M Lithium acetate in sterile transformation TE.
2.8.1.2 Transformation of competent yeast cells

0.3 ml of competent yeast cells were aliquoted into a 1.5ml eppendorf tube and 0.1-10 μg of transforming DNA was added and gently mixed. A no DNA control was also included. 0.7 ml of 50% PEG 4000 (less than 4 weeks old) was then added to the tube which was then mixed well by inverting several times. The tube was then incubated at 30°C for 60 minutes, followed by heat-shock at 42°C for 5 minutes. The next stage of the procedure was dependent upon the marker for which selection was performed. This could be either an auxotrophic marker (section 2.8.1.2.1) for example TRP1 or a dominant marker for example Chloramphenicol or G418 (section 2.8.1.2.2).

PEG 4000: 10g of PEG 4000 was weighed in a plastic universal and 20ml of Q water added to the 20ml mark. The solution was heated at 65°C to dissolve the PEG then filter sterilised.

2.8.1.2.1 Auxotrophic marker

Following heat-shock, the cells were pelleted at 4k for 5 minutes in a benchtop microfuge. The supernatant was then removed and the pellet gently resuspended in 400ul sterile Q water using a Gilson. The mixture was then divided between two minimal selection plates and the cells gently plated out using a flamed glass spreader. The plates were then incubated for 3-4 days after which time colonies should be observed.

2.8.1.2.2 Dominant marker

As for the auxotrophic marker, the cells were pelleted, but in this instance the cells were resuspended in 1ml YPD media. The cells were allowed to express at 30°C for 90 minutes if the G418 marker was employed and 60 minutes if the Chloramphenicol marker was employed. The cells were again pelleted at 4k 5minutes, the supernatant removed leaving approximately 200 µl and the cells gently resuspended using a Gilson. This was then divided between two selective YPD plates, which were incubated at 30°C. Colonies were normally apparent after 48h for the G418a marker and 4-7 days with the Cm³.
2.3.2 Yeast Total DNA preparation

The method employed for the preparation of total yeast genomic DNA was essentially as described by Cashmore et al. (1986) with further additional steps so that the DNA could be restricted. This method was also employed since it had previously been shown not to cause uneven losses of chromosomal or plasmid DNA and thus DNA produced in this way could be employed for plasmid copy number determinations.

A 400ml selective minimal or YPD culture contained in a 2l flask, was initially inoculated from either a 10ml culture or a colony from a transformation plate with a toothpick. This was incubated at 30°C with vigorous shaking until the culture was at a density of 1x10^7 cells/ml. The cells were then harvested at 9k 10 minutes in a Sorvall GS3 rotor, the pellet drained well and the cells resuspended in 10ml 1M Sorbitol. The cells were pelleted as before then resuspended in 2ml sphaeroplast buffer and 1mg solid Zymolyase (or lytic enzyme) added to the cell suspension. This was then incubated with gentle agitation at 30°C for a minimum of 60 minutes until sphaeroplast formation was complete determined under the microscope.

2 mg Proteinase K, 1ml EDTA and 3 ml 10% SDS were then added and the mixture gently mixed then incubated at 37°C 30 minutes. This was followed by a 10 minute incubation at 65°C to denature proteins present in the mixture and also to burst any remaining sphaeroplasts. The tube was then cooled on ice and the DNA phenol/chloroform extracted. The aqueous phase was then removed and the DNA precipitated as described 2, but in this instance 2M Sodium acetate pH 5.6. was employed. The DNA was pelleted by centrifugation at 10k 10 minutes, washed with 70% ethanol, then dried. The resultant pellet was resuspended in 10ml TE and 2 μl RNase T1 added and incubated at 37°C for 2h. This was followed by a 20 minute incubation at room temperature with 3 units of porcine α-amylase followed by 30 minutes at 55°C with 1 unit amyloglucosidase (from Aspergillus niger). 0.2ml 10% SDS and 40 μl 10mg/ml proteinase K was then added and further incubated at 37°C for 30 minutes. A phenol/chloroform extraction was performed as before and the resultant DNA pellet resuspended in 800 μl TE. The DNA concentration was then determined and the DNA stored at 4°C until required.

Sphaeroplast buffer : 1 M Sorbitol,
1M Potassium phosphate buffer pH 7.5,
10 μl β-mercaptoethanol.

2.6.3 Plasmid copy number determination

The copy number of a particular plasmid was determined by restriction of total yeast DNA with restriction endonucleases which would liberate an auxotrophic marker from the multi-copy plasmid and the chromosomal marker in single copy. Following hybridisation with the auxotrophic marker comparing the ratio of the signal obtained.

5 μg DNA prepared as in 2.8.2 was restricted with restriction endonucleases (2.6.5) which would cleave the chromosomal and plasmid DNA on either side of the yeast marker and liberate a fragment containing the same gene of a different size from the chromosome. The method is illustrated in Fig 2.8.3. To aid full restriction of the DNA, the restriction was performed in a 400μl volume using TE to dilute any impurities which could reduce the efficiency of enzyme restriction. Additional endonucleases which cleave outside the marker fragment region were also employed to break down the chromosomal DNA. The DNA was then restricted at 37°C preferably overnight, after which time additional enzyme was added and the restriction prolonged for a further several hours. Since the DNA was in a large volume, it was precipitated as in 2.6.2 and resuspended in 10 μl TE, loading buffer added and the sample electrophoresed as in 2.6.7. The gel was then southern blotted 2.8.5 and then hybridised (following prehybridisation) with 32P labelled yeast marker fragment. This marker fragment was liberated from purified plasmid DNA by digestion with appropriate endonucleases and treated as described in 2.6.5. Following hybridisation, the filter was sandwiched between two pieces of saran-wrap, and adhesive labels affixed around the periphery of the filter. Radioactive ink markings were then made on the labels with toothpicks, and once dry further saran-wrap used to cover the markings. The filter was then exposed to Kodak AR film at -70°C. After autoradiography, two bands were observed, which was usually arranged so that the lower band was the single copy chromosomal yeast band, whilst the upper band (larger fragment) was the marker on the plasmid. The X-ray film image was alligned over the saran-wrap covered filter using the radioactive ink markings and the edges of the band areas on the X-ray film pierced with a syringe needle, through to the filter. These holes were then used as a guide to excise the required filter areas, from which the saran-wrap was peeled prior to placing them into scintillation vials. 2.5 ml LKB 'Oriscint' a non-aqueous scintillation fluid was then added and the vials counted in a Beckman LS6800
Figure 2.8.3 Schematic diagram showing the process of plasmid copy number determination.

**Chromosome**

<table>
<thead>
<tr>
<th>Haploid gene</th>
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<tbody>
<tr>
<td>X</td>
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</tbody>
</table>

**RESULT**

- Isolation of DNA
- Digestion with endonuclease X
- Agarose gel/ Southern blotting
- Hybridise with 32P labelled marker fragment
  
  ```
  X   S
  ```

- Autoradiograph

<table>
<thead>
<tr>
<th>Multicopy plasmid gene</th>
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<tbody>
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<td>?</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Single copy (haploid) chromosomal gene</th>
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The ratio of haploid chromosomal gene signal to plasmid gene signal gives the copy number.

1
scintillation counter, ensuring that the vials had been treated with an anti-static device prior to counting. The counting process was repeated three times to ensure that static was not an interference in the count data obtained.

The ratio between the upper and lower counts was then calculated, which represented the average copy number per cell.

2.8.4 Yeast plasmid stability determination

The stability of plasmids transformed into yeast was determined in the following manner. A single transformant colony was inoculated into 10ml selective and/or non-selective media and grown until 10 doublings were reached. The percent plasmid containing cells was determined by plating an appropriate dilution, in duplicate, onto YPD agar plates to give approximately 100 - 200 cells per plate, incubating the plates then replica-plating the colonies as in section 2.7.2.1 onto selective plates. Following incubation, the number of colonies growing on the selective plates were taken as containing plasmid and the percent plasmid-containing cells calculated.

2.8.5 Southern blotting of agarose gels.

In most instances the Pharmacia LKB 2016 VacuGene vacuum blotting system was employed, which used a low pressure vacuum to transfer nucleic acids from a gel to a membrane. This was employed since it was much quicker and convenient than the conventional method. Hybond-N filter membrane was cut to the size of the gel and soaked in 4xSSC. The apparatus consisted of a plastic vacuum blotting unit with a porous support which fitted on the inner rim of the base unit. A plastic mask with a window area of a size just greater than the gel to be blotted was placed upon the support and the membrane placed within the window. Any gaps between the membrane and the window were sealed with nesco-film and the gel placed carefully on top of the membrane, ensuring no air bubbles were evident. The vacuum was then set at 40psi and denaturing solution gently poured onto the centre of the gel surface so that the gel surface was covered with solution, for 4 minutes. This was then removed by
tipping the apparatus to one side and syphoning the solution off. The gel was carefully blotted dry and neutralise solution poured, as before, onto the gel surface. After 4 minutes this was removed and replaced for a further 4 minutes with fresh neutralise solution. The gel was then blotted using 20 x SSC for 30-60 minutes depending upon the gel thickness and agarose concentration, ensuring that the gel surface was covered in solution for the duration of blotting. The vacuum was then switched off, residual solution removed and the gel carefully lifted from the filter surface. The filter was then blotted to remove excess liquid then cross-linked under short-wave U.V for 3 minutes.

<table>
<thead>
<tr>
<th>Denaturing solution:</th>
<th>1.5 M NaCl, 0.5 M NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutralising solution:</td>
<td>1 M Tris-HCl, 2 M NaCl pH 5.0</td>
</tr>
</tbody>
</table>

2.5.6 Southern Hybridisation

Southern hybridisation could be performed using a plastic container or a Hybaid hybridisation bag. The latter was normally employed since a smaller volume of buffer was required. In addition, a number of different hybridisation solutions could be employed. Initially, 5 x Denhardt\'s solution was employed but in some instances the background was not very clean. The hybridisation solution was therefore changed to Church Gilbert solution which gave consistently cleaner blots.

The U.V cross-linked filter (which may also be an E. coli transformation colony-blot), was placed into a Hybaid hybridisation bag and the bottom sealed using a heated bag sealer apparatus. Hybridisation solution was then added to the bag through one of the integral ports on the side of the bag using a 25ml syringe, which was also used to remove any air within the bag. The volume of buffer employed was dependent upon the size of the filter, but normally 20ml was sufficient. The filter was then prehybridised at 65°C in a shaking water-bath for 1-2 h, after which time the boiled probe was added to the bag via the integral ports as before. The filter was then hybridised for 18h at the same temperature, after which time it was washed in 4 x SSC, 0.1% SDS which was pre-warmed to 65°C. The hybridisation solution was initially poured from the bag through the integral ports into a radioactive sink area and the sealed bag opened using scissors. The filter was placed into a plastic container
and washed in the 65°C shaking water-bath for 30 minutes after which time the solution was replaced with fresh washing solution and the procedure repeated a further 5 times. Once washing was complete, the filter was blotted between two pieces of 3MM paper, then wrapped in Saran wrap and exposed to autoradiography, using XAR Kodak film, at -80°C overnight. The autoradiograph was then developed using a film processor and the filter exposed again if required.

**Denhardt's solution:**

- 1g/l BSA (fraction V),
- 1g/l Ficoll,
- 1g/l Polyvinylpyrrolidone in 4xSSC.

**Church Gilbert solution:**

- 0.5 M Potassium phosphate buffer pH 7.2,
- 10g/l BSA (fraction V),
- 1mM EDTA
- 70 g/l SDS.

The SDS was normally added as a 20 % stock solution, once the BSA had dissolved.

2.8.7 **Probe removal from Southern or Northern hybridised Hybond filters**

Northern or Southern blotted Hybond-N or nylon filters incubated with one radioactive probe could be employed for further probes by removal of the unwanted radioactive probe. This could be performed as long as the filter had not dried out after incubation. In most instances the filters are wrapped in Saran-wrap prior to autoradiographic exposure and thus are relatively damp. The filter was carefully removed from the Saran-wrap and placed into a plastic container of approximately the same size. Q-water at approximately 100°C was poured carefully over the filter and the container placed onto a moving platform to gently move the liquid across the filter. The Q-water was allowed to cool and then carefully poured away and the process repeated a further two times. A hand-held geiger counter was held over the filter to determine the extent of probe removal. The process could be repeated at this stage if a large amount of radioactivity was still present on the filter. The filter was then wrapped into saran wrap and exposed to film to determine if the probe had been removed. If removal was sufficient for further use the filter could then be re-employed.
2.9 RNA PREPARATION AND MANIPULATION

2.9.1 Yeast total RNA preparation.

100ml of selective minimal or YPD media was inoculated with culture from a fresh 10ml culture and incubated at 30°C until the cell count was 1-2 x 10⁶ cells/ml. The cells were then pelleted at 8,000 x g for 10 minutes in a Sorvall GS3 rotor and washed in sterile Q water. The cells were repelleted and resuspended in 1ml Kirby phenol mix and transferred to a 15ml screw-capped Sarstead tube. Acid washed glass beads were then added to just below the liquid meniscus and the tube vortexed 4x30 seconds, keeping the tube on ice between vortexes. Cell breakage was then checked under the microscope and if approximately 90% of cells were broken, a phenol/chloroform extraction was carried out. The tube was then mixed for 2 minutes and centrifuged in a Heraeus centrifuge at 5,000 x g for 10 minutes. The aqueous phase was then removed to a clean tube and extracted twice with phenol/chloroform. The resultant aqueous phase was transferred to a glass corex tube and the nucleic acids precipitated by the addition of 33 μl/mL 6M Lithium acetate and 5x volume of absolute ethanol and placed at -70°C for 30 minutes. The nucleic acids were subsequently pelleted at 10,000 x g for 15 minutes and resuspended in 1ml sterile Q water at 4°C. RNA was then precipitated by the addition of an equal volume of 6M Lithium acetate and placed at 0°C for 60 minutes. This was then pelleted at 10,000 x g for 10 minutes and washed twice in 3M Lithium acetate. The RNA was then resuspended in 400μl sterile Q and a final ethanol precipitation performed by the addition of 15μl 6M LiAc and 1ml absolute ethanol. The RNA was subsequently resuspended in 500μl Q and the concentration determined by scanning on a spectrophotometer. In this instance the Absorbance at 260nm was equal to 1 at 40μg/ml.

Kirby Phenol: 60 g/l 4-aminosalicylate,
10 g/l TPNS,
60 g/l Phenol,
50 mM Tris pH 8.4 in Q water.

Stored at 4°C in a light-proof container.
2.9.2 RNA gel electrophoresis.

This was performed essentially as described in Maniatis et al (1982). The RNA agarose gel was initially prepared by dissolving 0.75g Seakem agarose in 55ml Q water in a microwave oven. The mixture was allowed to cool to 70°C and 7.5ml 10 x MOPS and 13ml formaldehyde added. The agarose mixture was gently mixed and the gel poured in a fume hood until required. The RNA sample in a total volume of 5μl, was then prepared for loading onto the gel, by the addition of 1μl 10 x MOPS, 3.5μl Formaldehyde (38%) and 10 μl formamide (100%). This was incubated at 65°C for 10 minutes and 3.5μl RNA loading buffer and 1μl 1mg/ml EtBr, added. The sample was then stored on ice until required.

The agarose gel was then immersed in 1 x MOPS running buffer and samples loaded into the wells. The gel was then subjected to electrophoresis at 20-30 V overnight or 100 V 2-5 hours, after which it was viewed under short-wave U.V. and photographed. The electrophoresis tank employed was previously DEPC treated, by washing with Q containing 0.1 % diethylpyrocarbonate and leaving to stand at room temperature for at least 60 minutes.

**RNA loading buffer:** 50% Glycerol,
1mM EDTA,
0.4% Bromophenol Blue,
0.4% Xylene cyanol.

2.9.3 Northern blotting.

The method employed in most instances, involved the use of the LKB Vacublot system. The apparatus was set up as previously described (section 2.8.5) and the gel carefully placed onto the Hybond-N filter. The vacuum was set to 40 psi and 20 x SSC poured onto the gel, in sufficient volume to cover the gel surface. The gel was subsequently blotted for a minimum of 60 minutes depending upon its thickness, ensuring that during this time the gel was covered with 20 x SSC. On completion of blotting, the solution remaining was removed using a disposable pipette, the gel lifted from the filter and the filter dried between two pieces of 3MM paper. This was subsequently cross-linked under short-wave U.V for 4 minutes and either stored in Saran wrap until required or placed into a Hybaid hybridisation bag for hybridisation.
2.9.6 Northern hybridisation

Initially Northern hybridisation was performed using the following method. The filter to be hybridised was prehybridised in 20 mls of prehybridisation solution at 42°C for 2 hours. The prehybridisation solution was then poured from the bag via the integral ports and replaced by 20 mls hybridisation solution. The preboiled probe prepared as in , was then added and hybridisation performed in a shaking waterbath at 42°C for 48 hours. The filter was then removed from the hybridisation bag, rinsed in washing solution 3 x SSC, 0.1% SDS and placed into a suitable container. It was then washed 4 times for 30 minutes each wash, at 42°C, after which it was blotted dry between two pieces 3MM, wrapped in saran wrap, then exposed to Kodak XAR-5 film at -70°C.

It was also found that the Church-Gilbert hybridisation solution employed for Southern blotting, could also be employed for Northern hybridisation if the 1% BSA was omitted. The temperature employed for hybridisation in this instance was increased to 65°C and the hybridisation time decreased to 18 h. The use of this solution also eliminated the need to change the solution prior to probe addition. A further solution which could be employed was Blotto. Prehybridisation and hybridisation conditions were as described for Church-Gilbert.

| Northern prehybridisation solution | 50% formamide, 5 x SSC, 5 x Denhardt's, 100 µg/ml salmon sperm DNA. |
| Northern hybridisation solution    | 50% Formamide, 5 x SSC, 0.3% SDS, 1 x Denhardt's, 100 µg/ml salmon sperm DNA. |
| Church-Gilbert for Northerns      | 0.5 M potassium phosphate buffer pH 7.2, 70 g/l SDS, 1 mM EDTA. |
| Blotto hybridisation solution     | 1.5 x SSPE, 0.5 % Marvel, 1% SDS, |
2.10 PROTEIN PREPARATION AND MANIPULATION

2.10.1 Crude protein extraction from whole yeast cells.

The method employed for extracts for use in APT enzyme assays and PGK protein extracts was essentially as described by Hadfield et al (1987). 10ml of the required culture was grown to a mid-log density of 1x10^6 cells/ml in selective media at 30°C. The cells were then pelleted at 4k for 10 minutes in a Heraeus centrifuge, the supernatant discarded and the cells washed in 5ml ice-cold protein buffer. The cells were then repelleted, rewashed, then resuspended in 200μl of protein buffer.

The solution was then transferred to a 1.5ml eppendorf tube and acid-washed glass beads added to just below the liquid meniscus. The cells were then vortexed for 6x30 seconds, cooling the tube on ice between vortexes. Cell breakage was then examined under the microscope and if greater than 90% of the cells were broken, the glass beads were removed in the following manner. The eppendorf tube containing the protein solution was inverted and the base pierced with a hot syringe needle. This was then placed into a second eppendorf tube and both placed into a plastic universal container. This was subsequently centrifuged in a Heraeus centrifuge at 5k for 5 minutes after which time the glass beads remained in the upper eppendorf tube, whilst the protein and cell debris were in the lower tube. The latter was removed by centrifugation at 13k in a microfuge for 5 minutes and the cleared protein solution transferred to a clean eppendorf tube. The protein concentration in the sample was then determined as in or the sample stored at -20°C until required.

Protein Buffer: 0.4 M NaCl, 10 mM Tris-HCl pH 7.0, 10 mM MgCl₂, 1 mM DTT in Q water.

Prior to use 35mg/ml PMSF solution in absolute ethanol was added dropwise with adequate mixing.
25 ml of the culture required was grown at 30°C in selective minimal media, until it reached a cell density of 1x10⁷ cells/ml. The cells were harvested by centrifugation at 4k 5 minutes, the pellet drained and the wet weight of the pellet determined. The cells were then resuspended in 0.1M Tris-HCl (pH 8.5), 10mM EDTA, 10mM β-mercaptoethanol at 1ml/100mg wet weight of cells. 1ml of the cell suspension was then transferred to a 1.5 ml eppendorf tube, the cells harvested in a microfuge at 6k 2 minutes and the pellet resuspended, using a Gilson, in 100 µl of lysis buffer. 25 µl of 5x protease inhibitor mixture was also added, followed by the addition of glass beads (0.45mm diameter) to just below the liquid meniscus. The eppendorf tube was then vortexed for 6x30s cooling on ice between vortexes. This was repeated until cell breakage was 90% or greater and the glass beads removed as in the previous extract method.

**Lysis buffer**: 0.1M Tris-HCl pH 7.0, 2mM EDTA with 5µl/ml β-mercaptoethanol (added immediately prior to use)

**5 x protease inhibitors**: 20 mM EDTA, 20 mM PMSF and 10 µg/ml of pepstatin, leupeptin, chymostatin and antipain.

### 2.10.2 Protein assay

The protein content in crude cell extracts was determined by the method of Bradford (1976), using Biorad reagent.

The sample to be assayed was diluted in protein extraction buffer to a total volume of 800µl in an eppendorf tube. BSA standards were also set up to give a range of BSA concentrations from 1-25µg/ml in the same volume. 200µl of Biorad reagent was added to the samples which were mixed, then incubated at room temperature for 20 minutes. The absorbance of the samples was subsequently determined at 595nm. A calibration curve was plotted using the BSA data and the concentration of the protein samples determined. The protein concentration of the sample was taken as having half the value of the BSA standard since BSA has 2x the absorbance of most other proteins.
2.10.3 SDS-Polyacrylamide gel electrophoresis of proteins.

The method employed was based on that of Laemmli (1970). The final percentage of acrylamide employed for the electrophoresis was dependent upon the molecular weight range of the proteins to be resolved. As an approximation; 10% for 50-200 kD, 15% for 20-100 kD and 20% for 10-40 kD.

In most instances a Hoeffer baby gel system was employed giving 5ml gels of 100 x 50 x 0.75mm, with a stacking gel of 20mm. The gel apparatus consisting of two glass plates with 2 mm spacers between them was assembled prior to making the gel solution. The glass plates were carefully cleaned using IMS and Acetone and one plate siliconised using sigmacote. The plates were then assembled with the spacers, using the supplied Bulldog clips. The base of the gel apparatus was sealed with molten agarose and the acrylamide solution could now be made.

The resolving gel was made up as outlined for a 10% gel. 5ml of stock acrylamide solution, 1.88 ml of resolving gel buffer stock, 0.15 ml 10% SDS, 0.75 ml 1.5% ammonium persulphate (freshly made in Q water) and 7.23 ml Q water. All solutions were mixed in a Buchner flask, degassed, then 15 μl TEMED added. This was then carefully poured between the glass plates of the assembled apparatus, until within 20 mm of the top of the glass plate and a small amount of water saturated butanol layered above this to aid polymerisation. The stacking gel was then made up, which had the same composition in all gel systems. 1.25 ml stock acrylamide solution, 5 ml stacking gel buffer stock, 0.1 ml 10% SDS, 1ml 1.5% ammonium persulphate, 5.65 ml Q water and 15 μl TEMED. The butanol was removed from the resolving gel surface by washing with Q water and the stacking gel poured above it. A well-forming comb was placed into the top of the stacking gel and the gel allowed to polymerise.

Once polymerisation was complete (30 - 60 minutes) the apparatus was placed into a vertical gel tank containing running buffer and the wells flushed out using a syringe. The protein samples were prepared for running on the gel by the addition of an equal volume of sample buffer and the samples boiled for 2 minutes in a PEG bath at 105°C. The gel was then ran at 60V through the stacking gel and up to 150 V in the main gel, until the dye front reached the bottom of the gel.
**Stock acrylamide:** 30g acrylamide and 0.8g bisacrylamide in 100 ml Q water.

**Resolving gel buffer stock:** 3 M Tris-HCl pH 8.8

**Stacking gel buffer stock:** 0.5 M Tris-HCl pH 6.8

**Running buffer:**
- 144g Glycine
- 30g Tris
- 5g SDS

**Sample buffer:**
- 25 mM Tris-HCl pH 6.8,
- 2% SDS,
- 10% glycerol,
- 0.2 M β mercaptoethanol,
- 0.002% bromophenol blue.

### 2.10.4 Staining protein gels.

Protein gels were stained by two different types of stain, the first described was employed more frequently. The staining solution was made by dissolving 0.1 g of Coomassie blue in a solution containing 50 ml methanol, 40 ml Q water and 10 ml acetic acid. The protein gel attached to one glass plate, was immersed into the staining solution contained in a suitable container for approximately 2 h, ensuring the gel was adequately covered by placing the container on a slowly shaking platform. The stain was then carefully poured off the gel and replaced with destain composed of 50 % methanol and 10 % acetic acid until the background staining had sufficiently decreased to allow protein bands to be observed.

The second stain occasionally employed was Kenacid blue, which was made by dissolving 0.1g Kenacid Blue in 45ml methanol, 10ml acetic acid, 45ml Q water. The staining time and the destain employed was as for Coomassie blue staining.

### 2.10.5 Alcohol dehydrogenase assay.

The assay employed for the determination of alcohol dehydrogenase activity was derived from a number of methods in Methods in Enzymology (pers. comm. A.Carter). The assay followed spectrophotometrically the conversion of NADH to NADP by Alcohol dehydrogenase and relied on the initial enzyme reaction rate.
The amount of protein employed in the assay was dependent upon the ADH activity of the extracts, but varied from 50-100 μg of protein in most instances. If the initial enzyme rate was too fast, the sample was diluted and the assay repeated. The extract was added to protein extraction buffer to give a sample volume of 0.1ml. This was added to 7ml of Tris-HCl pH 9.5 and 0.1 ml 1.5 mM NADH in a plastic cuvette. A blank sample was also made, but in this instance it contained 8 ml Tris-HCl. The samples were placed into a Shimadzu spectrophotometer set on time scan mode, with a paper speed of 10mm/min on an absorbance range of 335-340 nm. The samples were blanked for 2 minutes then 0.1 ml of 3M ethanol added, the cuvette was carefully mixed and the reaction allowed to proceed until the reaction began to plateau out. The change in absorbance/minute was then calculated from the initial enzyme reaction rate, which was the linear portion of the scan.
CHAPTER THREE.

EXPRESSION OF BACTERIAL AMINOGLYCOSIDE PHOSPHOTRANSFERASE IN YEAST AS A MODEL HETEROLOGOUS GENE AND DOMINANT MARKER
3.1 INTRODUCTION

Following the introduction of methods to transform foreign DNA into yeast (Beggs, 1978; Hinnen et al, 1978) a means of recovering cells containing the foreign DNA was required. Auxotrophic markers such as LEU2 (Hinnen et al, 1978), TRP1 (Hitzeman et al, 1981) and URA3 (Struhl et al, 1979) were initially employed, but were dependent upon the complementation of a recessive auxotrophic mutation in the yeast strain employed. As a consequence, the genetic background of the strain had to be defined which would be difficult with wild-type or industrial yeasts. This problem could be overcome, however, by the use of dominant selectable markers, since yeast cells could be transformed independently of their phenotype.

Although dominant markers were widely employed for the selection of plasmid transformed cells in E.coli, most were and still are, predominantly antibiotic resistance genes, conferring resistance to, for example, ampicillin, tetracycline (Bolivar et al, 1977), and hygromycin B (Ohmae et al, 1979). This type of selection would be very difficult in yeast since the antibiotics commonly employed were not inhibitory to S. cerevisiae. It was found, however, that S. cerevisiae was inhibited by the antibiotic Geneticin G418 which is a 2-deoxystreptamine antibiotic that is structurally related to gentamycin (Jiminez and Davies, 1980). The antibiotic inhibits translocation on bacterial ribosomes by binding to both the 30S and 50S subunits of 80S ribosomes, inhibiting protein synthesis and mRNA reading, the extent to which both are affected varies, which is dependent upon antibiotic concentration. At low concentrations of gentamycin, protein synthesis is inhibited, but little mRNA misreading occurs. As the concentration of gentamycin increases, mRNA misreading increases and inhibition is reversed whilst at high concentrations inhibition of synthesis increases. Gentamycin and the other aminoglycoside antibiotics, kanamycin and neomycin, however, did not have inhibitory activity indicating that the difference between them must reflect relative uptake permeability of the yeast cell wall.

Resistance to the aminoglycoside antibiotics is provided by two types of the enzyme 3'-O-aminoglycoside phosphotransferase (designated I and II), which are encoded by the bacterial transposons Tn903(601) and Tn5 respectively. The 3'-O-aminoglycoside phosphotransferase I is effective in yeast (Jiminez and Davies, 1980) and catalyses the transfer of a phosphate group from cellular ATP, to the 2-deoxystreptamine moiety at the 3' hydroxyl side group of...
the antibiotic which renders the antibiotic inactive (Haas and Dowding 1975).

Tn903, encodes three high molecular weight polypeptides, two of which are thought to be associated with the transposition functions, whilst the third encodes the APT enzyme (Davies et al, 1978, Cohen and Shapiro, 1980).

Geneticin G418 resistance had been successfully employed as a dominant marker for use in E.coli (Rao and Rogers, 1979), plant cells (Herrera-Estrella et al, 1983), and mammalian cells (Colbere-Garapin et al, 1981), but in yeast, poor transformation efficiency limited its use and it was therefore not widely employed. The poor transformation efficiency was illustrated by work of (Jiminez and Davies, 1980) in which a small ColE1 DNA molecule, into which Tn903 had been transposed (pAO43), was co-transformed with a LEU2 based plasmid into S. cerevisiae. In this form, only 8% of the LEU+ progeny obtained were resistant to G418, indicating that the transformation of G418 was very inefficient. This would be expected since the transformation efficiency of plasmid DNA without a replication origin is very low, as discussed in section 1.4.1. This work did, however, demonstrate that S. cerevisiae will express the APT gene and become resistant to G418. Further work by Hollenberg (1982), using Tn903 on a multi-copy plasmid, again indicated that G418 transformants could be selected. In this instance however, only 15% of the colonies were found to be authentic transformants, which was attributed to poor expression of the APT enzyme. As a consequence, a low concentration of G418 had to be employed for selection and as a result high background was a problem. In both instances, therefore, direct selection of resistant transformants was not feasible.

The problem of high background and the inability to directly select G418 transformants was overcome by Webster and Dickson, (1983). A vector containing a single copy of pAO43 (Parent plasmid containing Trn903) integrated into a LEU2 based plasmid and transformed into S. cerevisiae, could yield G418 transformants, if 12-18h post-transformation growth on YPD agar was allowed, prior to antibiotic application as an overlay on the plates. Transformant number was critically dependent upon the length of the post-transformation growth, but numbers were still lower than those obtained when selecting for an auxotrophic marker, ranging from 1-11% less. This poor transformation efficiency could be attributed to a low level of production of the APT enzyme within the yeast cell.
The APT gene had potential as a useful heterologous gene, in the form of a dominant marker and reporter gene, since it had already been well characterised and in addition an enzymatic assay was available for measurement of expression levels. This latter point was important since not many sensitive heterologous assays were available. In addition, the APT gene had an additional advantage over some commonly employed dominant markers, in that it could be employed for selection in \textit{E. coli} simplifying gene manipulations.

To be useful as a dominant marker, however, expression of the APT gene required improvement, since this would eliminate the need for long post-transformation incubations which were a prerequisite to obtaining G418 resistant transformants. Additionally this would lead to an increase in the transformation efficiency of the marker. The method employed by Webster and Dickson \cite{Webster1983}, to obtain resistant transformants required plating the cells after transformation, onto agar plates and overlaying the cells with further agar. Following 18h of growth at 30°C, G418 was spread over the agar surface and the plates reincubated for 3-4 days, after which time transformed cells should be apparent. Improving expression should reduce the time required to obtain transformants and also simplify the process. A preferential procedure would be that following transformation, cells could be incubated in YPD medium for a specified post-transformation incubation time (which would be less than the 18 h previously required) and then plated directly onto agar plates containing G418.

Once APT expression had been optimised in yeast, the APT gene could be used as a reporter gene (discussed in section 1.6.4) since APT activity could be determined. The assay methods that were employed, were time-consuming and laborious, but it was considered that improvements could be made to provide a more sensitive, quick and reliable method to quantitate activity.

A major aim of the work in this chapter was to improve the expression of the APT gene in yeast and additionally the assay method. In doing so it was hoped that a very useful yeast dominant marker and reporter gene could be produced, providing an alternative to those currently employed.
3.2 The Isolation of the APT coding sequence

It was considered that the major factor limiting expression of the APT gene in the previously described transposon form, was the presence of bacterial sequences. The sequences which control the expression of E.coli genes are very different to those controlling expression in yeast cells. Increased expression of the gene would be expected, if these sequences were removed and the gene, under the control of yeast expression signals, incorporated into a multi-copy plasmid.

In order to isolate the gene, a transcriptional fusion was made rather than a translational fusion, since this would ensure that the same APT protein effective in yeast would be produced. The transcriptional fusion, could be easily made by use of an Xhol site, present on the APT gene. Initially the APT coding region was isolated from the bacterial transposon Tn903 (Jimenez and Davies 1980) and placed under the control of a PGK1 promoter and into a multi-copy plasmid (reported in Hadfield et al, 1990). Vierira and Messing (1982), had isolated a 1.43 kb APT HaelI restriction fragment of Tn903 which cleaved the Tn903 cartridge at the two inverted repeats (Figure 3.2.1a). This fragment containing the APT coding sequence was GC tailed and cloned into a pUC family plasmid (Vierira and Messing 1982) via a PstI polylinker site. This produced the coding sequence cartridge termed APT-4K, which contained several ATG and other possible start codons 5' of the APT encoding open reading frame. The APT-4K cartridge was cloned into a pEMBL8+ plasmid (Dente et al, 1983), primarily so that sequencing could be performed and further manipulations carried out. The aforementioned potential false start codons were removed by Bal31 deletion to within 7 bp of the 5' APT initiator codon. A Bell linker was subsequently added and the resultant cartridge termed APT0. The 3' end was further trimmed back with Bal31 to remove the poly GC tail and a Bell linker inserted creating APT1 and plasmid pCH116. In all instances the sequence of the APT cartridges were confirmed.

Further modifications of APT1 at its 5' end were also performed. A synthetic oligonucleotide was inserted between the Bell and Xhol sites which increased the size of a short bacterial 70S ribosome binding site (Shine and Dalgarno 1974) by 3 bases, creating the APT2 cartridge. This would improve the capability of translation of the APT transcripts in E.coli. In addition, the DNA sequence flanking the ATG start codon was altered so that it more closely resembled the consensus for translation initiation in yeast as documented by Cigan and Donahue (1987).
IR : indicates inverted repeats.

Restriction sites: B = BamHI, Bc = BcII, Bg = BglII, H2 = HaeII, P = PstI, S = Sall, X = Xhol

Double-stranded, blunt ended oligonucleotide linkers had the following sequences; for the introduction of a BcII site, 5' CTGATCAG 3' and for BglII, 5' CAGATCTG 3'.
Figure 3.2.1b  Sequence characteristics of the APT-coding sequence cartridges

fr1 and fr2 refer to different translational reading frames
RBS = Ribosome binding site
The codon usage within the first 10 codons was also changed so that minor codons were replaced by major ones, improving the overall yeast codon bias index (Bennetzen and Hall 1982) from 0.07 for APT1 to 0.1 for APT2.

3.3 Construction of the multi-copy APT determinant

3.3.1 Promoter and terminator isolation.

The yields of heterologous proteins, expressed from *S. cerevisiae* may largely be determined by the choice of promoter employed (Hitzeman *et al.*, 1981, Tuite *et al.*, 1982). The strongest yeast promoters appear to be those of the glycolytic genes since the glycolytic enzymes give rise to greater than 50% of the total cell protein. Commonly employed glycolytic genes include PGK1 (Holland and Holland 1978; Dobson *et al.*, 1982), ADH1 (Bennetzen and Hall 1982, Hitzeman *et al.*, 1981) and PYK (Burke *et al.*, 1983). The chromosomal copy of the PGK gene directs synthesis of about 1% total cell protein as PGK (Holland and Holland 1978), which is increased to 50-80% of the total cell protein, when it is incorporated onto a high-copy-number plasmid (Kingsman *et al.*, 1985). In addition, the PGK promoter has also been extensively characterised (Dobson *et al.*, 1982).

Due to the aforementioned factors, the PGK1 promoter was selected to direct the expression of the APT cartridge on a multi-copy plasmid employing the PGK1 terminator. The promoter and terminator regions of the gene, were generated from a plasmid-cloned 3.0kb HindIII fragment containing the PGK1 gene (G.H.J. Pretorius) as described in the figure legend. This produced a pUC based plasmid containing the PGK1 promoter and terminator, with an *E. coli* origin of replication (plasmid pCH137; Appendix). The resultant PGK1 promoter provided a mRNA initiation site but not a translation initiation site which had been deleted by exonuclease digestion and a BglII linker inserted (Fig 3.3.1a).

The APT1 cartridge described in section 3.2.1, was isolated from pCH116 DNA that had been cultivated in a dam-*E. coli* strain, to allow restriction with BclI. The BglII and BclI fragment was isolated as described in section 2.2.9 and ligated into the pCH137 plasmid at the BglII site located between the PGK1 promoter and terminator (Fig 3.3.1b), to give plasmid pCH147 (Appendix). This could occur since both the BclI and BglII ends were compatible. The APT cartridge therefore effectively replaced the PGK1 coding region. The
PGK1 was cut at a unique KpnI site within the coding region and 500 bp of DNA deleted towards the promoter by Bal31 exonuclease. This removed the translation start codon. The ends were made flush by treatment with Klenow DNA polymerase I and a BgIII linker ligated onto the ends, which was 18 base pairs downstream from the transcription start. Restriction with BglII cleaved the linker at the promoter side and the BglII site present upstream of the PGK1 terminator and the two ends were ligated together.
AGTAATTATCTACTTTTTTCAG ATCT

mRNA start

Bg linker
Figure 3.3.1b  Construction of the heterologous APT cartridge

The APT1 and APT2, BglII and BCIll ended cartridges were inserted into the BglII site between the PGK1 promoter and terminator, thereby replacing the absent PGK1 coding sequence.

Restriction sites:  Bg = BglII, H = HindIII, Xn = XmnI
pr = promoter, t = terminator
pPGK1  APT  tPGK1

H  Xn  Bg  H

......AGTAATTATCTACTTTTCAGATCAGGGGTGTT ATG.......  APT1
mRNA  Bg\Bc  junction
start

......AGTAATTATCTACTTTTCAGATCAGGAGGTTACC ATG.......  APT2
mRNA  Bg\Bc  junction
start
orientation of the APT cartridge was checked by restriction with SmaI, present at one end of the cartridge, so that the correct (sense) orientation would be in the same direction as the promoter. In the correct orientation, the first initiator codon was AUG, the strongest translation initiation codon in yeast (Sherman and Stewart 1982); translation should initiate optimally and correctly at the APT initiator codon.

3.3.2 Incorporation of a yeast replication origin onto the heterologous APT cartridge plasmid

Plasmids to be employed for yeast transformation, are usually constructed so that they can be propagated in either E.coli or yeast. For this reason they normally include pBR322 sequences for replication and selection in E.coli and sequences which allow replication in yeast, in addition to a yeast selectable marker. In the form of plasmid pCH137, the plasmid would be unable to be replicated in yeast.

A number of replication origins have been incorporated into plasmids, but the most commonly employed is the 2μm circle replication system, which enables plasmids to be stably maintained. Plasmids have been designed which incorporate the entire 2μm sequence, for example the vector pDB219 (Beggs 1978), due to the large size of these vectors, most vectors contain only those sequences essential for autonomous replication, namely the 2μm origin and STB region. The latter region is required for efficient partitioning of plasmid DNA between the mother and daughter cells during cell division. The proteins required for plasmid replication are provided in trans from the nuclear genome and the endogenous 2μm plasmid within the yeast strain (Broach and Hicks 1980). For this reason, the yeast strain employed to harbour the plasmid must contain 2μm; termed cir⁺.

The plasmid pCH147 containing the PGK1 promoter/APT coding sequence/terminator cartridge described in section 3.3.1, was modified so that it contained a 2μm origin of replication and a STB locus, in addition to a LEU2 auxotrophic marker. These would allow the plasmid to be maintained, replicated and selected for in yeast; the pUC region (containing pBR322 derived sequences) provided sequences for replication in E.coli and ampicillin resistance for selection.
Figure 3.3.2.1  Multi-copy plasmids containing the APT cartridge

Plasmids pBEJ16 and pBEJ17 are identical plasmids except that the LEU2 marker and 2μm sequences are in the opposite direction. The plasmids were constructed by the insertion of a 3.7kb PstI fragment from plasmid pYRG47, containing the LEU2 auxotrophic marker and a 2μm fragment containing ori and STB into the PstI site of a pUC19 based plasmid containing the PGK1/APT coding cartridge. Transformants were isolated containing the fragment in both orientations, identified by fragment size differences on an agarose gel.

Plasmid pBEJ15, was constructed in the same manner as the other plasmids except the PstI fragment was cloned into a plasmid containing the PGK promoter and terminator but lacking the APT coding sequence.

Distances between the restriction sites are shown in bp.

Restriction sites: A= A\text{val}, B= \text{BamHI}, Bg= \text{BglII}, E= \text{EcoRI}, Ev= \text{ECoRV}, H= \text{HindIII}, K= \text{KpnI}, P= \text{PstI}, S= \text{SalI}, Sm= \text{SmaI}, Sp= \text{SphI}, Ss= \text{SstI}, Xb= \text{XbaI}, Xn= \text{XmnI}
a

pBEJ17

LEU2
ori
STB

898

686

386

930

8.02 Kb

pPGK1

1410

2.7 Kb

pUC19

b

pBEJ15

8.02 Kb

pPGK1

1410

2.7 Kb

pUC19
A 2 μm ori-STB region, accompanied by a yeast LEU2 marker was initially isolated from plasmid pYRG47 (P. Meacock) by PstI endonuclease restriction to yield a 3.7 kb fragment. This was ligated into the PstI site present in the pCH147 plasmid. Its orientation was determined by EcoRI restriction, which yielded two fragments of 7.1 kb and 0.9 kb. This plasmid was designated pBEJ16 (Fig 3.3.2.1). The PstI fragment was also inserted in the opposite orientation for comparison purposes, which was differentiated from the other orientation since EcoRI restriction resulted in two fragments of 5.4 kb and 2.6 kb. This was designated plasmid pBEJ17. The PstI fragment was also ligated into plasmid pCH137 which lacked the APT cartridge to give plasmid pBEJ15 (Fig 3.3.2.1).

3.4 Performance of the PGK1 promoter driven APT expression construct as a G418 resistance determinant in E. coli and S. cerevisiae

To ascertain whether the G418 resistance shuttle marker could function effectively, transformations were performed in S. cerevisiae and then into E. coli to determine if the marker could be successfully employed.

3.4.1 Transformation of the vectors in yeast.

To determine to what extent a dominant selectable marker phenotype could be obtained with the heterologous APT gene construction, the plasmids pBEJ16 and pBEJ17 were transformed into S. cerevisiae. YPD medium was employed to select colonies for G418 resistance since minimal medium contains salts which appear to inhibit antibiotic uptake into the yeast cell (Webster and Dickson 1983), resulting in the failure to suppress the growth of non-transformed cells. Minimal medium, as a result cannot be employed for the selection of G418 resistant transformants.

Natural resistance to G418 of haploid laboratory S. cerevisiae strains is found to be very similar, but industrial S. cerevisiae show differing sensitivity. Growth of the untransformed S. cerevisiae strain S150-2B employed for the transformation, was found to be effectively and completely inhibited by G418 at a concentration of 500 μg/ml in YPD media. This was also found to be the concentration which inhibited the growth of S. cerevisiae haploid
TABLE 1  Growth of *S. cerevisiae* strains on YPD agar containing different concentrations of G418

a) **Haploid laboratory strains**

<table>
<thead>
<tr>
<th>mg/ml G418</th>
<th>S158-2B</th>
<th>DBY746</th>
<th>MDY48-4c</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.25</td>
<td>( \approx 10^2 )</td>
<td>( \approx 10^2 )</td>
<td>( \approx 10^2 )</td>
</tr>
<tr>
<td>0.5</td>
<td>( 10^8 )</td>
<td>( 10^8 )</td>
<td>( 10^8 )</td>
</tr>
<tr>
<td>1.0</td>
<td>( 10^8 )</td>
<td>( 10^8 )</td>
<td>( 10^8 )</td>
</tr>
</tbody>
</table>

b) **Industrial Strains**

<table>
<thead>
<tr>
<th>( \mu g/ml ) G418</th>
<th>ALE (top)</th>
<th>ALE (Bottom)</th>
<th>DISTILLING</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>25</td>
<td>( \approx 10^2 )</td>
<td>( \approx 10^2 )</td>
<td>0.1</td>
</tr>
<tr>
<td>50</td>
<td>( 10^9 )</td>
<td>( 10^9 )</td>
<td>( 10^9 )</td>
</tr>
<tr>
<td>100</td>
<td>( 10^9 )</td>
<td>( 10^9 )</td>
<td>( 10^9 )</td>
</tr>
</tbody>
</table>
laboratory strains DBY746 and MD40-4C (Table 1). Brewing strains, however, were inhibited by G418 levels of only 25 μg/ml (Sakai and Yamoto, 1986).

Plasmids pBEJ16 and pBEJ17 were transformed into S. cerevisiae strain S150-2B by the lithium acetate method described in section 2.4.1.2, including plasmid pMP81 (Hollenberg 1982), containing the unmodified bacterial transposon Trn903 (Fig 3.4.1.1) as a control. To determine the relationship of expression time with the number of G418 resistant transformants, a number of identical transformations were set up so that a range of incubation times could be investigated. In determining this relationship, the incubation time required to obtain the optimum number of transformants could be determined.

Following transformation, the cells were divided into two equal volumes. To one volume, 2 volumes of YPD broth were added and the cells incubated at 30°C for the required time, after which time the cells were plated onto YPD plates containing G418 at 500 μg/ml. The plates were subsequently incubated at 30°C. 2 volumes of YPD were employed rather than 3 or 4 volumes since the results obtained with these differing volumes were the same and 2 volumes were easier to handle.

In addition to G418 selection, transformants were also selected for the LEU2 auxotrophic marker on the plasmid. In this instance the other volume of cells were incubated for the same length of time as the YPD containing cells, after which an equal volume of sterile water was added to the cells to aid plating. The cells were plated onto minimal plates lacking leucine and incubating at 30°C.

Resistant transformant colonies were found to appear on the YPD/G418 plates after 36-48h. The frequency of G418 resistant colonies appearing after various post-incubation times is shown in Fig 3.4.1.2a. The number of G418 resistant transformants apparent on selective plates after a 2 hour post-transformation incubation with YPD together with data obtained for the auxotrophic marker is shown in Table 2.

It was apparent that G418 resistant colonies could be obtained by direct selection and that transformants could be obtained after as little as 30 minutes, although to obtain the maximum number of transformants a post-expression time of 1 hour or more was necessary. As a
Figure 3.4.1.1  Plasmid pMP81

This plasmid contains the unmodified bacterial transposon Tn903, from which the APT coding sequence is derived. It is based on *E. coli* plasmid pCR1.

(Hollenberg, 1982)

Figure 3.4.1.2

**Frequency of G418 resistant transformants with varying post-transformation expression time**

![Graph showing the frequency of G418 resistant transformants with varying post-transformation expression time.](image)

TABLE 2  The number of G418 resistant transformants obtained after a post-expression time of 2 hours and auxotrophic transformants

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>NUMBER OF TRANSFORMANTS/µg DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G418&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pBEJ16</td>
<td>784</td>
</tr>
<tr>
<td>pBEJ17</td>
<td>612</td>
</tr>
<tr>
<td>pMP81</td>
<td>0</td>
</tr>
</tbody>
</table>

* : All values shown are the average of three repetitions

Variation = ±10
consequence a post transformation expression time of 1.5 hours was subsequently employed in all future APT transformations rather than 1 hour, to ensure that the number of transformants was indeed optimal. On comparison of the transformation efficiencies of the G418 marker with those of the LEU2 auxotrophic marker, the efficiencies were very similar although the G418 marker did provide slightly more transformants. A slight difference was found between the pBEJ16 and pBEJ17 plasmids with both the G418 resistance marker and the LEU2 marker, which presumably is due to the conformational difference in the plasmids composition.

LEU2 resistant transformants were obtained with the control pMP81 plasmid containing the unmodified Tn903, but no directly selectable G418 resistant transformants were obtained with up to 3 hours post-transformation expression in YPD. A secondary G418 resistance phenotype was obtained when LEU2 resistant colonies were replica-plated onto YPD/G418 plates, which indicated some APT expression. This reflects the previous work of Webster and Dickson (1983) in which the cells required greater than 18h post-transformation incubation to produce G418 resistant transformants.

The ability to directly select G418 resistant transformants with only a 60 minute post-transformation incubation in addition to a comparable number of transformants to the auxotrophic marker, indicated that increased APT expression had been obtained.

3.4.2 Transformation of the vectors into E. coli

Since E. coli cells are not inhibited by G418, to enable selection of cells containing the APT gene, kanamycin, a related aminoglycoside antibiotic had to be employed.

During construction of the pBEJ16 and pBEJ17 plasmids, the plasmids were transformed into E.coli strain JM83 and selected with the Ampicillin resistance determinant present in the pUC19 portion of the plasmid. If transformants were selected directly on kanamycin plates, containing 10 µg/ml kanamycin, resistant colonies were not obtained. This may have been a consequence of failure to express the APT gene in E.coli, due to the absence of fortuitous E. coli promoter activity. Alternatively, expression of the APT gene may occur, but was not sufficient to provide a directly selectable kanamycin resistance phenotype. To test this
Transformants from the parent plasmid pBEJ15, which lacked the APT cartridge (acting as a control) and those from plasmids pBEJ16 and pBEJ17, were initially selected via the ampicillin resistance marker present on the plasmids. 100 colonies from the resultant ampicillin resistant transformants for each plasmid, were then picked onto kanamycin plates, which were subsequently incubated at 37°C overnight. No kanamycin resistant colonies were obtained from pBEJ15 as expected, since it did not contain the APT gene, but all colonies carrying pBEJ16 and pBEJ17 were kanamycin resistant. This indirect secondary kanamycin resistance phenotype indicates the build-up of a resistance phenotype indicative of the accumulation of the APT enzyme due to a low level of expression. Importantly, however, this secondary resistance phenotype could be used to score recombinant clones in subsequent manipulations involving the marker, an advantage over other markers available.

3.4.3 Growth, stability and copy number of G418 resistant transformants.

In producing a heterologous APT marker, it would be important to establish that growth of cells containing the marker were not affected by expression of APT protein and additionally to determine the number of copies of the plasmid which are present in the cell. A further important factor would be to determine that the rate of loss of the plasmid containing the APT gene was not excessive. This would ensure that during selective growth, cells containing the plasmid would not be lost from the cell population, an important factor for a dominant marker. For this reason, the following work was performed.

3.4.3.1 Growth and viability of cells containing the APT gene.

*S. cerevisiae* G418 resistant transformants were grown in YPD/G418 medium alongside an untransformed $S_{150}$-2B control. All were found to have a doubling time of 90 minutes (Fig 3.4.3.1). A comparison of the cells under the microscope, also revealed no apparent differences between both the transformed and untransformed cells. It was therefore concluded that the expressed APT protein had no adverse effect on the host.
3.4.3.2 Plasmid stability

The stability of pBEJ16 and pBEJ17 transformed cells was investigated in rich YPD medium containing 500 μg/ml G418, minimal selective medium and also non-selective medium over 10 generations. Results obtained are shown in Table 3.

As in the transformation data, the pBEJ17 plasmid was found to be slightly less stable than the pBEJ16 plasmid. The pBEJ16 plasmid was found to be most stable in rich selective medium, being lost at a rate of 0.2% per generation, which increased to a 1.2% loss per generation in non-selective medium. Losses of 2μm plasmids, containing all of 2μm, has been reported to be at a rate of 0.01% for every cell division (Armstrong et al, 1989), although the addition of other yeast sequences is known to make plasmids slightly more unstable (Futcher and Cox, 1983).

3.4.3.3 Determination of the plasmid copy number of pBEJ16 and pBEJ17

In determining the copy number of plasmids, the method employed needs to be both accurate and reproducible. The copy number determination method outlined in section 2.8.3, is accurate but there are a number of steps at which inaccuracies could result. The initial isolation of total genomic DNA, is an important step and it is critical that the method employed for isolation, does not result in the loss of plasmid DNA. The method employed by Cashmore et al (1988), described in section 2.8.2 and used in the copy number determinations throughout this work, has been shown not to result in plasmid DNA loss. A further important stage is DNA restriction, since the DNA must be fully restricted to enable accurate determination. Full restriction of this type of DNA can be difficult owing to the presence of carbohydrate, but it can be achieved by the use of enzymes to break down any isolated carbohydrate and large excesses of restriction enzymes. A very critical part of the method used for copy number determination is the determination of the ratio between the plasmid and chromosomal bands on the filter onto which the DNA was transferred and probed. Following autoradiography of the filter at various exposures, two procedures can be undertaken to measure the ratio, which will be dependent upon the clarity of the resultant autoradiograph. The autoradiograph can be densitometer scanned, scanning each track in turn then calculating the ratio of the peaks obtained, which would correspond to the bands on the autoradiograph. An alternative method described in section 2.8.3, involves using the
### TABLE 3  
Stability of plasmids pBEJ16 and pBEJ17, in selective and non-selective media

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>% PLASMID STABILITY OVER 10 GENERATIONS *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SELECTIVE MEDIA</td>
</tr>
<tr>
<td></td>
<td>YPD/G418 medium</td>
</tr>
<tr>
<td></td>
<td>YPD medium to YPD/G418</td>
</tr>
<tr>
<td>pBEJ16</td>
<td>97.7</td>
</tr>
<tr>
<td>pBEJ17</td>
<td>96.2</td>
</tr>
</tbody>
</table>

* : All values shown are the average of two repetitions on two different transformants. Variation = ± 5%

**Figure 3.4.3.1**

- Growth of cells containing plasmid BEJ16 in YPD/G418 medium compared to untransformed S150-2B cells in YPD
autoradiograph, to mark the band areas on the filter which can then be excised and scintillation counted. Both methods can be prone to errors. The densitometer method may only be employed for autoradiographs that are clean, which will therefore be dependent on the DNA, its restriction, and the hybridisation. The band areas should not be too overexposed or too faint otherwise again inaccuracies can result. In the scintillation method, it is critical that the autorad is correctly orientated over the filter so that the correct band areas are excised, if not an inaccurate count would be derived. It is also important that the filter is clean. Smearing or a lot of background will decrease count accuracy. On scintillation counting, it is known that static can interfere with the counting so all vials must have static removed prior to counting, additionally the bands should be counted at least three times. In both cases, copy number should be measured at least three different times.

The copy number of the pBEJ16 and pBEJ17 plasmids was determined (essentially as described in section 2.8.3), probing the filter with a $\gamma^{32}P$ labelled 2.2kb LEU2 fragment, isolated by PstI restriction of plasmid pYEP13 (Appendix). The resultant autoradiograph is shown in Fig 3.4.3.2. The ratio of the plasmid band to the chromosomal band was then determined by both the densitometer procedure and the counting procedure.

Copy number data obtained for plasmids pBEJ16 and pBEJ17 using both methods, indicated little difference between both. On repeating the process for another filter containing the same plasmids again a similar result was obtained. Data is shown in table 4. This indicated that with a good autoradiograph, reproducible data could be obtained. The copy number of pBEJ16 and pBEJ17 was found to be 18 in both minimal and rich selective media.

Additional plasmid copy number determinations for other plasmids described in later chapters were also performed using both scintillation counting and autoradiograph scanning and these are also shown in Table 4.

3.5 Determination of APT enzyme protein levels

For use of the APT gene as a reporter gene, it would be necessary to have a means by which levels of APT enzyme could be determined. Additionally, determination of the protein levels would enable a better understanding of the amount of APT protein required to provide
Figure 3.4.3.2  Plasmid copy number determination of cells transformed with plasmid pBEJ16 and pBEJ17

Southern blot of total genomic DNA from *S. cerevisiae* S150-2B cells transformed with plasmids pBEJ16 and pBEJ17 and grown in either YPD/G418 medium or minimal selective medium (HUT). DNA was restricted with *SalI* and *XhoI*, separated on a 0.8% TAE/agarose gel then blotted as described in section 2.8.5 onto a Hybond-N filter. This was then hybridised overnight at 65°C in Church Gilbert hybridisation solution with a labelled 2.2kb LEU2 fragment probe. The LEU2 fragment was isolated by *SalI* and *Xho* restriction of pYEP13.

In the tracks shown, the lower band (1.4kb) represents single-copy chromosomal LEU2; the upper band (2.2kb) represents the multicopy plasmid LEU2. Regions corresponding to the bands were excised from the filter and scintillation counted.

Lane 1, 6 = untransformed S150-2B
Lane 2, 7 = pBEJ16 YPD/G418
Lane 3, 8 = pBEJ17 YPD/G418
Lane 4 = pBEJ16 HUT
Lane 5 = pBEJ17 HUT
TABLE 4  A comparison of plasmid copy number data obtained by scintillation counting a probed filter and by scanning an autoradiograph, for plasmids pBEJ16 and pBEJ17 and also other plasmids described in later chapters.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Plasmid copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scintillation Counted</td>
</tr>
<tr>
<td>pBEJ16 (HUT)</td>
<td>18</td>
</tr>
<tr>
<td>pBEJ16 (G418)</td>
<td>20, 19</td>
</tr>
<tr>
<td>pBEJ17 (HUT)</td>
<td>16, 18</td>
</tr>
<tr>
<td>pBEJ17 (G418)</td>
<td>19</td>
</tr>
<tr>
<td>pBEJ32</td>
<td>21, 17</td>
</tr>
<tr>
<td>pBEJ44</td>
<td>18</td>
</tr>
<tr>
<td>pBEJ38 (G418)</td>
<td>18, 20</td>
</tr>
<tr>
<td>pBEJ38 (HUL)</td>
<td>17, 18</td>
</tr>
</tbody>
</table>
resistance to G418. Initially protein gels were employed in order to determine the amount of protein.

Protein extracts, were prepared (as in section 2.10.1) from S150-2B cells transformed with pBEJ16, and separated on a 12.5% SDS-Polyacrylamide gel (section 2.10.3), alongside an untransformed control extract. After Kenacid-blue staining (section 2.10.4) and destaining only a very faint 30.9 kd APT protein band was discernible, which was absent in untransformed cells (Fig 3.5.1). This represented only a very small percentage of the total cell protein and indicated that the level of expression was very low. Protein gels were therefore insufficient for the determination of the amount of APT protein expressed in the cell and a more accurate measure would be required. For this reason, it was necessary to develop an assay which could be easily employed to provide accurate APT expression data.

3.5.1 **Development of the APT enzyme assay.**

A number of assays had been reported for use with aminoglycoside phosphotransferase activity first described by Ozanne et al (1969). The assays were based on the mode of action of the enzyme, in that, they assayed the transfer of labelled $\gamma^3P$ phosphate to the 2-deoxystreptamine moiety at the 3' hydroxyl side group of the antibiotic. The antibiotics possess a positive charge and are therefore able to bind to ion-exchange paper such as phosphocellulose. The labelled antibiotic remains bound to the paper whilst the unreacted cofactors, for example $\gamma^3P$ ATP are washed off. This method was found to be the most sensitive and convenient assay to use on comparison with other available techniques (Davies and Smith, 1978).

The initial documented method of Haas and Dowding (1975) was found to be only semi-quantitative, since the ATP consuming reactions present in crude extracts were not eliminated and thus interfered with the assay. To eliminate the ATPase activity, the APT enzyme was partially purified by passing the extract down a Sephadex G200 column (Jimenez and Davies, 1980). This would however, prove particularly laborious and costly if a number of assays were performed. An alternative approach performed was to run crude protein extracts on a
200μg of total soluble cell protein isolated from cells containing untransformed S150-2B cells and cells transformed with plasmid pBEJ16 were subject to SDS-Polyacrylamide gel electrophoresis, using a 15% resolving gel. The size markers employed were rabbit phosphorylase a (92 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29.5 kDa) and trypsin inhibitor (20 kDa).
denaturing polyacrylamide gel, which was subsequently covered with agarose containing kanamycin and α<sup>32</sup>P ATP (Reiss et al., 1984). The resultant labelled derivative was immobilised on phosphocellulose paper which was exposed to autoradiography. Again, the method was laborious, but it was determined that the amount of label incorporated into the Kanamycin bound onto the P81 paper, directly correlated with the amount of α<sup>32</sup>P used.

In addition to the APT being employed as a marker, it has also the potential to be employed as a reporter gene. The enzyme assay method should therefore be easy to perform and relatively quick, but more importantly quantitative and reproducible. The previous documented methods do not fit these criteria and an alternative approach was considered. The method, again exploited the positive charge on the antibiotic and its immobilisation on phosphocellulose (P81) paper, but considered an alternative approach for the removal of the contaminating substrates. It was considered that the most troublesome component in the assay would be the protein kinases and other ATPases. Protein kinases could be eliminated, if present, by protease treatment resulting in their degradation, whilst any remaining background activity could be detected by including an untransformed protein extract as a control.

The APT assay reaction mix contained:

10mM Tris-Cl pH 7.5,
10mM MgCl₂,
1mM DTT,
400mM NaCl,
30mM NH₄Cl,
0.3mg/ml G418 and 1mM ATP pH 7.0.

The latter contained labelled γ<sup>32</sup>P ATP (supplied by Amersham, specific activity 3000 Ci/mmol) and cold ATP in the ratios of 1 γ<sup>32</sup>P ATP to 1976 cold ATP. This was incubated with 10 μg protein extract in a total volume of 33μl, i.e. 20μl reaction buffer + 3μl G418 solution + 10μl protein extracts. In all instances the protein extract was diluted in APT extraction buffer to give a 1μg/μl solution. It was found that it was important to keep the protein concentration constant.

The reaction mixture was incubated at 35°C for 20 minutes which was found, over time-course reactions, to be the optimal incubation time and the time over which the reaction rate was found to be linear. The reaction was stopped by the addition of 5μl of 10% SDS and 5μl
10 mg/ml proteinase K which had to be freshly made to ensure maximum activity, and the incubation continued at 35°C for a further 30 minutes.

Following the incubation period, the reaction mixture was spotted onto 2x 1 cm² squares of Whatman P81 phosphocellulose paper. The squares were then allowed to air dry for 30 seconds and then washed in sterile water heated to 80°C for 2 minutes. This was followed by 3 further washes in room temperature water. The paper squares were then dried under a heat lamp for 20 minutes and were then placed into scintillation vials containing non-aqueous scintillation fluid. The cpm obtained for the background control were then subtracted from the cpm of the transformed protein samples and subsequently converted to cpm/μg cell protein/min reaction time. If high background cpm were obtained with the untransformed S150-2B sample, this could indicate that the proteinase K step was not functioning correctly. The values obtained were then converted to APT units, representing pmols of phosphate transferred to G418 per minute, per mg cell protein using cpm per pmole ATP as a conversion factor. This was obtained using the concentration of radioactivity provided by the supplier, scintillation counting a known amount, then calculating the cpm per pmole thus obtained.

3.5.2 APT activity of the heterologous plasmids.

The above assay was employed to determine the APT enzyme activity of the pMP81 plasmid construction and plasmids pBEJ16 and pBEJ17. The assay was performed on three separate isolates and the data obtained shown in Table 5.

As expected, APT activity in pMP81 is very low reflecting the inability to obtain directly selectable G418 resistant transformants. Plasmids pBEJ16 and pBEJ17 on the other hand, gave very good enzyme activity, found to be greatest in rich selective medium. As reflected in the transformation and stability data, pBEJ16 gives the greatest APT activity; the amount expressed in minimal medium is the equivalent to that obtained in rich medium with pBEJ17, whilst the activity in minimal is approximately 4 fold more than pBEJ17. Again, why this should be so is not clear.

The data obtained, therefore indicated that although good APT activity was obtained, the protein is not very abundant.
TABLE 5  APT expression of cells containing plasmids pBEJ16 and pBEJ17 in minimal and YPD/G418 media

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>APT UNITS/mg CELL PROTEIN</th>
<th>YPD/G418 medium</th>
<th>Minimal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMP81</td>
<td>15</td>
<td></td>
<td>nd</td>
</tr>
<tr>
<td>pBEJ16</td>
<td>3010</td>
<td>2384</td>
<td></td>
</tr>
<tr>
<td>pBEJ17</td>
<td>2149</td>
<td>670</td>
<td></td>
</tr>
</tbody>
</table>

nd = Not done

*: All values shown are the means of three repetitions
3.5.3 Determination of the relationship between APT units and the % of total cell protein equivalent to APT

It was considered that although the APT unit data obtained from the assay, provided a means of estimating the APT enzyme activity in the cell, the ability to equate these units to the percentage of total cell protein which this constitutes, would provide more useful information. In order to determine this relationship, it would normally be necessary to purify the enzyme, then assay the activity of varying dilutions of the pure enzyme. This would however be very time-consuming and as a result the strategy adopted was to overexpress the APT enzyme, which could be separated on an acrylamide gel, stained, then densitometer scanned. A value would be obtained, which would relate the amount of APT protein to the total cell protein obtained. Dilutions of the APT protein extract could be made and these assayed to provide APT units, which could ultimately be equated to the percent total protein. As a final step the percentage APT protein could be plotted against APT units.

3.5.3.1 Overexpression of the APT protein

To facilitate the overexpression of the APT protein, which was the strategy outlined previously, an E. coli vector, pTTQ18 (Stark 1987) was employed, which had been constructed to allow the regulated expression of genes.

The pTTQ18 vector contains a polylinker/lacz α region flanked by a strong trp-lac (tac) promoter. Expression from the promoter is prevented until it is induced by IPTG (isopropyl-β-D-thiogalactopyranoside) due to the presence of a lacI² allele for the lac repressor. The APT2 cartridge (described in 3.2.1) was employed for the overexpression, since it had an improved translation capability for APT transcripts in E. coli, owing to a larger bacterial 70S ribosome binding site. Translation will initiate at the same point as in yeast and thus the protein which is produced will be the same as that which is expressed in yeast. The APT2 cartridge was on a BglII/BclI fragment and was cloned downstream of the tac promoter utilising the BamHI site present on the vector (Fig 3.5.3.1a). The resultant plasmid was subsequently transformed into E. coli strain 5K. The APT gene was induced in a number of transformants by the addition of IPTG into the growth medium and crude protein extracts prepared from the transformed E. coli cells. 10μg of the extract was separated on an SDS-
Figure 3.5.3.1

a) Plasmid pTTQ18 employed for the overexpression of APT protein

To overexpress the APT protein, the APT2 cartridge on a BgII/BglII fragment was inserted into the BamHI site of the pTTQ18 plasmid (M. Stark), which was downstream of the tac promoter.

Restriction sites: B = BamHI, Bc = BclI, Bg = BglII, E = EcoRI, H = HindIII, K = KpnI, P = PstI, S = SalI, Sm = SmaI, Sp = SphI, Ss = SstI, Xb = XbaI

b) Analysis of *E. coli* total cell protein, from untransformed cells and cells transformed with pTTQ18-APT2 induced with IPTG

SDS-polyacrylamide gel electrophoresis of APT protein in soluble cell protein from *E. coli* cell extracts, using a 12.5% resolving gel. 25 μg of total soluble cell protein from the untransformed cells and 10 μg of transformed cell extract was loaded per track

Track 1 = pTTQ18-APT2 transformed cells induced by IPTG
Track 2 = untransformed *E. coli* 5K cells.

The size markers employed were rabbit phosphorylase a (92 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29.5 kDa) and trypsin inhibitor (20 kDa).
A

274  Sm  240  H  462

Bc  APT 2  Bgl

E  Ss  K  Sm  B  Xb  S  P  Sp  H

pTTQ18

Amp R

ori  ptac  lacI  AosI

t2  t1

Hpal  Ev

lacIQ

2.70 Kb

B

1  2

APT→

-45.0

-29.5

-20.0
polyacrylamide gel, alongside 25μg of an untransformed *E. coli* control with appropriate size markers. On staining, the over-expressed 29.9kD APT protein could be observed, which was absent in the untransformed control (Fig 3.5.3.1b). The gel was subsequently scanned on a densitometer and the APT protein determined as being present at 15.5% of the total cellular protein.

**3.5.3.2 APT assay to equate APT units to % APT protein**

To determine the relationship between APT units and % APT protein, a range of dilutions of the overexpressed *E. coli* extract were made, using the data previously determined, to give a range of APT protein levels in each sample.

The samples were subsequently assayed and APT units obtained. In order to give reproducible results in the APT assay, the protein content of the samples were kept constant by the addition of untransformed crude *E. coli* protein extract to give a 1μg/μl protein solution. It was also necessary to determine if the APT activity was affected by the presence of *E. coli* proteins and whether the data obtained could be directly compared to yeast APT data. If this was unable to be performed, it would be necessary to purify the APT protein. Consequently, non-yeast proteins were also included in the assay reaction by using these for dilution, which in addition to the *E. coli* protein was acetylated Bovine serum albumin and also untransformed *S. cerevisiae* protein extract. Data obtained from the assays is shown in Table 6. The results indicated a slight variation between the differing proteins but an underlying pattern could not be observed, indicating that the differences may have resulted from dilution error.

It was therefore concluded that the APT assay data was unaffected by the background proteins and thus the same APT protein expressed in either yeast or *E. coli* could be compared via enzymatic activity.

The data obtained from the dilution series was employed to plot a log graph of APT units /mg protein against percent APT (Fig 3.5.3.2). The graph was employed to equate the APT units obtained for the assay of pBEJ16 and pBEJ17 to the percent APT protein. This was found to constitute only 0.2 - 0.25% of the total cell protein in rich selective medium much lower than
TABLE 6 Results of an APT assay employing standard amounts of APT protein and varying types of protein extract for dilution

<table>
<thead>
<tr>
<th>µg APT PROTEIN</th>
<th>S150</th>
<th>BSA</th>
<th>NM522</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>22.0</td>
<td>23.0</td>
<td>24.5</td>
</tr>
<tr>
<td>0.5</td>
<td>17.0</td>
<td>25.0</td>
<td>38.0</td>
</tr>
<tr>
<td>0.1</td>
<td>9.0</td>
<td>18.0</td>
<td>11.0</td>
</tr>
<tr>
<td>0.05</td>
<td>5.0</td>
<td>6.5</td>
<td>8.5</td>
</tr>
<tr>
<td>0.01</td>
<td>3.4</td>
<td>4.65</td>
<td>1.85</td>
</tr>
<tr>
<td>0.005</td>
<td>2.85</td>
<td>0.85</td>
<td>0.55</td>
</tr>
<tr>
<td>0.001</td>
<td>0.65</td>
<td>0.40</td>
<td>0.35</td>
</tr>
</tbody>
</table>

* : All values shown are the means obtained from two determinations
Figure 3.5.3.2

Relationship between % APT and APT Units/mg cell protein

[Graph showing the relationship between % APT and APT Units/mg cell protein]
one would expect to be obtained from the PGK1 promoter. This was found not to be a consequence of the stability of the APT protein, since work by Hadfield et al., (1991), determined that the low APT product yield was not a consequence of rapid turnover of APT protein, since the half-life of the protein was found to be 7.5 h, similar to that of the PGK1 protein which has a half-life of 6-8 hours (Mellor et al., 1985). This was therefore eliminated as a reason for the low APT product yield.

3.6 Transcript analysis of plasmid pBEJ16

Low APT product yield could not be attributed to the stability of the protein but the amount of transcription emanating from the plasmids had not been determined. As a result, RNA was extracted from cells containing pBEJ16 (section 2.9.1) and untransformed cells and equal amounts run on an RNA agarose gel (2.9.2). The gel was Northern blotted (section 2.9.3) and the subsequent filter probed with an APT probe, isolated from plasmid pCH116. A discrete abundant transcript of 1.5kb (Fig 3.6) was obtained from plasmid pBEJ16, which indicated that transcription was not a cause of the low product yield.

3.7 A comparison of different translation initiation codons

Since the mRNA levels from the plasmids were not the major cause of reduced protein levels, the reduction may be a consequence of poor translation initiation coupled with a poor codon bias of the APT gene, which is 0.01.

Kozak, (1984) determined that of 95% of the messages examined, ribosomes initiate translation almost exclusively at the 5' proximal AUG codon. Previously it was considered that the first AUG provided the signal for ribosomes to recognise this region, but it was determined that the sequence context surrounding the AUG codon modifies the efficiency of the initiation at the codon. Yeast initiation regions maintain a conserved nucleotide sequence flanking the AUG and for optimal recognition and efficient utilisation of the AUG, a purine, normally an A residue is present at position -3. This was apparent in 75% of the messages examined (Werner 1987, Baim and Sherman 1988, Cigan and Donahue, 1988). A consensus sequence for yeast was therefore proposed, which differed slightly to the eukaryotic.
Figure 3.6 Transcript analysis of plasmid pBEJ16

Northern blot of total RNA isolated from untransformed S150-2B cells (-) and from cells transformed with plasmid pBEJ16. The APT transcript is shown by the arrow and its size from an RNA ladder was approximately 1.5 kb.

7μg of RNA was loaded per track and the gel was blotted onto Hybond-N nylon filter. The filter was then hybridised using a formaldehyde hybridisation solution and an APTl fragment probe.

a. (i) APT

\[ 1.5 \]
consensus. The consensus sequence for yeast is;

\[
5' \ A/Y \ A \ A/U \ A \ A \ U \ G \ U \ C \ U \ 3' \quad \text{(Cigan and Donahue 1987)}
\]

The APT1 gene employed on the plasmids was found to have the sequence;

\[
5' \ U \ G \ U \ U \ A \ U \ G \ A \ G \ C \ 3'
\]

Baim and Sherman (1988), observed that a U residue at position -1 and an A at -3 in the consensus sequence of an isocytchrome C gene reduced expression by 10-20\%. On analysis of the APT1 cartridge, a U residue was found to be present at -1 (although an A residue was not present at -3) and in addition the sequence was found to be very different to the consensus. This poorer translation initiation environment may be a reason for the poor translation. On isolation of the APT1 gene cartridge, an additional APT cartridge had also been isolated (section 3.2), which although having a U residue at position -1, was found to have a sequence that showed closer similarity to the yeast consensus. This sequence was;

\[
5' \ U \ A \ C \ C \ A \ U \ G \ U \ C \ U \ 3'
\]

To determine if translation initiation played a role in the poor translation of the APT gene, translation levels were compared on use of the more optimal consensus sequences.

3.7.1 Effect of the translation initiation environment on translation.

The expression of the APT protein from the APT1 and APT2 cartridges with the previously described sequences, was analysed to determine if the sequences did in fact have an effect upon translation initiation and translation efficiency. A comparison of the amount of APT protein obtained from both cartridges would give some indication of translation differences which could exist. The APT1 cartridge was therefore replaced by the APT2 cartridge in the pBEJ16 and pBEJ17 plasmid and also in a further plasmid pBEJ24 (described in Chapter 4), ensuring that only the one parameter had been changed. Following transformation, the transformed cells were grown in YPD/G418 medium and minimal selective medium, to a density of 1x10^7 cells/ml, including those cells containing the APT1 cartridge for comparison. An APT assay was then performed and data obtained is shown in Table 7.

On comparison of the amount of APT protein expressed from the plasmids containing the APT1 cartridge and APT2 cartridge, no significant differences were apparent and thus the sequence of the APT1 cartridge had no effect on translation.
3.8 An analysis of secondary structure

Since translation initiation was found not to cause a lower expression of the apt gene, it was considered that some other aspect of translation was responsible. A number of workers (Baim et al., 1985, Baim and Sherman 1988 and Cigan and Donahue, 1987), had determined that the introduction of sequences capable of forming hairpin loops into the CYC1 and HIS4 genes, caused the mRNA of the genes to be translated less efficiently, illustrating that secondary structure could be an important constraint in translation and could ultimately affect expression.

The extent to which translation is affected, is also dependent upon the stability of the hairpin and also its position relative to the AUG. For example a hairpin structure immediately downstream of an AUG, reduced translation two-fold (Baim and Sherman, 1988). A very stable secondary structure, with a thermal stability of -58 kcal/mol, inhibited translation completely since migration of the 40S ribosomal subunit was halted, whereas a weaker one of -7.6 kcal/mol was only partially inhibitory.

Since the previous observations indicated that translation could be affected if a secondary structure was present within the APT sequences, the possibility of potential secondary structure formation in these sequences were analysed by computer. The computer predictions revealed that neither cartridge had any significant potential secondary structure.

The low expression of the APT protein could not be explained in terms of poor transcription, poor translation, unstable protein or unstable plasmid and thus some other factor may be responsible. This lower heterologous protein expression had been previously observed by a large number of workers and for this reason further investigation was warranted. The APT gene cartridge was employed as a model heterologous gene for this purpose.

3.9 DISCUSSION

The bacterial transposon Tn903, encodes the G418 resistance determinant, aminoglycoside phosphotransferase, which provides resistance to aminoglycoside antibiotics, neomycin, kanamycin, in addition to geneticin G418. The resistance is provided by the inactivation of the antibiotic by the transfer of a phosphate group from cellular ATP, to the 3' hydroxyl
TABLE 7  A comparison of APT expression from plasmids containing either the APT1 or the APT2 cartridge

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>APT CARTRIDGE</th>
<th>APT UNITS/ mg CELL PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G418 medium</td>
</tr>
<tr>
<td>pBEJ16</td>
<td>1</td>
<td>3010</td>
</tr>
<tr>
<td>pBEJ18</td>
<td>2</td>
<td>3000</td>
</tr>
<tr>
<td>pBEJ17</td>
<td>1</td>
<td>2149</td>
</tr>
<tr>
<td>pBEJ19</td>
<td>2</td>
<td>2223</td>
</tr>
<tr>
<td>pBEJ24</td>
<td>1</td>
<td>3240</td>
</tr>
<tr>
<td>pBEJ25</td>
<td>2</td>
<td>2958</td>
</tr>
</tbody>
</table>
Tn903 had been employed in an unmodified form, on a multi-copy plasmid pMP81 (Hollenberg 1982), but on transformation into yeast, no primary G418 resistant transformants were obtained. This indicated that any fortuitous expression from the bacterial sequences was occurring at a very low level. This was confirmed by APT assay data which indicated a very low level of APT expression. This effect had been noted previously when the bacterial sequences on the Hygromycin B gene were not capable of driving sufficient levels of expression of the gene product in yeast (Kaster et al, 1984) and was also found when expressing the E.coli β-lactamase enzyme encoded by the Bla gene. The use of Saccharomyces promoters overcame this problem and increased gene expression in yeast resulted. The ADH1 promoter hooked to the β-lactamase gene resulted in a 100-fold increase in expression (Reipen et al, 1982), whilst increased expression of chloramphenicol acetyltransferase was also obtained when the CAT gene was put under the control of the ADH1 promoter (Hadfield et al, 1986). The elimination of bacterial sequences from a gene could also increase expression. This was observed when bacterial sequences from a Bacillus subtilis gene which included part of a 5' flanking region and a putative β-glucanase promoter, were removed and previously unseen expression of the gene was obtained (Cantwell et al, 1986). As a consequence of this information, the strategy adopted to express the APT gene, was to eliminate any unrequired bacterial sequences and in addition employ a yeast promoter to direct the expression.

The Tn903 transposon was successively trimmed down to produce a number of APT cartridges, which eliminated the unrequired bacterial sequences but retained the APT coding region. This was then cloned between the strong PGK1 promoter and terminator region. The promoter and terminator region had previously been employed, in this way to produce prochymosin, pre-prochymosin and chymosin from a multi-copy plasmid (Mellor et al, 1985). The terminator was employed to ensure the correct processing of the 3' end of the mRNA since this could affect mRNA stability and the overall level of gene expression (Zaret and Sherman, 1982). The latter point had been illustrated during expression of met prochymosin, which increased 10 fold on the addition of the PGK1 terminator (Mellor et al, 1985).

Following the incorporation of the APT1 coding region into a multi-copy plasmid construction and the subsequent transformation into E. coli, it was apparent that kanamycin resistant transformants could not be directly selected. This was considered to be due to the slower initiation of transcription by the PGK1 promoter than the Tn903 promoter in E. coli. It was
originally considered that eukaryotic promoters do not function in *E. coli*, since eukaryotic promoters require 3 RNA Polymerases compared to 1 in *E. coli* (Maniatis *et al.*, 1982), which would explain the poor activity. Recent data, however, demonstrated that this was not the case and a number of eukaryotic promoters could in fact operate in *E. coli*, although a yeast promoter was not considered (Antonucci *et al.*, 1989).

The presence of a yeast promoter in a bacterial environment, could therefore result in the slower establishment of the resistance phenotype, which was found to be the case. This was illustrated in that transformants initially selected for ampicillin resistance when replica-plated onto kanamycin medium, were found to be kanamycin resistant. Although the marker could not be used directly in *E. coli*, it could be employed as a secondary screen for transformant colonies.

In *S. cerevisiae*, the situation was different. It was found that transformation of the multi-copy plasmids into yeast, resulted in the ability to select G418 resistant transformants directly. Previous work using the Tn903 cartridge, had required a minimum post-transformation incubation of 16 hours to enable G418 resistant transformants to be selected (Webster *et al.*, 1983). In the multi-copy form, transformants could be selected directly after only 30 minutes post-transformation incubation in YPD medium, reaching a maximum efficiency after 2 hours incubation. The number of G418 resistant transformants selected in this way was found to be equivalent to that obtained when the auxotrophic LEU2 marker was employed. Previously, other antibiotic markers had resulted in transformation efficiencies 10 fold lower than the auxotrophic marker, for example the hygromycin B marker and the Tn903 cartridge; an exception was the sulphometuron methyl resistance marker. Resistant transformant colonies were additionally found to appear within 36 - 48 hours after incubation at 30°C, again in contrast to the hygromycin B marker which requires a 72 hour incubation (Gritz and Davies, 1983). Growth of the resistant transformants in G418 medium, indicated that the production of the APT protein, had no adverse effect on the cell, since the pattern obtained was equivalent to that of the untransformed *Saccharomyces* strain. The marker was also relatively stable, being lost at a rate of 0.2% per generation in selective medium, which increased to a loss of 1.2% per generation during non-selective growth. In most instances the marker would be employed under selective conditions and is therefore sufficiently stable for this use.

The use of APT1 as a dominant marker and a reporter gene, was also aided by the ability to
quantitatively determine the amount of APT enzyme expressed by the transformant colonies, easily and accurately. The assay developed was based on that of Haas and Dowding (1975), but by the addition of proteinase K and the use of untransformed control extracts, additional purification steps previously employed to eliminate contaminating protein kinases were not required. The proteinase K incubation would degrade any labelled kinases, which would ultimately be washed off the P81 paper, whilst the inclusion of an untransformed S. cerevisiae protein extract would detect any residual background activity. In addition to the assay data, the percent APT protein to which this related, could be provided via the relationship between the units and the percentage of the total protein, determined by dilution of a known amount of APT protein.

APT assay data of the two heterologous constructions, pBEJ16 and pBEJ17 indicated that the APT activity of pBEJ16 was greater than the corresponding construction pBEJ17. This was rather surprising, since the only difference between both, was the orientation of the LEU2 marker and the ori and STB region. In addition, no plasmid rearrangement had occurred. A further interesting point noted was that the activity in minimal medium against that obtained for the rich selective medium was not greatly different. It could be expected that the presence of G418 in the medium would result in increased APT expression to inactivate the antibiotic, which is observed in the case of the CAT gene which is inducible in the presence of sub-inhibitory concentrations of antibiotic (Dick and Matzura, 1990). This is, however not apparent, indicating that the APT enzyme is constitutively expressed.

The difference in activity of both constructions could not be attributed to a gene dosage effect, since both were found to be identical, nor to a difference in the stage at which the cells were harvested for protein extractions, since both were harvested at a cell number of 1x10⁶. Plasmid stability was also eliminated as a factor, since the difference in stability was insignificant and would not contribute to differences in the assay data obtained. No difference in copy number was observed between both plasmids and between the plasmids in differing media, being 18 in both cases. This was again eliminated as a reason for the APT protein level differences.

By use of the relationship between the APT activity and the percent APT protein, it was established that the protein expressed from the heterologous constructions was only in the range of 0.2-0.25% of the total cell protein. The PGK1 promoter had previously been
employed to direct expression of human interferon α2 (IFN2) on a high-copy number plasmid (Mellor et al, 1985), and yields of 1-3% of the total cell protein had been obtained. The amount of APT protein expressed from the BEJ16 and BEJ17 plasmids was therefore 4-12 fold lower, which was not attributable to a rapid protein turnover or greatly reduced mRNA levels. Levels of APT transcript from both plasmids was also investigated and it was determined that transcription form both plasmids was abundant and since discrete transcripts were also obtained, accurate transcription initiation occurred. This indicated that the low protein expression was a consequence of poor translation efficiency.

Translation of a message, is a complex event, which is considered to proceed via a scanning model proposed by Kozak, (1978, 1989). In its simplest form, the 40S ribosomal subunit after association with Met tRNA\textsuperscript{Met}, GTP and several initiation factors binds to a CAP structure at the 5' end of the mRNA, and migrates 3' along the 5' untranslated leader, until it finds an AUG, where it will then join with the 60S subunit and translation will commence. Kozak, (1984) determined that of 95% of the messages examined, ribosomes initiate translation almost exclusively at the 5' proximal AUG codon. Consideration of the translation initiation environment of the APT gene, revealed that the APT1 cartridge employed, did not have an optimal recognition sequence for efficient translation from the AUG codon, considered to be an A residue at -3 from the initiating AUG. An additional APT cartridge, namely APT2, had been constructed which contained this optimal sequence, but would still produce the same APT protein. Subsequent analysis of the constructions revealed that no significant differences in expression level were obtained, which indicated that in this instance, the initiation environment was not a major factor governing the expression level. In addition, secondary structure was not a problem in either cartridge.

The heterologous APT cartridges were found to provide an efficient G418 resistance phenotype in multi-copy form, but for wider use it would be advantageous to use the marker in an integrated single-copy form. Work by Hadfield et al, (1990), confirmed that the APT cartridge could be successfully employed in this form and G418 resistant transformants could be selected directly. An assay of APT activity indicated that expression varied dependent on the integrating construction and in addition revealed that the minimum APT activity required to enable direct selection of G418 resistant transformants, was 60 units. This represented only approximately 0.006% of the total cell protein as APT. Lower APT levels such as 15 units, as obtained by plasmid pMP81, enabled resistant transformants to be obtained indirectly, but
growth in selective medium was found to be impaired.

APT assay data obtained, indicated that the same heterologous APT cartridge, expressed varying amounts of APT protein, dependent upon how it was employed. In the single copy form, APT expression varied from 60 units, to a maximum of 700 units (Hadfield et al, 1990). APT expression from 20 copies of the plasmid was 3000 units, which for comparison purposes represented only 150 units of APT per cell copy. It is widely expected that on using a multi-copy plasmid to express a heterologous gene, expression is in excess of that obtained from an integrated copy of the gene. This data indicates that this may not always be the case. It could be that by increasing the number of copies of a heterologous gene in the cell that trans-acting factors are titrated out, resulting in a negative effect on expression, or that the location and orientation of the gene influences expression, some locations being better than others. These factors and other factors affecting gene expression will be considered in later chapters.
CHAPTER FOUR

DISCOVERY AND INITIAL CHARACTERISATION OF AN INHIBITION EFFECT ON EPISOMAL PLASMIDS CAUSED BY STRONG CHROMOSOMAL GENE PROMOTERS
The amount of protein expressed from a gene on a vector is dependent upon a large number of factors (discussed in section 1.3), one of which is the strength of the promoter used to direct expression of the gene. Promoter strength directly reflects the amount of mRNA produced as a result of transcription initiation at the promoter, which is in turn governed by the UAS (as discussed in section 1.2.1.1). The promoter regions of a number of *S. cerevisiae* genes such as PGK1 (Tuite et al, 1982; Dobson et al, 1982), ADH1 (Bennetzen and Hall, 1982; Hitzeman et al, 1981), GAP3 (Holland and Holland, 1980) and PYK (Burke et al, 1983), have been isolated and employed to express a number of heterologous genes, since expression of glycolytic gene products is high. The glycolytic proteins constitute 50% of the total cell protein within the cell. It had been observed, by a number of workers, however, that the addition of a strong promoter to drive heterologous gene expression does not guarantee high yields of the protein. It was found by Chen et al, (1984) and Mellor et al, (1985), that the expression of a heterologous gene using the PGK1 promoter, was a fraction of that obtained with the homologous PGK1 gene, with expression of interferon-α2 using the PGK1 promoter on a *leu2-d* (Erhart and Hollenberg, 1983) plasmid being less than 5% of total cell protein (Mellor et al 1985). This was also illustrated when using the PGK1 promoter to express APT protein, described in the previous chapter, when the maximum amount of protein obtained was only in the order of 0.2% of the total cell protein. PGK protein expressed when the PGK gene is present on a multi-copy plasmid is at least 50-fold more.

This lower expression, has been widely observed when such "strong" promoters have been used to direct expression of heterologous proteins. It was for this reason that further analysis of gene expression was undertaken to investigate why "strong" promoters employed to direct expression of heterologous proteins on plasmids in the cell were not able to direct higher levels of expression. The APT gene was employed, since results from Chapter 3 had indicated that this gene could be employed as a reporter gene, owing to three very useful reporter characteristics. Firstly, it can be expressed without a detrimental effect on the host yeast cell. Secondly, its expression can be easily detected by a powerful and sensitive radiolabelled enzymatic assay and finally, it also provides a dominant selectable marker in the form of resistance to the antibiotic G418, which therefore enables expression and plasmid vector performance to be monitored in rich selective medium, in addition to minimal selective medium.
Initial work investigated the effect of a number of promoters on the expression of the APT gene on the vector plasmid. During the course of this work, it became apparent that the promoters, in addition to causing an effect on expression, were also causing an effect on plasmid transformability and copy number.

4.2 Investigation of the effect of the promoters.

Since variable expression levels of a number of proteins had been found using different promoters to direct expression, the effect of this parameter on APT expression was investigated.

The APT1 coding sequence cartridge had been initially incorporated into a previously constructed plasmid, pCJ17 (C. Jones, unpublished) (Fig 4.2.1), which contained a TRPI auxotrophic marker in addition to a 2μm origin and STB region and a CYC1 terminator, giving plasmid pCJ18. This plasmid formed the basis of further studies as it contained upstream polylinker sites for insertion of promoters.

4.2.1 Promoters investigated.

Promoters investigated were from PGK1, ADH1 and CYC1. The ADH1 promoter (Bennetzen and Hall, 1982) directs the expression of yeast alcohol dehydrogenase iso-enzyme I, giving rise to 1-2% of total cellular mRNA in glucose-grown S. cerevisiae cells, whilst the CYC1 promoter (Guarente and Mason, 1983) directs the expression of iso-1-cytochrome c and is regulated by physiological signals such as heme and catabolite repression, being expressed at a greater level in lactate and glycerol than glucose or galactose medium (Struhl, 1986). The promoter fragments are illustrated in Fig 4.2.1.1. Two versions of the PGK1 promoter were employed, which differed in that one contained approximately 800 bp of DNA upstream of PGK1, which was absent from the other version. Promoters were all upstream of the APT cartridge and constructions were made as described in the figure legend of Fig 4.2.1.2. All the promoter fragments lacked a translation start codon, which was provided by the APT cartridge as a consequence of its design.
Plasmid pCJ18 was produced by ligation of a BellI and BellI fragment containing the APT1 gene into the BellI polylinker sites of plasmid pCJ17.

Restriction sites: A = Avail, B = BamHI, Bc = Bell, Bg = BellI, E = EcoRI, Ev = EcoRV, H = HindIII, K = KpnI, P = PstI, Pv = PvuII, S = SalI, Sm = Smal, X = Xhol.

Distances between restriction sites are shown in bp.
Figure 4.2.1.1   Sequence and structure of the promoters analysed.

The sequence of the promoter fragments which were investigated upstream of the APTl coding sequence are shown.

Arrows denote direction of transcription, smaller arrows denote transcription initiation sites.
Figure 4.2.1.2 Structure of the differing promoter constructions

Plasmid pBEJ24 was constructed by ligation of a BamHI - BglII fragment from pCH147 (Appendix), containing the PGK1 promoter and APT1 coding sequence into the BamHI site of plasmid pCJ17.

Plasmid pCH211 was constructed by ligation of an XmnI - BglII fragment from pCH147, containing a shorter PGK1 promoter and APT1 coding sequence, into the SmaI and BglII sites of plasmid pCJ17.

Plasmid pBEJ11 was constructed by ligation of a BglI - BamHI fragment from pCH116 containing the APT1 coding sequence, into the BamHI site of plasmid pCH97 (Appendix). This site was between the ADH1 promoter and CYC1 terminator of that plasmid.

Plasmid pBEJ1 was constructed by ligation of a SalI - BamHI CYC1 fragment isolated from plasmid pLG669-ATG (Appendix) (Guarente and Ptashne, 1981) into the SalI and BamHI sites of plasmid pCJ18.

In all instances correct orientation was confirmed by relevant enzyme restriction.
4.2.2 **Effect of promoters on transformability, expression, plasmid copy number and stability.**

Studies of the promoter containing plasmid constructions were embarked upon, to examine the influence of the different promoters on transformability, expression, stability and copy number. As a control and for comparison purposes the plasmid pCJ18, lacking a promoter was included in the analysis.

4.2.3 **Comparison of the transformability**

All the previously described plasmids were transformed into *S. cerevisiae* strain S150-2B using either the auxotrophic TRP1 marker or the heterologous APT1 gene for transformant selection. For the selection of TRP+ transformants, cells were plated immediately after heat shock, onto minimal TRP plates. For selection of the APT1 marker a post-transformation incubation in YPD medium of 90m prior to plating the cells on selective medium, was allowed for expression of the G418-resistance phenotype. The incubation time selected had been determined in section 3.4.1, using varying incubation times.

Data obtained from transformation is shown in Fig 4.2.3.1a. and is the average from more than three different transformations. In all cases the transforming DNA had been prepared using Caesium chloride gradients.

It was apparent with pCJ18 (no promoter), that a directly selectable G418^ phenotype could not be obtained with a 90m post-transformation incubation. A longer incubation of 6 h or 16 h, provided directly selectable G418^ transformants, with a greater number being obtained on the 16 h incubation (Fig 4.2.3.1b). In addition, all the transformants selected via the auxotrophic marker were found to be G418 resistant when picked onto plates containing 0.5 mg/ml G418. This indicated that APT enzyme was expressed within the cell, although at a low level and in addition, that the marker could be employed as a secondary selectable phenotype. This promoter-free expression must have resulted from background transcription (Marczynski and Jaehning, 1985).
**Figure 4.2.3.1a**  A comparison of the transformability of plasmids without a promoter and with varying promoters

![Bar graph showing the transformability of plasmids with different promoters](image)

Data shown is the average of at least 3 transformations. Variation is ±2%.

**Figure 4.2.3.1b**  Frequency of G418 resistant transformants with varying post-transformation expression time obtained with plasmid pCJ18
When expression of the APT gene was driven by the PGK1, CYC1 or ADH1 promoters, G418 resistant transformants could be directly selected after 90m incubation, indicating greater expression due to the presence of the yeast promoter. It was apparent however, that the number of resistant transformants obtained varied, depending upon which promoter was employed to direct APT expression. The greatest number of transformants were obtained with the PGK1 promoter, whilst only one transformant was obtained when the plasmid contained the CYC1 promoter. The ADH1 promoter construction also gave rise to very few resistant transformants. The shorter fragment PGK1 promoter construction yielded fewer transformants than the longer fragment version, possibly indicating interference with promoter activity due to absence of the upstream DNA, which may contain transcriptional terminators.

In the absence of a promoter, the number of auxotrophic transformants obtained was found to be in excess of $1 \times 10^9$ per µg of DNA. In the presence of a promoter, however, there was a marked reduction in the transformability of the plasmids on minimal TRP selection. This was found to be decreased by 5-10 fold, in all instances. The extent of the reduction varied between the promoters, but the trend was different to that observed for G418$^\circ$. The greatest reduction was found with the CYC1 promoter in this instance, with the least with the ADH1 promoter, which was however, still in the region of a 5-fold reduction. In addition, all of the TRP1$^+$ transformants selected were also G418$^\circ$. Several repetitions of the transformation were performed, and on each occasion the same effect was evident. This was not a consequence of the plasmid preparation since, although the actual number of transformants was slightly variable, the same trend was also observed using different plasmid preparations.

It was therefore evident, that somehow the promoters were influencing the transformability of the heterologous constructions, in a way that was affected by the culture medium. The reduction effects may have been caused by some type of interference owing to APT expression, since in promoter absence, transformability was improved. One obvious possibility is product toxicity upon the yeast cells. If this factor was responsible for the reduced transformability, it would be expected that growth of cells containing plasmids with the APT1 promoter-driven cartridge, would be much reduced. Growth of cells containing the APT1 cartridges was therefore examined.
4.2.4 Transformed cell doubling-time.

Cells transformed with the APT containing plasmids were inoculated into YPD/G418 medium and the doubling times for growth in this medium measured. The doubling time for cells transformed with plasmids pBEJ24 and pCH211 were found to be normal (90 minutes), with that of pBEJ1 increased slightly (100 minutes). Cells transformed with pBEJ1 failed to grow, which was found not to be due to plasmid loss or rearrangement. Since cell growth was normal in the presence of plasmids expressing large amounts of APT protein, product toxicity was discounted as an explanation for the reduced transformability.

The doubling time of cells containing pCJ18 (no promoter), in YPD/G418 medium was found to be 90 minutes, following an initial lag period of approximately 10-14 hours. This lag would be expected, since expression of APT on this plasmid was lower than those containing the promoter and thus the cells would need to accumulate APT protein before they could grow in this medium. Two transformants were grown in YPD/G418 medium, one selected using the TRP1 marker and the other selected on YPD/G418 plates, following a post-transformation incubation of 6 hours. It was evident that growth was very similar in both instances and surprisingly growth of the TRP+ selected transformant was slightly better. Since a lag period was required for growth of the APT selected transformant, this could indicate that the lag was not required for plasmid amplification, but indeed for protein accumulation. Absorbance readings of plasmid pCJ18 transformants inoculated into minimal and YPD/G418 media are shown in Fig 4.2.4.

4.2.5 APT expression from the promoter containing gene constructions.

To determine if the observed effects on plasmid transformability could be a consequence of expression levels from the various plasmid constructs, APT expression levels were determined for all plasmids in both minimal and rich selective medium. Three individual transformants were selected for each plasmid and grown to a density of 1x10^5 cells per ml, in both minimal selective medium and YPD/G418 medium. Cell-free soluble protein extracts were prepared and used in the APT enzyme assays (as described in section 3.5.1). The results are shown in Fig 4.2.5.
Figure 4.2.4

Growth of cells containing the promoterless APT gene containing plasmid pCJ18 in minimal and YPD/G418 media

* Transformant was selected using the TRP+ marker, others selected using the APT marker in G418 media
Figure 4.2.5

APT expression from plasmids containing different promoters

Data shown is the average of at least 3 repetitions
Variation is - 5
It was apparent that plasmid pCJ18 (lacking a promoter) expressed APT enzyme in both minimal and G418 medium, with expression in the latter being 5-fold greater. Expression levels in YPD medium without G418 (after initial growth in minimal selective medium), were found to be equivalent to that obtained in G418 medium, indicating that the presence of the antibiotic had no effect on expression yield. By use of the relationship between APT units and % APT protein (Section 3.5.3.2), expression from pCJ18 was found to be equivalent to approximately 0.002% of the total cell protein. This confirmed that background APT expression could occur without a yeast promoter, although the yield of product is rather low.

APT expression levels were found to differ dependent on the promoter employed, but was found to be 10 to 20-fold greater than that obtained in the plasmid not driven by a yeast promoter. 2-3 fold more APT enzyme was produced using the "long" PGK1 promoter than the shorter version in both rich and minimal medium, indicating that 5' non-PGK1 sequences influence expression level, possibly by acting as a block to incident background transcription. In YPD/G418 medium, expression with the ADH1 promoter, another trimmed-down promoter, was similar to that obtained with the short PGK1 promoter. Transformant plasmids with the CYC1 promoter failed to grow in YPD/G418 medium and so analysis of expression in rich medium could not be determined.

Performance was also found to be greatly influenced by the culture medium. Expression in minimal medium indicated that both the ADH1 and CYC1 promoters gave 5-10 fold more APT than that obtained from the PGK1 promoter. Additionally, expression from the ADH1 promoter was greater in minimal medium than in YPD/G418 medium, which was the converse to the other promoter constructs.

Expression levels in the rich selective medium, corresponded with the number of directly selectable G418\(^*\) transformants, which had been previously observed by Zhu et al, (1985). This further suggests that the APT product is not toxic to the host cells. Levels of APT expression in minimal medium, did not reveal any pattern in terms of amount of expression and the number of TRP* transformants.

Thus, the amount of APT within the cell did not correlate with the reduced transformation frequency. As no trend was apparent, some other factor such as plasmid stability and copy number would seem to be responsible.
4.2.6 The copy number and stability of cells containing the APT gene with different promoters.

4.2.6.1 Determination of plasmid copy number

Plasmid copy numbers were determined for cells grown under TRP\(^+\) minimal medium selection and also under YPD/G418 selection, by Southern hybridisation of total genomic DNA, as described in section 2.8.6. A 1.45 kb TRP1 fragment was employed as the hybridisation probe. This was isolated from plasmid pYRp7 (Appendix) by EcoRI restriction and labelled as described in section 2.6.8.1. Plasmid copy numbers were then determined as described in section 2.8.3. In all instances, copy numbers were determined for at least 3 individual transformants. Data obtained is shown in table 1 and some individual genomic DNA tracks in Fig 4.2.5.1.

The plasmid copy number for pCJ18, without the promoter driven APT cartridge, was determined to be in the order of 54 copies per cell in rich selective medium and approximately 25 copies per cell in minimal medium. Preliminary data in YPD medium without G418, indicated that plasmid copy number in this medium was 60 copies per cell. In addition, the plasmid copy number of pCJ18 did not increase when the concentration of G418 in the growth media was increased (C. Hadfield, unpublished). On promoter addition, the copy numbers of the plasmids were found to be significantly reduced and only in the order of 10-19 copies per cell. This number varied between the constructions, which could not be attributed to differences in the composition of the plasmids, since all the considered plasmids were based upon the same basic vector. This therefore indicated that the promoters may be responsible for the copy number reduction. Comparison between copy number reduction and expression (Fig 4.2.4.1), however, showed no correlation between the extent of the copy number reduction and the level of heterologous product (i.e. relative promoter strength).

4.2.6.2 Determination of plasmid stability

The stability of the plasmids was investigated (as described in section 2.8.4), by growth over 10 generations, which was considered sufficient to determine whether there was a significant effect on plasmid stability. Cells were grown in selective YPD/G418 and minimal medium and also in non-selective YPD. The results obtained are shown in Table 2.
TABLE 1  Plasmid copy number of cells containing APT plasmids with differing promoters in selective rich and minimal media

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>PLASMID COPY NUMBER*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YPD/G418</td>
</tr>
<tr>
<td>pCJ18</td>
<td>54</td>
</tr>
<tr>
<td>pBEJ24</td>
<td>18</td>
</tr>
<tr>
<td>pCH211</td>
<td>18</td>
</tr>
<tr>
<td>pBEJ1</td>
<td>fg</td>
</tr>
<tr>
<td>pBEJ11</td>
<td>19</td>
</tr>
</tbody>
</table>

* Copy numbers shown are the means of at least 3 copy number determinations, using both densitometer scanning and scintillation counting and represents the average plasmid copy number per cell. Variation was ±3 in most cases
Copy number determination of the promoter containing constructions.

Total genomic DNA from S150-2B plasmid-transformed cells, was restricted with EcoRI and Sall, then separated on a 0.8% TAE/EtBr gel. This was subsequently blotted onto Hybond-N filter membrane using the method of Southern and hybridised at 65°C overnight in hybridisation solution, with a 1.45 kb labelled TRP1 probe isolated by EcoRI restriction from plasmid pYRP7 (Appendix).

Numbered tracks contain genomic DNA from cells transformed with the named plasmids and grown in the stated medium.

- Track 1, 7 = pCJ18 YPD/G418 medium
- Track 2, 6 = pBEJ1 HUL medium
- Track 3 = pBEJ11 YPD/G418 medium
- Track 4 = pBEJ11 HUL medium
- Track 5 = pBEJ24 YPD/G418 medium
# TABLE 2  Plasmid stability of A\(^{+}\)T plasmids with differing promoters in selective and non-selective media

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>% PLASMID STABILITY OVER 10 GENERATIONS *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SELECTIVE MEDIA</td>
</tr>
<tr>
<td></td>
<td>YPD/G418 Medium</td>
</tr>
<tr>
<td>pCJ18</td>
<td>97.5</td>
</tr>
<tr>
<td>pBEJ24</td>
<td>76.4</td>
</tr>
<tr>
<td>pCH211</td>
<td>84.6</td>
</tr>
<tr>
<td>pBEJ1</td>
<td>fg</td>
</tr>
<tr>
<td>pBEJ11</td>
<td>95.8</td>
</tr>
</tbody>
</table>

fg = Failed to grow

*: All values shown are the average of at least three repetitions (± 1% variation)
The data indicated that although some variability between the constructions was evident, no construction was highly unstable. In all instances loss of the plasmid varied from 0.8%-2.4% per generation, which was not dependent on plasmid copy number. However, the promoter-free plasmid pCJ18 was very slightly more stable, which agreed with the observation of Panchal (1987), that there is a positive correlation between stability and copy number.

On the basis of these results, it is clear that plasmid instability was not a major factor in the reduced transformability and it would not appear sufficient to be responsible for the reductions in copy number and transformability observed. The presence or absence of G418 had no notable effect either, suggesting that the antibiotic was not toxic to the cells, even in the absence of a promoter (pCJ18) when the resistance enzyme APT was markedly lower.

4.3 DISCUSSION.

The transformation, into \textit{S. cerevisiae} of plasmid pCJ18, which contained the APT gene without an associated yeast promoter, was found to be very efficient when the TRP1 auxotrophic marker was employed for selection. In most instances, in excess of $10^7$ transformants per \(\mu\)g DNA were obtained. Addition of a yeast promoter upstream of the gene, however, resulted in a 5-10 fold reduction in the numbers of transformants obtained. Although the exact numbers varied, which reflected the competence of the \textit{S. cerevisiae} cells employed in the transformation, the same degree of reduction was consistently observed. This reduction in transformation efficiency varied in accordance with the associated yeast promoter, being reduced in the order

\[ \text{CYC1} > \text{PGK1} > \text{ADH1}. \]

which could implicate promoter strength as an influencing factor on the degree of the reduction. Subsequent APT expression levels, determined for the various constructions in minimal selective medium however, were found to be in the order;

\[ \text{ADH1} > \text{CYC1} > \text{PGK1} \]

and did not correlate in any way with the effects, since the construction giving the greatest APT expression did not give the largest reduction.

All plasmids, with the exception of the CYC1 promoter containing plasmid, transformed
when selected on YPD/G418 medium, but much lower frequencies than those in minimal medium were obtained. All the promoter containing constructs however, express more than enough APT to facilitate a direct G418 resistance phenotype and thus greater frequencies should result. As before, the transformation efficiency varied dependent on promoter, with; 

\[
\text{PGK} > \text{ADH} > \text{CYC} (\text{CYC} \text{ none}),
\]

which reflected APT expression in this medium. This effect had been noted by Zhu et al (1985), when the use of a stronger Actin promoter to direct expression of a dihydrofolate reductase cDNA, resulted in both increased expression and increased transformation efficiency in comparison to the use of a CYC1 promoter.

No transformants were obtained with the CYC1 promoter construct at a 1 \( \mu \)g transforming DNA concentration, although increasing the concentration of DNA, did result in a transformant being obtained. In the case of plasmid pCJ18, lacking a promoter, increasing the post-transformation expression time to 6h resulted in a number of G418\(^{th}\) transformants being obtained.

When a promoter was added to an APT gene lacking an associated promoter, an important effect observed was a large reduction in plasmid copy number. In the absence of an associated promoter, the plasmid copy number was found to be in the region of 54 copies per cell. Subsequent promoter addition lowered this to 10-20 copies per cell, which was dependent on the promoter employed. Analysis of the copy number reduction and the promoter strength revealed that both parameters were unrelated, since the copy number reduction of the ADH1 and PGK1 promoters were equivalent, although expression levels with both promoters did differ.

In terms of expression of the heterologous APT gene,

\[
\text{PGK} > \text{ADH} > \text{promoter} \quad (\text{YPD/G418 medium}),
\]

\[
\text{ADH} > \text{PGK} > \text{CYC} > \text{promoter} \quad (\text{minimal medium})
\]

confirming that promoter strength alone was not responsible and some other aspect of promoter function was involved. A number of workers have established that plasmid copy numbers do vary depending upon the gene being expressed and also the promoter employed to direct the expression. Data obtained in the expression of the APT gene, was in agreement
to some extent, to work of Janes et al. (1990), in which the expression of the hirudin protein was investigated using a number of GAP promoter variants which differed in their transcriptional efficiencies. Analysis of plasmid copy number indicated that when the burden of expression was low, the plasmids attained the highest copy number, whilst high promoter efficiency forced the cells to establish a lower copy number. This was also observed by Piper and Curran (1990), since plasmid copy numbers of homologous PGK plasmids under selection for PGK⁺, in a pgk⁻ strain, were lower in cells having the greatest expression. The high copy number of plasmid pCJ18, which had the lowest expression, was in agreement with the previously described work. When considering the promoter-containing constructions, however, no correlation between lower copy number and high expression was evident and thus copy number of the promoter containing plasmids was not related to expression.

Erhart and Hollenberg (1983), had indicated that cells containing a plasmid with a defective LEU2 marker grown under leucine selection, resulted in an amplification of plasmid copy number. This is apparent since the LEU2 marker employed, produces only 5% of the wild-type level of β-isopropyl malate dehydrogenase. To compensate for the reduced expression, plasmids containing the marker under LEU2 selection increase their copy number to approximately 100 copies to provide a greater amount of enzyme. In non-selective conditions, plasmid copy number is much lower. This type of effect was also observed by Zealey et al. (1988), on expression of a Herpes simplex thymidine kinase (TK) gene on a plasmid containing a normal LEU2 marker. Under leucine selection the copy number of the plasmid was found to be 15 copies per cell, which was increased to 100 copies when selection for the TK gene was employed. The copy number was additionally controlled by the stringency of selection, since increasing the concentration of the selection resulted in an increase in the copy number and the TK expression.

The copy number of plasmid pCJ18 was not as high as those previously described and was not a consequence of plasmid amplification due to the selection pressure, evidence for which was provided by a number of points. The first of which was that the plasmid copy number without selection in YPD medium was also found to be elevated, which was in contrast to the work of Erhart and Hollenberg (1983). Expression of the APT1 gene in YPD medium lacking G418, was found to be equivalent in the presence of G418, which would not be expected if plasmid amplification occurred. In addition, increasing the concentration of G418, did not result in an increase in pCJ18 plasmid copy number, which had been observed in the work
of Zealey et al. (1988) and also APT expression in concentrations of G418 up to 1mg/ml, remained very similar (Reid, 1987). Growth of pCJ18-transformed cells in YPD/G418 medium was found to be slower initially, but doubling time was then equivalent to promoter-containing constructions. This lag period, occurred regardless of whether the pCJ18-transformed cells were selected for the APT gene (following a 6h post-transformation expression time) or the TRP1 marker. Growth of the TRP selected transformant in YPD/G418 media was found to be marginally better than that selected for APT, which was surprising since APT expression in minimal medium is lower than in YPD/G418 medium. The lag period merely indicated that APT protein needed to accumulate within the cells before growth could proceed normally, occurring after a certain number of doublings. If amplification was responsible for the increased copy number, it would be expected that growth of the TRP selected transformant would be slower than that selected with G418. Thus factors other than the selective pressure determined the copy number. This was supported by work of Baldari et al. (1987), in that a plasmid containing both a leu2-d and URA3 marker, showed an increased copy number on URA3 selection.

The documented effects were also not a consequence of interference with endogenous 2μm, since it had been noted by Futcher and Cox (1984), that interference between the 2μm based plasmid and the endogenous 2μm present in the same cell can result in a reduction in copy number of both. This is due to a shortage of transacting proteins, and both plasmid types competing for the same cellular machinery. The plasmid constructions considered in this work, contained the 2μm origin of replication and also the region encompassing STB, which included REP3, whilst the other REP functions required by the cell, were provided in trans by the endogenous 2μm plasmid. Since all the plasmids studied, however, required trans-acting factors from the endogenous 2μm, the presence or absence of a promoter should have no influence.

Product toxicity was also not responsible for the reduction in copy number, since although APT expression was high, plasmid stability remained at a level not consistent with toxicity and growth was not affected.

Analysis of plasmid stability in minimal and rich selective medium and also non-selective medium, indicated that in all instances, stability was very high and unaffected by the copy number reductions. High stability was expected since the loss of 2μm based plasmids is
documented as being approximately 1% per generation (Armstrong et al, 1989). In light of the copy number reduction, however, it was expected that stability would be affected. In the constructions employed, losses of plasmid over 10 generations was found to be in the region of 0.06 %-2.2 % in selective and non-selective medium, although over a greater number of generations this would be expected to increase. Stability of plasmid pCJ18 however, was found to be greater in non-selective medium, than selective, but the difference between both was not significant. Some variation of plasmid stability was evident within the constructions, although the reduction in plasmid copy number played no role. No factor could be deemed responsible for the variation.

Growth of all plasmid containing cells in minimal media was found to be normal. In YPD/G418 medium, cells containing plasmids with the PGK1 promoter and ADH1 promoter grew normally, having a doubling time equivalent to that of the untransformed strain, although that of the ADH1 promoter construction was slightly longer (approximately 100m). Plasmids containing the CYC1 promoter, however, failed to grow in this medium, indicating a plasmid defect, although growth in YPD without G418 and in minimal medium was found to be normal. This effect was not due to plasmid loss or rearrangement nor to the presence of G418. The latter could be discounted since cells containing plasmid pCJ18 grow normally in G418 medium, following a lag phase, yet the plasmid produces only a fraction of the resistance enzyme, compared to the promoter containing plasmids.

Expression data obtained in YPD/G418 medium, indicated that the relative strength of the promoters in question were: PGK1 > ADH1. The difference in expression between the shorter PGK1 promoter fragment and the ADH1 promoter (which was also a truncated promoter) was not significant. In comparison to the longer PGK1 promoter fragment, which contains an additional 800bp of 5' non-PGK promoter DNA upstream of the PGK1 promoter, however, a 2-fold reduction in expression was evident, which clearly indicates that the additional sequences are required for more maximal expression. This is considered a result of the presence of transcriptional terminators in the 800 bp region, which are also lacking in the ADH1 promoter fragment.

In minimal selective medium, APT expression from the PGK1 promoter containing constructions, was found to be approximately 4-5 fold lower than that obtained in G418 medium, which was expected. In the constructions containing the CYC1 and ADH1
promoters, however, expression was 2 and 3-fold greater, respectively, than the longer PGK1 promoter containing construction. This was in agreement with Cantwell et al., (1986), since expression of a β-glucanase protein was greater using an ADH1 promoter than when the gene was under the control of a CYC1 promoter. With the APT1 gene under the control of the ADH1 promoter, however, expression was 2-fold greater in minimal medium than YPD/G418 medium. This effect was unexpected and reasons for the effect were unclear, since both media contained glucose and thus ADH1 promoter activity should be the same under both conditions. It could be possible, however, that the salts present in the minimal medium were having some regulatory effect on the promoter. The greater expression of APT obtained with the CYC1 promoter than that from PGK1 was surprising since the former promoter is a weaker promoter in an homologous environment and also less efficient in glucose medium. Thus, it would be expected that the CYC1 promoter would be less efficient than the PGK1 promoter which was not observed, indicating that some other factor is influencing expression.

Auxotrophic transformants obtained from plasmid pCJ18, containing the promoterless APT gene, were found to grow when picked onto G418 medium. Further analysis, had indicated that an incubation of 6 hours in YPD was sufficient to enable G418\(^\text{R}\) transforms to be obtained directly. Both results indicate that APT expression must occur, but at a low level and in order to allow growth in the presence of the antibiotic, the enzyme has to accumulate within the cell. APT assay data in both minimal and YPD/G418 media, indicated that APT expression was evident, but 20-fold less in YPD/G418 medium than the long PGK1 promoter containing construction. Expression was 2-fold greater in YPD/G418 than in minimal medium, but this difference was attributable to a difference in plasmid copy number, since expression per gene copy in both media was identical. By use of the relationship of APT units against % APT protein determined in Chapter 3, APT expression in YPD/G418, was only 0.0025% of the total cell protein.

To determine how expression from the plasmid occurred in the absence of a promoter, total RNA was extracted from the transformants and Northern blotted. By use of a single stranded riboprobe complementary to APT, 5 APT sense-strand transcripts were detected. Subsequent stripping of the blot and reprobing with immediately upstream pBR322 DNA, identified the same transcripts, indicating that they were in fact initiating in the pBR322 DNA. Initiation at a number of the pBR322 bacterial sequences present on a plasmid had previously been reported by Marczynski and Jähning (1985). Sumrada and Cooper (1985), had observed that
the pBR322 sequences placed upstream of the \textit{CAR1} TATA sequence could in fact activate transcription of the \textit{CAR1} gene, whilst Sidhu and Bolton (1990) found that the pBR322 sequences were responsible for the alteration of the regulation of the \textit{PHOS} gene. The effect in pCJ18 was therefore analogous to reported events.

Further transcript analysis, determined that these \textit{APT} transcripts were very abundant, which was not reflected in the level of APT enzyme detected, indicating that translation from these fortuitous transcripts was very inefficient. Translation of the fortuitous transcripts is considered to occur by a process of downstream initiation, which is a much rarer and less efficient event (Sherman and Stewart, 1982).

The effects on copy number, described in this chapter, represent a loss of potential product-encoding genes and may therefore be considered as one of the factors limiting foreign gene expression in yeast.
CHAPTER FIVE

FURTHER INVESTIGATION OF THE PROMOTER-MEDIATED
PLASMID INHIBITION
5.1 **INTRODUCTION**

Results described in the previous chapter, showed that the addition of a "strong promoter" of chromosomal origin to a plasmid-borne gene construction caused reductions in plasmid copy number and transformation efficiency, with little effect on plasmid stability. Preliminary data indicated that these effects were not a consequence of the strength of the promoter, but some other factor. Jayaram *et al.* (1983) found that transcription into the REP3 and ori region of a plasmid by an induced GAL10 promoter, reduced plasmid copy number and stability. Similarly, Murray and Cesarini (1986) reported that an induced GAL promoter, transcribing directly across the 2μm origin of replication, caused an inhibition of plasmid replication. It was considered possible that the results observed on promoter addition, were a consequence of the same phenomenon. The copy number differences obtained between the differing promoters would therefore be a consequence of the promoter strength; the stronger the promoter, the greater the amount of transcription through the origin. However, in the situation reported in Chapter 4, the promoters were not transcribing directly into the origin, but were positioned approximately 2kb away. Furthermore, there were also intervening transcription terminators from CYC1 and 2μm A genes. Thus, if transcription from the promoters was the cause of the inhibitory effects, the terminators would have to be allowing a substantial volume of transcription to pass through without termination. To determine if transcription was in fact a cause of the observed effects, the effects of addition of a second terminator or inverse orientation of the gene constructs were analysed. Additionally transcripts emanating from a number of differing constructions were investigated by Northern blotting to determine whether they crossed the replication origin of the plasmid.

The plasmids considered in the initial work in Chapter 4 all contained the 2μm origin of replication and STB element. Despite the reductions in copy number, the plasmids remained highly stable. This presumably reflects the functioning of the 2μm partitioning system through the cis-acting element STB. Plasmids containing an ARS origin (and lacking STB) may therefore show greater instability due to the promoter-containing gene construction affecting copy number. This possibility was therefore also examined.
5.2 The influence of alternative plasmid configurations on the plasmid inhibition effect

5.2.1 Plasmid-borne gene constructions with alternative or additional terminators.

The following gene constructions were created that contained alternative or additional terminators to CYC1 at the 3' terminus of the heterologous gene.

pCH208: This plasmid contained a PGK1 promoter-driven APT cartridge, but the CYC1 terminator was replaced by that of PGK1, so that the terminator originated from the same yeast gene as the promoter (Fig 5.2.1.1). This construction examines the possibility that transcription may leak through a terminator unless it is correctly matched to the promoter.

pCH209 and pCH218: These constructions contain the PGK1 and ADH1 promoter-driven APT cartridge, respectively, and an additional inserted transcription terminator. Thus, at the 3' end of the heterologous gene, these constructs contain the PGK1 and CYC1 terminator-containing fragments in tandem (Fig 5.2.1.1). These plasmids were constructed to examine the possibility that substantial transcription leaks through the first terminator and subsequent 2μm A terminator and thereby interferes with origin function. Presence of the additional terminator would therefore be expected to relieve the inhibitory effects by reducing any such through transcription.

pCH108: This construct contains the APT0 coding-sequence cartridge, as opposed to APT1 in the other constructs, driven by an ADH1 promoter (Fig 5.2.1.1 and 3.2). APT0 contains a poly (GC) tract at its 3' terminus, which appears to act as a transcription terminator (see later). This construct is analogous to pCH208 and pCH218, in adding an additional terminator.

Plasmids pCH208 and pCH209, both contained a full length functional PGK1 promoter, but 800 bp of 5' non-promoter DNA, upstream of the promoter had been deleted, to give a "trimmed" promoter fragment.
Figure 5.2.1.1 Structure of plasmids of alternative configuration.

**Plasmid pCH208**: A pIC19R plasmid (Appendix) containing a HindIII partial digest of a PGK1 promoter-APT-PGK terminator cartridge cloned into the HindIII site (pCH148; Appendix), was restricted with XmnI and HindIII, to liberate the PGK1 promoter-APT-terminator cartridge which was cloned into the SmaI and HindIII site of plasmid pCJ17, replacing the CYC1 terminator.

**Plasmid pCH209**: As plasmid pCH208, but the XmnI-BamHI fragment was cloned into the SmaI and BglII site of plasmid pCJ17, upstream of the CYC1 terminator.

**Plasmid pCH218**: An ADH1 promoter fragment was isolated from pCH97 (pYcDE-2 containing a BamHI linker between the ADH1 promoter and CYC1 terminator; Appendix) by restriction with Sail and BamHI. Plasmid pCH209 was restricted with Sail and XhoI to delete the PGK1 promoter, which was replaced with the ADH1 fragment.

**Plasmid pCH108**: The APTO cartridge was isolated as described in section 3.2 as a BglII-BclI fragment. This was then cloned into the BglII site of plasmid pCH98 (pYcDE-2 containing a BglII linker between the ADH1 promoter and CYC1 terminator; Appendix).

**Plasmid pCH211**: pCH147 (Appendix) was restricted with XmnI and BglII to liberate the PGK1 promoter and APT1 cartridge which was then cloned into the SmaI and BamHI sites of plasmid pCJ18.

**Plasmid pCH155**: Plasmid pCH148 (Appendix) was restricted with Sail and EcoRI and the fragment cloned into the Sall and EcoRI sites of plasmid pYcDE-2, substituting the ADH1 promoter.

**Plasmid pCH210**: 5'PGK DNA was deleted from plasmid pCH155 with Bal31 to remove sequences upstream of the XmnI site of the PGK1 promoter. The CYC1 terminator was deleted by Bcl restriction and an EcoRI linker added.

**Plasmid pBEJ26**: A pIC19R plasmid containing an ADH1 promoter-APT-CYC1 terminator cartridge cloned into the XhoI and HindIII site of the plasmid was restricted with BamHI and
Figure 5.2.1.1b  Structure of plasmids pBEJ16 and pBEJ17

Plasmid construction is described in chapter 3, section 3.3
These additional plasmid constructions were investigated in terms of their transformability into yeast, APT expression, copy number and stability. Results for transformation and APT expression are summarised in Fig 5.2.1.2a and b respectively. Stabilities in selective and non-selective media are shown in Table 1 and copy numbers in Fig 5.2.1.3 and Table 2.

Changing the terminator of the APT gene from CYC1 (pCH211 Fig 4.2.1.2) to a PGK terminator as in pCH208, had no effect on plasmid copy number, but improved APT expression in rich medium. Both effects were also observed in the presence of an additional terminator in both media, although, again no improvement in plasmid copy number resulted. This was also evident with the ADH1 promoter-driven construct, although in this instance transformability was improved, and expression reduced. Replacing APT1 with the APTO cartridge (3.2), was found to give no additional benefit and was in fact a poorer expression cartridge than APT1. An alternative or additional terminator therefore provided no further benefit in overcoming replication inhibition, suggesting that transcription was not responsible. However, this does not discount the possibility that primary transcription extends beyond the terminators for some distance, before being trimmed and processed at the 3' end. Under such a scenario, transcriptional inhibition of the origin of replication could still occur, despite the presence of additional promoters.

5.2.2 Constructions with the plasmid-borne gene in opposite orientation

The following constructions were created so that they were analogous to those previously described, but differed in the orientation of the heterologous gene cartridge, which would be directed towards the pBR322 sequences of the plasmids. In the previously described constructs, the heterologous cartridge was directed towards the 2μm portion of the plasmid and thus the described plasmids would examine the possibility that interference with the 2μm sequences was the cause of the replication inhibition.

pCH210 : This construct was analogous to plasmid PCH208, but the PGK1 promoter-driven APT cartridge was in the orientation described above (Fig 5.2.1.1).

pCH185 : This construct was identical to pCH210, but an additional terminator was inserted upstream of the promoter, which would prevent transcription into the PGK1 promoter (Fig
Figure 5.2.1.2

(a) Transformability of plasmids with differing promoters and constructions

- G418 selection
- Minimal selection

(b) APT expression obtained with plasmids having differing promoters and constructions

- G418 medium
- Minimal medium

Data illustrated in a) and b) is the average of at least 3 repetitions
Variability is approx 3% in a) and 10% in b)
### TABLE 1  Plasmid stability of AFT plasmids with differing promoters and construction in selective and non-selective media

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>% PLASMID STABILITY OVER 10 GENERATIONS °</th>
<th>SELECTIVE MEDIA</th>
<th>NON-SELECTIVE MEDIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>YPD/G418 medium</td>
<td>Minimal medium</td>
</tr>
<tr>
<td>pCH208</td>
<td>68.8</td>
<td>90.9</td>
<td>88.6</td>
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<td>pCH209</td>
<td>93.8</td>
<td>80.9</td>
<td>69.4</td>
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<td>97.2</td>
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<td>96.7</td>
<td>95.2</td>
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<td>94.8</td>
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<td>91.1</td>
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<tr>
<td>pCH210</td>
<td>76.6</td>
<td>91.8</td>
<td>91.8</td>
</tr>
<tr>
<td>pBEJ26</td>
<td>nd</td>
<td>98.6</td>
<td>nd</td>
</tr>
<tr>
<td>pBEJ16</td>
<td>97.7</td>
<td>94.8</td>
<td>92.9</td>
</tr>
<tr>
<td>pBEJ17</td>
<td>96.2</td>
<td>92.8</td>
<td>85.8</td>
</tr>
</tbody>
</table>

nd = Not done

*: All values shown are the means of three stability determinations

Variation = ± 5%
Figure 5.2.1.3 Copy number determination of plasmids of alternative configuration

Total genomic DNA from S150-2B cells transformed with the plasmids was restricted with EcoRI and Sall then separated on a 0.8% TAE/EtBr gel. This was subsequently blotted onto Hybond-N filter membrane using the method of Southern and hybridised at 65°C overnight in 3xDenhardt's hybridisation solution (Section 2.8.7), with a 1.45kb labelled TRP1 probe isolated by EcoRI restriction from plasmid pYRP7 (Appendix).

Numbered tracks contain genomic DNA isolated from cells transformed with the stated plasmids. Unless stated all cells were grown in YPD/G418 medium.

Tracks shown are from a number of different filters, hybridised and probed at different times.

Track 1,8 = pCH155
Track 2 = pCH208
Track 3 = pCH209
Track 4 = pCH210
Track 5 = pBEJ26
Track 6 = pCH218
Track 7 = pCH218 HUL medium
Track 9 = pBEJ16 HUT medium
Track 10 = pBEJ17 HUT medium
Track 11 = pBEJ16
Track 12 = pBEJ17

Arrows indicate the position of the chromosomal marker bands.
<table>
<thead>
<tr>
<th>PLASMID</th>
<th>CONSTRUCTION</th>
<th>PLASMID COPY NUMBER*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>YPD/G418 Medium</td>
</tr>
<tr>
<td>pCH211</td>
<td>5' trimmed PGK1 promoter, CYC1 terminator</td>
<td>18</td>
</tr>
<tr>
<td>pCH208</td>
<td>5' trimmed PGK1 promoter, PGK1 terminator</td>
<td>18</td>
</tr>
<tr>
<td>pCH209</td>
<td>5' trimmed PGK1 promoter,CYC1 and PGK1 terminators</td>
<td>18</td>
</tr>
<tr>
<td>pCH210</td>
<td>opposite orientation of pCH208</td>
<td>20</td>
</tr>
<tr>
<td>pCH155</td>
<td>As pCH210, but terminator upstream of promoter</td>
<td>20</td>
</tr>
<tr>
<td>pBEJ11</td>
<td>ADH1 promoter, CYC1 terminator</td>
<td>19</td>
</tr>
<tr>
<td>pCH218</td>
<td>ADH1 promoter,CYC1 and PGK1 terminators</td>
<td>19</td>
</tr>
<tr>
<td>pBEJ26</td>
<td>opposite orientation to pBEJ11</td>
<td>18</td>
</tr>
<tr>
<td>pBEJ16</td>
<td>LEU2 based plasmids/PGK1 promoter and terminator</td>
<td>18</td>
</tr>
<tr>
<td>pBEJ17</td>
<td>opposite orientation of pBEJ16</td>
<td>19</td>
</tr>
</tbody>
</table>

nd = Not Done

* Copy numbers shown are the means of at least 3 copy number determinations and represents the average plasmid copy number per cell. Variation was approximately ± 2
5.2.1.1). Thus in this construction, read-through transcription should be eliminated.

pBEJ26: This construct was identical to pCH210, but contained an ADH1 promoter driven APT cartridge (Fig 5.2.1.1).

Opposite orientation of the heterologous cartridge resulted in increased APT expression (Fig 5.2.1.2b) and, additionally there was a slightly improved plasmid copy number in the PGK promoter driven construct (pCH210) (Table 2). Inclusion of an additional terminator upstream of the promoter (pCH155) gave no further benefit and in fact resulted in a reduction in transformability (5.2.1.2a), but much better expression. Conversely with the ADH1 promoter-containing construction, plasmid copy number was not increased. Thus, inversion of the gene to point away from the origin did not eliminate the promoter-mediated inhibition effects on the vector plasmid.

5.2.3 Altered plasmid construction configuration

One possibility was that the promoter-mediated inhibition may be specific for one type of plasmid construction, rather than being a more generalised effect. To determine if plasmid inhibition could be overcome by altering plasmid configuration, a differently configured plasmid was investigated. Plasmids pBEJ16 and pBEJ17, (Fig 5.2.1.1b), constructed in Chapter 3, employed LEU2 as the auxotrophic marker and with the 2µm origin positioned to be isolated as much as possible from possible stray transcription. In pBEJ16 the PGK promoter-driven APT cartridge points in the direction of the 2µm origin, but 5.7 kb of pUC and LEU2 DNA intervenes, with LEU2 in the opposing orientation. Plasmid pBEJ17 differs in that LEU2 and the APT cassette both face away from the origin in the same direction, with pUC19 and STB DNA intervening before ori.

These alternative configurations, showed improved transformability (5.2.1.2a) particularly in YPD/G418 medium with a greater number of transformants in this medium than all other constructions. APT expression (Fig 5.2.1.2b) was improved, but plasmid copy numbers were no different to those obtained with the other constructions (Table 2). Thus, although there were improvements in transformability with these plasmid configurations, the underlying factor influencing copy number still appeared to be evident.
Investigation of a plasmid identical to pBEJ16 described above (pBEJ15 chapter 3), but lacking the APT coding sequence, indicated that this plasmid was also affected by the inhibition effect. Plasmid copy number of this plasmid in minimal selective medium, was found to be 16, less (although not significantly), than pBEJ16 and pBEJ17. In addition, transformability was reduced almost 3-fold. This implies that it is promoter function which is responsible for the effects and not the APT product.

Plasmid stability of all previously described constructions was found to be very high in the majority of cases, similar to the other promoter-containing constructions analysed in Chapter 4. Only slight and not significant variability between the constructions was evident.

5.3 Transcript analysis of the various constructions

In order to directly investigate whether the plasmid inhibition effect resulted from transcripts interfering with the 2μm origin, transcription produced from the various plasmids (including those considered in Chapter 4) was investigated by Northern blotting.

Total RNA was extracted from the plasmid-containing cells and equal quantities Northern blotted and serially hybridised with various probes. As a start-point, probing with labelled DNA of the contained plasmid revealed all of the plasmid-encoded transcripts (Fig. 5.3.1a). This revealed a number of interesting features. Firstly, in the absence of a promoter (pCJ18), a very large amount of transcription was evident from the plasmid in both YPD and minimal medium. A comparison of the amount of transcription in minimal and YPD medium, (Fig 5.3.1) show that for the same total amount of RNA, there was about a 10-fold reduction in pCJ18-specific transcriptional activity in minimal medium compared with that in YPD. The transcripts observed included abundant, discretely sized species, as well as heterogenous transcripts covering a large size range. Longer exposures indicate that the same transcripts are produced in minimal medium, but are merely less abundant overall. Addition of a PGK promoter (pBEJ24, pCH208, pCH209, pCH155 and pBEJ16), ADH1 promoter (pBEJ11 and pCH108) or a CYC1 promoter (pBEJ11), eliminated virtually all of the background transcripts, with only two abundant, discretely-sized transcripts of 2.5 kb and 1.65 kb or 1.5 kb (dependent on the terminator employed) remaining, which were apparent in both
In all cases 7 \( \mu g \) of total RNA was separated on a 1\% agarose/formaldehyde gel and the gel blotted overnight at room temperature onto Hybond-N filter membrane. The filter was hybridised overnight at 42\(^\circ\)C in Northern hybridisation solution (Section 2.9.4).

Lanes containing RNA from cells transformed with the various plasmids as marked, grown in either YPD/G418 medium (Y) or minimal selective medium (M).

RNA sizes are as marked.

Panel A shows the autoradiograph after the filter was probed with the relevant labelled plasmid to reveal all transcripts.

Panel B shows the autoradiograph after probing with a labelled apt fragment probe.

Panel C shows the autoradiograph after probing with a labelled TRP1 fragment.
rich and minimal medium. To determine the identity of the transcripts, the filter was stripped (as in section 2.4.8), then reprobed with an APT (Fig 5.3.1b) or TRP1 fragment probe (Fig 5.3.1c). This revealed that the smaller transcript was in fact the APT message and the larger one the TRP message. Intriguingly, however, although addition of a "strong" promoter eliminated background transcription of the plasmid, the amount of APT message produced was less than the amount of background transcription. To some extent this may correspond with the decrease in plasmid copy number. In addition it was evident that approximately 10-fold less APT mRNA was observed in minimal medium compared with YPD.

The sizes of the APT transcripts, indicated that the mature transcript had 3' termini located within the terminators; a difference in size being obtained when the CYCl terminator was present (the transcript was 1.65 kb), to that of 1.5 kb when a PGK terminator was present, reflecting the different fragment sizes. The transcript obtained in the presence of two tandemly arranged terminators, as in pCH209, was of the same size as that obtained in the presence of the most 5' terminator, indicating that transcription terminated at the first terminator. No transcript of longer length, which would correspond to termination at the second terminator could be detected, indicating that the terminators were highly efficient at actually stopping transcription. This therefore confirmed that all the APT transcripts emanating from the strong promoters, terminated short of the 2μm origin. Transcription across the origin could therefore be discounted as a reason for the copy number reduction effect.

With plasmid pCH108, containing the poly (GC) rich tract, transcription terminated earlier than expected, even though the cartridge contains 200 bp more DNA. This would however be consistent with the poly (GC) tract behaving as a terminator. One possibility is that this portion of the DNA may form a region of left-handed DNA which RNA polymerase would find difficult to unwind.

On employing a TRP1 probe, two discrete transcripts were observed with all the plasmids. A smaller transcript of 1.6 kb, the size of which indicated that it terminated upstream of 2μm ori (at the proximal side of STB), and a larger more abundant transcript of 2.5 kb, which terminated close to the 2μm-CYC1 terminator junction and, as a consequence, must have passed through STB and ori. The TRP marker employed on the plasmids, did not contain a terminator, which partly accounted for its extended transcription, although in all cases, it
passed unimpeded through the STB terminators, which are composed of both an efficient terminator and a "silencer" with terminator-like properties (Murray and Cesarini, 1986). The TRP1 transcription could not be responsible for the copy number reduction, however, since it was also evident in the promoter-free control plasmid, pCJ18, which had a higher copy number.

It was also noted that the plasmid inhibition effect, which was associated with promoter addition was not as severe with minimal medium as with rich medium. Transformation frequencies were slightly higher with minimal medium, which indicated that growth medium may have a role. In addition, transcription in minimal and YPD medium, (Fig 5.3.1) show that for the same total amount of RNA, there was about a 10-fold reduction in transcriptional activity in minimal medium compared with that in YPD.

The yield of transcripts from the PGK1 and the ADH1 promoters was found to be surprisingly low compared with that from the supposedly weaker TRP1 promoter (Figs 5.3.1b and c). This again suggests that it is not the yield of transcription from the strong promoters that correlates with the inhibition phenomenon. A better correlation would be in the extent of "suppression" of the background transcription associated with the presence of a strong promoter, rather than transcription per se.

5.3 The effect of promoter addition on ARS based plasmids.

The observations of plasmid inhibition described so far, which were associated with the addition of promoters from strongly expressed chromosomal genes, all involved 2μm derivative plasmids. These contained both the origin of replication and the cis-acting stability locus of the 2μm plasmid. Although the inhibited plasmids showed reduced copy number, their inherent stability was unaffected, which could reflect the influence of the 2μm partitioning system. It was therefore decided to investigate the effect of promoter addition to plasmids containing an ARS replication origin and therefore not acted upon by the 2μm partitioning system.

ARS plasmids are highly unstably inherited compared to the equivalent 2μm counterpart (Zakian and Kupfer, 1982) and thus if the copy number reduction effect observed in the 2μm plasmids acted upon the ARS containing plasmids, a further decrease in plasmid stability
might be expected.

5.4.1 ARS plasmid constructions.

ARS plasmids carrying APT1 gene constructs are shown in Fig 5.4.1 and their construction described in the figure legend.

They formed two sets which were identical in all respects, with the exception of the orientation of the TRP/ARS cartridge. Plasmids pRCM6 and pRCM9 contained the PGK1 promoter/APT1 cartridge/PGK terminator, whilst plasmids pRCM7 and pRCM10 contained an ADH1 promoter/APT1 cartridge/ CYC1 terminator. Promoterless APT1 cartridge/ CYC1 terminator plasmids, pRCM5 and pRCM8 were used as controls.

5.4.2 Transformability, stability and APT expression of the ARS/APT containing plasmids

The ARS based plasmids were transformed into S. cerevisiae strain S150-2B and transformants selected using either the TRP1 auxotrophic marker or the APT1 G418-resistance marker, as described previously. The results are summarised in Fig 5.4.2.1.

With one orientation, in which the ARS sequence was directed towards the APT sequence, the addition of a promoter caused an increase in transformation efficiency in YPD/G418 medium, with the converse in the opposite orientation. This contrasts with the 2μm based plasmids, since the addition of a promoter to ARS plasmids had only a minimal effect on transformability, and orientation of the TRP/ARS sequence had a more major effect. A further difference was the much lower frequencies of auxotrophic transformation of the ARS plasmids, even in the case of the promoter free-control. This is not an unusual effect, however, and is generally found with ARS plasmids (Tschumper and Carbon, 1980). Thus, with respect to transformation, addition of a promoter has an effect, but it is manifest in a different way, to the 2μm plasmids discussed previously.

To determine if transformability was related to APT expression in these ARS plasmids, expression was investigated in both minimal and rich selective medium as previously described in section 2.5. The results obtained are summarised in Fig 5.4.2.2
Two sets of plasmids were constructed by Dr R. Mount, which differed only in the orientation of the TRP/ARS sequence.

The heterologous promoter/gene cartridges were cloned into the BamHI site of either plasmid pRCM11 or pRCM12, upstream of the TRP/ARS sequence, confirming cartridge orientation by careful restriction analysis.

Restriction sites: As before with K = KpnI, Sp = SphI

Arrows show the direction of transcription
Figure 5.4.2.1

ARS based plasmid transformability

ARS/TRP sequence is directed away from APT

ARS/TRP sequence is directed towards APT

Data shown is the average from at least 3 transformations.
Variation is -10 in minimal selection and -5 in G418.
Figure 5.4.2.2

APT expression obtained from the APT gene on ARS based plasmids

ARS/TRP sequence is directed away from APT

ARS/TRP sequence is directed towards APT

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>YPD/G418 medium</th>
<th>Minimal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRCM5</td>
<td>1,897</td>
<td>912</td>
</tr>
<tr>
<td>pRCM6</td>
<td>1,628</td>
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<tr>
<td>pRCM7</td>
<td>842</td>
<td>265</td>
</tr>
<tr>
<td>pRCM8</td>
<td>1,070</td>
<td>181</td>
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<td>pRCM9</td>
<td>7,249</td>
<td>7,537</td>
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<td>pRCM10</td>
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</tr>
<tr>
<td>pCJ18</td>
<td>170</td>
<td>91</td>
</tr>
</tbody>
</table>

Data shown is the average from at least 3 determinations
Variation is -1%
In was evident that the APT gene without an associated promoter on an ARS plasmid, expressed a large amount of APT protein, being nearly equivalent to that obtained in a number of the 2μm based plasmids containing a promoter (see Chapter 4). APT expression was found to vary considerably in plasmids containing the same promoter, but a different orientation of the TRP/ARS sequence. The plasmid containing the TRP/ARS sequence pointing towards the APT cartridge, was found to give considerably more expression than the other orientation. APT expression with the ADH1 promoter was found to be greatest in YPD/G418 medium in these constructs, which again was the opposite of that found with the 2μm based plasmids, in which greatest expression with this promoter was in minimal media. The orientation of the TRP/ARS sequence was therefore having an effect on APT expression, which reflected the transformation results.

Stability of the plasmids was also investigated, over 10 generations of growth in either rich or minimal selective medium or non-selective medium, the initial inoculum for the latter being obtained from a minimally grown culture. The plasmid stability results are shown in Fig 5.4.2.3a/b.

Stability of the ARS plasmids was found to have a very different pattern to that of the 2μm plasmids. The plasmids were more stable in rich selective medium (ranging from 98.8%-12.8%) than in minimal selective medium (ranging from 74.7%-2.8%) (Fig 5.4.2.3a), but much less stable in non-selective YPD medium (Fig 5.4.2.3b). It has been documented that in selectively grown cultures 5% to 25% of cells harbour the ARS plasmid (Murray and Szostak, 1983) and thus in these instances more stable plasmids were evident. Inclusion of the PGK1 promoter (pRCM6 and pRCM9), rather than the ADH1 promoter (pRCM7 and pRCM10), notably reduced stability, in selective and non-selective media, with the converse being noted with the 2μm-based plasmids. The differing stability of the PGK1 and ADH1 promoter containing ARS plasmids was reflected in the more sectored appearance of the auxotrophic and G418^ resistant transformatant colonies containing plasmids with the PGK1 promoter. Cells containing the ADH1 promoter plasmid were of normal appearance.

Thus, it was evident that addition of the PGK1 promoter to the ARS plasmids did cause a decrease in stability in selective YPD/G418 medium and also non-selective medium, although increased stability was evident on addition of the ADH1 promoter. Although stability was
Figure 5.4.2.3
Stability of plasmids containing the APT gene cartridge and an ARS origin over 10 generations

a) In selective media

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>YPD/G418</th>
<th>Minimal</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRCM5</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>pRCM6</td>
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<td>pRCM7</td>
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<td>pRCM8</td>
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<td>pRCM10</td>
<td>98.8</td>
<td>10.6</td>
</tr>
</tbody>
</table>

b) In non-selective media

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>YPD/G418</th>
<th>Minimal</th>
</tr>
</thead>
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<td>pRCM5</td>
<td>9.2</td>
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<td>0.75</td>
</tr>
<tr>
<td>pRCM10</td>
<td>24.9</td>
<td>24.9</td>
</tr>
</tbody>
</table>

a) Cultures grown in YPD/G418 or minimal media for 10 generations, plated onto YPD, then replica plated onto YPD/G418 or minimal plates.

b) Transformant grown in YPD media for 10 generations, plated onto YPD then replica-plated onto YPD/G418 or minimal plates.

Data shown is the average of 2 repetitions. Variation = -5
reduced as anticipated if the copy number reduction effect was evident, the fact that TRP/ARS sequence orientation had a significant effect on plasmid performance, made any conclusions difficult to draw.

Copy number data was not obtained with the ARS constructions as it was clear from the transformation and stability results, that the ARS plasmids behaved quite differently to the 2μm-based plasmids, showing very little inhibitory effects of the promoters and a stronger effect of orientation. It was concluded that the inhibitory effects of "strong" promoters is primarily manifest with the 2μm based vectors, which would seem to implicate 2μm-specific attributes.

The 2μm derived plasmid used, contain the cis-acting STB locus, but not the inverted repeats, which could suggest that the inhibitory effects of reduced transformation efficiency and copy number (but not stability) are influenced by the partitioning system. It could be postulated that promoter presence interferes with the plasmid partitioning mechanism lending to reduced replication.

5.5 DISCUSSION

From the preliminary analysis reported in Chapter 4, the likely cause of the promoter-mediated plasmid replication inhibition seemed to be transcription interfering with the 2μm origin of replication, in the manner reported by Murray and Cesarini (1986). For this to occur, the transcription terminators employed on the heterologous gene cartridge would need to terminate transcription very inefficiently. In this chapter, analyses of constructs containing the same terminator as promoter, two terminators in tandem and also the heterologous cartridge in the opposite orientation so that it was no longer directed towards the 2μm sequences, revealed that this was not the case. On the contrary, the transcripts from the "strong" promoters, were efficiently terminated and did not reach the origin region.

It was evident, however, that a TRP transcript emanating from the TRP1 marker on the plasmid, did pass unimpeded through the 2μm origin. However, the TRP1 fragment used in these plasmids eliminated both some upstream sequence (resulting in only one transcript being produced) (Kim et al, 1986) and some 3′ sequence, apparently deleting the transcription terminator. However, since copy number of the promoterless plasmid pC118, was high in the
presence of this through-transcription, it would appear that this was not a significant factor in the inhibition effect. Alteration of the orientation of the heterologous APT cartridge, so that it pointed away from the origin and also no longer clashed with the TRP1 transcription also failed to relieve the inhibition effects.

Improved transformability with alternative plasmid configurations containing the LEU2 marker, initially resulted in some improvement in plasmid performance, but copy number was not increased. The improved transformability may reflect that the LEU2 marker which was transcribed at a lower level than TRP1 and had a correctly terminated transcript, may be more favourable as an auxotrophic marker than TRP1. Thus, a better comparison than the TRP1 based plasmid pCJ18, would have been an equivalent LEU2 promoterless plasmid. These plasmids were constructed, but time constraints prevented investigation. Subsequent work would have involved determining the copy number of the promoterless version of the plasmids and determining if the copy number and transformability of these plasmids reflected that of plasmid pCJ18.

Unlike data obtained in Chapter 4, it was evident that the increased number of G418\(^\text{R}\) transformants did not correlate with greater APT expression. It was additionally noted that in the TRP1 based plasmids, better transformability was obtained in minimal medium, compared with YPD/G418 medium. This implicated that growth medium may have a role in the plasmid inhibition effect, although plasmid copy number was not significantly increased in this medium.

Transcript analysis revealed that addition of a promoter eliminated a large amount of background heterogenous transcription. What was also unexpected, was that the amount of APT transcription was less than the background transcription. This could be accounted for, to some extent, although not entirely, by the copy number reduction observed. Thus, whilst copy number decreased about 2.5-fold in YPD on addition of a promoter, overall transcription decreased about 10-fold. Furthermore, the amount of APT transcription was less than the TRP transcription, which is known to be 500-fold less efficient at directing expression in comparison to the PGK1 promoter (Panchal 1987). This low transcription by the "strong" promoters, is presumably a consequence of the heterologous gene constructions. Such constructs have been reported to produce considerably reduced transcription compared with homologous genes (Chen et al, 1984).
Plasmid inhibition caused by the strong promoters, was found to be limited to 2μm based plasmids, since the addition of the same promoters to plasmids containing an ARS origin of replication and lacking the 2μm partitioning system, indicated a different effect. In this case, orientation of the TRP/ARS sequence on the plasmid exerted a much stronger effect on plasmid performance than the addition of a promoter. Auxotrophic transformation efficiency of the promoterless ARS plasmids was much reduced in comparison to the 2μm based counterparts, with subsequent promoter addition resulting in an increase or decrease in efficiency, depending on TRP/ARS orientation. Similarly, orientation affected APT expression and plasmid stability in the same way.

Stability of the ARS plasmids as expected, was much reduced in comparison to the 2μm based plasmids, owing to the lack of a partitioning function. It was of interest to note that the least stable ARS plasmids, in both orientations, contained the stronger PGK1 promoter, which was reflected by the very sectored appearance of these colonies. It is also known that the ARS plasmids integrate with high frequency and thus more stable plasmids may reflect a greater number of integrants in the cell population. If the very variable plasmid stability was taken into account on looking at the APT expression data, then the expression levels would be very different.

Promoterless plasmids containing the ARS origin, were found to express equivalent levels of APT to the promoter-driven cartridges in the 2μm plasmids (discussed in Chapter 4), being in excess of 100-fold more than the equivalent promoterless 2μm based plasmid. Transcripts initiating in the bacterial pUC DNA upstream of the APT cartridge, however, would be poorly translated (illustrated by data from the promoterless 2μm plasmid) and thus the high expression levels observed, must be due to the presence of a large number of copies of the plasmid within the cell. Zakian and Kupfer (1982), had noted that in a population of ARS-containing transformants, the copy number could be 100 in some cells, which is a consequence of the high rate of nondisconjunction (Murray and Szostak, 1983), which allows the copy number to increase in a minority of cells. This could explain the increased expression, although in plasmid pCJ18 copy number was 54 and expression was much greater than 2-fold less. Thus some other factor was also responsible.

The observed effects of ARS/TRP cartridge orientation on transformability, expression and stability, were initially considered a consequence of interference of ARS function due to the
proximity of the promoter directing APT expression. This is known to occur even with a transcriizational terminator between the promoter and the ARS (Snyder et al., 1988). The ARS has a number of flanking sequences which contain two ABF1 binding sites and have been shown to be nuclear scaffold attachment sites. Thus the transcription complex may sterically inhibit ARS binding proteins from binding to this region, preventing the initiation of DNA replication. However, the effects observed in this instance were more severe when the ARS sequence was further away from the promoter-APT cartridge, (except with the promoterless plasmid), indicating an additional effect. On closer examination of the constructions, when the ARS was further away from the APT coding sequence, pUC19 DNA is immediately upstream, whereas in the opposite orientation TRP1 sequence intervenes. Transcription in the pUC DNA may therefore play an additional role. As an anomaly, however, in the promoterless APT plasmid, this orientation performs the worst, indicating that the presence of a promoter does exert an additional effect.

In light of this work, ARS plasmid performance is dependent upon the plasmid construction and ARS/marker orientation and is more complex than expected. The promoters employed to direct expression of the gene play only a minor role in the levels of gene expression obtained. Although copy numbers were not determined, it would seem that addition of a promoter to these plasmids has little effect, unlike the 2μm based plasmids, which infers some effect of the partitioning system on the inhibition.

In summary, results presented in this chapter indicate that the plasmid inhibition effects which are observed on the addition of a promoter, are not a consequence of transcription, since additional terminators and alternative configuration do not have a significant effect. In addition, it would also seem that the effect may be limited to 2μm based plasmids, inferring some involvement of partitioning. In all however, the effects apparent on changing plasmid configuration, construction and replication origin are very significant and must be important considerations in designing a plasmid to express foreign genes.
CHAPTER SIX

HOMOLOGOUS GENES AND THE
EFFECT OF PROMOTER DELETIONS ON PLASMID INHIBITION
6.1 INTRODUCTION

The plasmid inhibition effects described in the previous chapters were observed in constructions expressing the same heterologous gene. A number of questions are therefore raised as to whether the effect is;

i) a consequence of that particular gene,
ii) a consequence of the expression of a heterologous gene in general,
iii) due to the presence of the promoters, independent of gene construct.

In order to address some of these possibilities, the transformability and copy number of two plasmids containing different homologous genes were investigated.

As the plasmid inhibition effects were not a consequence of the strength of the promoter, nor due to interference of plasmid origin function resulting from through transcription, it was speculated that the inhibition may be caused by some other aspect of promoter function. One possibility is that regulatory proteins and transcription factors which bind to the promoters, could in so doing, inhibit the progression of replication forks along the plasmid DNA, thereby resulting in fewer plasmid copies and reduced establishment at transformation. Brewer (1988), indicated that in a replicating E. coli chromosome, if a gene is occupied by an RNA Polymerase as a replication fork approaches, the replication complex may be stalled, leading to replication delays. A similar effect may be evident in Saccharomyces. In any promoter, a number of proteins are involved in transcriptional activation, forming a transcription complex. The number and types of regulatory proteins involved will vary depending upon the promoter and its regulatory characteristics and thus the extent to which the replication fork is stalled, could reflect the sum of all the different proteins bound.

6.2 Transformability and copy number of plasmids containing homologous genes

Plasmids containing either the ADH1 or PGK1 homologous gene were constructed by cloning both genes into TRP1 based plasmids (Fig 7.2.1.2 and Fig 6.3 respectively), to investigate if the plasmid inhibition effect was evident in plasmids expressing a different gene with a homologous promoter.
The homologous gene containing plasmids were constructed as follows:

pBEJ40: The whole PGK1 gene was isolated from a plasmid supplied by A. Chambers, by HindIII restriction. The fragment was subsequently cloned into the HindIII site of pC19R (Appendix). The PGK1 gene was then restricted with BamHI and XhoI and cloned into the BamHI and SalI site of pCH215, confirming orientation by restriction with BglII. (Fig 7.2.1.2)

pBEJ32: The whole ADH1 gene was isolated by BamHI restriction, from plasmid pAC2 (A. Carter) containing the complete gene. The fragment was then cloned into the BglII site of pCJ17 and orientation confirmed by XbaI restriction. (Fig 6.3)

The described plasmids were transformed into S. cerevisiae S150-2B, including the parental plasmids as controls, and transformants selected using the auxotrophic TRP1 marker on minimal media plates. Data obtained are shown in Table 1. In addition plasmid copy number was determined in minimal medium and again data is shown in Table 1.

In comparing the data obtained for plasmids containing either the heterologous or homologous gene, it was evident that in the presence of the PGK1 promoter, there was little difference in terms of transformation efficiency, but some, although not significant increase in copy number in the homologous plasmid. In the case of the ADH1 promoter, transformability was reduced in the presence of the homologous gene, but copy number was no different. Thus it would seem that in the homologous gene situation, the PGK1 promoter was slightly less inhibitory.

6.3 The effect of promoter deletions on plasmids expressing homologous and heterologous genes.

It had been speculated in the previous chapter, that the observed effects on plasmid copy number were a consequence of proteins binding at the promoter. In an attempt to examine this possibility, the effects of varying promoter deletions on the plasmid copy number of plasmids containing homologous and heterologous genes were investigated.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Coding Sequence</th>
<th>No. transformants /μg DNA in Minimal Media</th>
<th>Plasmid Copy Number in Minimal Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBEJ24</td>
<td>APT1</td>
<td>229*</td>
<td>18</td>
</tr>
<tr>
<td>pBEJ40</td>
<td>PGK1</td>
<td>360</td>
<td>20 (± 3)</td>
</tr>
<tr>
<td>pBEJ11</td>
<td>APT1</td>
<td>470*</td>
<td>19</td>
</tr>
<tr>
<td>pBEJ32</td>
<td>ADH1</td>
<td>176</td>
<td>20 (± 2)</td>
</tr>
</tbody>
</table>

The plasmid copy number shown is the average copy number per cell. Data was obtained from at least 2 individual copy number determinations for plasmids pBEJ24 and pBEJ11 and at least 3 for pBEJ40 and pBEJ32.

* Transformation data was obtained using a different batch of *S. cerevisiae* competent cells, although competence controls were very similar. Transformation data variation was ± 10
6.3.1 Effects of promoter deletions on plasmids containing homologous genes

The PGK1 gene was selected for investigation, since the promoter has been extensively characterised (Stanway et al. 1987, 1989, Chambers et al., 1988) and a number of sequence elements involved in transcriptional activation had been identified and located.

Additionally, a number of plasmids had been constructed by Chambers et al. (1988), which contained a number of varying PGK1 promoter deletions, upstream of the PGK1 coding region. These plasmids were 2μm based plasmids, but the wild-type LEU2 marker had been replaced with the defective leucine marker, leu-2d, which was known to artificially raise the plasmid copy number (Beggs, 1978; Erhart and Hollenberg, 1983). Since the defective marker would influence plasmid copy number and mask other effects, the entire PGK genes on the plasmids were excised from these plasmids and cloned into the vector plasmid pCH215 (Fig 6.3a) containing the TRP1 marker, as described in the figure legend. All the deletions considered are shown diagrammatically in Fig 6.3b.

6.3.1.1 Plasmid transformability, copy number and stability

The deletion plasmids were transformed into S. cerevisiae S150-2B and transformants selected using the auxotrophic TRP1 marker on minimal media plates. Results are shown in Table 2.

The transformation efficiency of plasmids containing the PGK1 promoter deletions was less than the plasmid containing the intact promoter. Deletion of the Heat Shock Element (HSE) (pBEJ44) had little effect on transformation frequency. Large differences were observed between the differing promoter deletions, but no pattern between the deletion and thus the strength of the promoter and plasmid transformability emerged. Deletions in the promoter region of the PGK1 gene therefore affected the plasmid and thus its transformability.

To determine if these effects ultimately affected plasmid stability and copy number, both were determined in minimal selective medium. Three individual transformants were examined and results are shown in Fig 6.3.1.1 and Table 2.
Plasmids containing the promoter deletions were constructed as follows; Plasmids obtained from Dr A. Chambers containing the deletions were restricted with HindIII to remove the PGK1 gene and the promoter deletion. The fragments were separated on acrylamide gels, eluted and then cloned into the HindIII site of plasmid pC19R (Appendix). The PGK1 fragments were subsequently removed by restriction with SmaI and partial XhoI. The fragments were then cloned into the SmaI and SalI site of plasmid pCH215 and orientation confirmed by restriction.
TABLE 2  Transformability, plasmid stability and plasmid copy number of plasmids containing various PGK1 promoter deletions in minimal selective medium

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter Binding site Deletion</th>
<th>No. of transformants/µg DNA*</th>
<th>% Stability over 10 Generations*</th>
<th>Plasmid Copy Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBEJ40</td>
<td>-</td>
<td>360</td>
<td>95.3</td>
<td>20 (± 3)</td>
</tr>
<tr>
<td>pBEJ41</td>
<td>ABFl</td>
<td>194</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>pBEJ42</td>
<td>RAP1</td>
<td>3</td>
<td>94.0</td>
<td>21 (± 5)</td>
</tr>
<tr>
<td>pBEJ43</td>
<td>RAP1, ABFl</td>
<td>45</td>
<td>92.4</td>
<td>24 (± 5)</td>
</tr>
<tr>
<td>pBEJ44</td>
<td>HSE</td>
<td>333</td>
<td>nd</td>
<td>19 (± 1)</td>
</tr>
<tr>
<td>pBEJ45</td>
<td>HSE, pTATA</td>
<td>85</td>
<td>99.5</td>
<td>nd</td>
</tr>
<tr>
<td>pBEJ46</td>
<td>RAP1, ABFl, HSE</td>
<td>45</td>
<td>88.5</td>
<td>20 (± 2)</td>
</tr>
<tr>
<td>pBEJ47</td>
<td>RAP1, pABFl, HSE, pTATA</td>
<td>66</td>
<td>94.2</td>
<td>20 (± 3)</td>
</tr>
<tr>
<td>pBEJ48</td>
<td>RAP1, pABFl, HSE, TATA</td>
<td>196</td>
<td>91.4</td>
<td>18 (± 3)</td>
</tr>
</tbody>
</table>

* : Values shown for transformability and stability are the average of at least 2 repetitions. The plasmid copy number shown in bold is the mean of at least 3 determinations and the standard deviation is shown in brackets. The value represents the average copy number per cell. Variation for transformation data is ± 10 stability ± 5%
Figure 6.3.1.1 Copy number determination of cells in minimal medium containing PGK1 promoter deletion plasmids

Tracks shown are from a number of different filters, hybridised and probed at different times.

In all instances, genomic DNA was isolated from S. cerevisiae S150-2B transformed cells grown in minimal selective medium (HUL) and restricted with EcoRI, Sall and BamHI. DNA was separated on 0.8% TAE/EtBr agarose gels and blotted using the LKB Vacugene Vacublot system (Section 2.8.5) onto Hybond-N nylon membranes. The filters were then hybridised overnight at 65°C in Church Gilbert solution (section 2.8.7) using a TRP1 labelled fragment probe as described previously.

Arrows indicate the position of weaker chromosomal TRP1 bands.
Plasmid stability was found to be very high, and not significantly affected by the differing promoter deletions. In most instances, plasmid loss was only in the region of 0.05% - 0.9% per generation although that of plasmid pBEJ46 (RAP1, ABF1 and HSE deletion), was marginally higher at 1.2% per generation, which could not be attributed to the promoter deletion.

Plasmid copy number was very similar with all deletions, although in the absence of the ABF1 and RAP1 binding site (pBEJ43) a slight improvement was evident. However, when the deletion additionally encompassed the HSE, this effect was not observed. The largest promoter deletion (pBEJ48), was found to have the lowest copy number.

Thus, it would seem from the preliminary data, that loss of parts of the promoter tended to have a negative effect on plasmid performance. This was not what was expected if the protein binding sites inhibitory to replication had been deleted, although this could reflect the difference of a homologous plasmid.

6.3.1.2 Transcript and protein analysis

Transcription and protein product levels were analysed as part of the characterisation of the effects of the promoter window deletions. The amount of transcription obtained from some of the deletions has been documented, but only with *leu-2d* constructs (Ogden et al, 1986).

Total RNA was extracted from cells grown in minimal medium to a density of 1x10⁷ cells/ml, which was subsequently Northern blotted (section 2.9.3). The filter was then probed with a 2.95 kb *PGK1* probe isolated by HindIII restriction of a plasmid based on pUC which contained the whole *PGK1* gene. The filter and the corresponding gel photo are shown in Fig 6.3.1.2.1.

Although the loadings of the blotted gel were not very consistent (Fig 6.3.1.2.1b) it was sufficient to check if relative transcription levels were similar to the *LEU2* plasmids as the *leu2-d* plasmids already reported. The amount of RNA in lanes 3, 4, 7 and 8 were almost equivalent visually, with that in lanes 2, 5, 6 and 9 approximately 3-fold less than that of the previous lanes. RNA in lanes 1 and 10 were a further 1-fold lower.
Figure 6.3.1.2.1  Northern blot analysis of yeast cells transformed with the PGK1 promoter deletion plasmids.

Panel A: Autoradiograph of filter containing 7μg RNA isolated from transformed cells grown in minimal selective medium and probed with a PGK1 gene fragment. RNA was separated on a 0.8% agarose/formaldehyde gel and then blotted using the LKB Vacublot system for 4 hours. The filter was then hybridised overnight at 65°C in Blotto solution (Section 2.9.4).
The binding site deletion is shown in brackets.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untransformed S150-2B</td>
</tr>
<tr>
<td>2</td>
<td>pCH215</td>
</tr>
<tr>
<td>3</td>
<td>pBEJ40 (whole promoter)</td>
</tr>
<tr>
<td>4</td>
<td>pBEJ44 (HSE)</td>
</tr>
<tr>
<td>5</td>
<td>pBEJ43 (ABF1 and RAP1 binding sites)</td>
</tr>
<tr>
<td>6</td>
<td>pBEJ4/7 (partial ABF1, RAP1, HSE, partial TATA)</td>
</tr>
<tr>
<td>7</td>
<td>pBEJ45 (HSE and TATA)</td>
</tr>
<tr>
<td>8</td>
<td>pBEJ48 (partial ABF1, RAP1, HSE, TATA)</td>
</tr>
<tr>
<td>9</td>
<td>pBEJ41 (ABF1)</td>
</tr>
<tr>
<td>10</td>
<td>pBEJ42 (RAP1)</td>
</tr>
</tbody>
</table>

Panel B: Photograph of gel used for Northern blotting in Panel A showing the loadings.

Panel C: Autoradiograph of filter containing RNA from cells transformed with plasmid pBEJ46, not included on previous filter. All conditions described in Panel A were employed.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untransformed S150-2B</td>
</tr>
<tr>
<td>2</td>
<td>pCH215</td>
</tr>
<tr>
<td>3</td>
<td>pBEJ40 (whole promoter)</td>
</tr>
<tr>
<td>4</td>
<td>pBEJ44 (HSE)</td>
</tr>
<tr>
<td>5</td>
<td>pBEJ46 (ABF1, RAP1, HSE)</td>
</tr>
<tr>
<td>6</td>
<td>pBEJ41 (ABF1)</td>
</tr>
</tbody>
</table>
A 1.45 kb PGK1 transcript was detected in all tracks except 1 and 2, in addition to a larger transcript. On longer exposure this larger transcript was evident in track 2, but not in track 1 (untransformed control). This was a consequence of some pUC DNA contamination present in the PGK1 probe, resulting from poor separation of the PGK1 gene from the remaining plasmid. This was confirmed when the parent plasmid which contained pUC DNA but lacked PGK1 DNA, produced the transcript, but the S150 untransformed control lacking the plasmid did not (Not shown).

The PGK transcript was found in greatest abundance in plasmids containing the whole PGK gene (pBEJ40), pBEJ44 (HSE deleted) and pBEJ45 (HSE and TATA). Poorer transcription was evident when the ABF1 and RAP1 sites were deleted (pBEJ41, pBEJ42, pBEJ43, pBEJ47 and pBEJ48).

Thus, the analysis showed a similar pattern from these LEU2 based constructs as published for leu2-d, in that although transcription was reduced when the ABF1 and RAP1 sites were deleted it was not abolished. It has been documented that the PGK1 promoter has no requirement for TATA sequences (Ogden et al, 1986; Stanway et al, 1989) and additionally the CT block is not required for transcription to occur (Stanway et al, 1989). Data obtained indicated that this was in fact the case. To complete the analysis, the amount of protein expressed by the deletions was examined.

Protein was extracted from transformants containing the deletion plasmids, as in section 2.10.1 and separated on an SDS-polyacrylamide gel (section 2.10.3). The gel was stained in Coomassie Blue stain (2.10.4) and the proteins compared (Fig 6.3.1.2.2). Two separate gels were analysed with one containing differing protein samples (Panel A and B). Although the gel would not give an accurate measure of protein concentration, those deletions producing good yields of protein could be identified.

Examination of the protein gels (Fig 6.3.1.2.2), indicated that the 44kd PGK1 protein band was only observed in protein cell extracts containing plasmids pBEJ40 (containing whole PGK1), pBEJ45 (ABF1 deletion) and pBEJ44 (HSE deletion) and not in the other samples, which in some instances could be due to the lack of sensitivity of the protein gels in detecting PGK1 protein. It was of interest to note that protein was detected in the ABF1
Figure 6.3.1.2.2 Analysis of total cell protein isolated from cells transformed with PGK1 promoter deletion containing plasmids.

2μg of total cell protein was run on a Hoeffer gel system, using a 10% resolving gel and staining in Comassie Blue stain for 4 hours. Binding site deletion is shown in brackets.

Panel A

Lane 1 = pBEJ42 (RAP1)
Lane 2 = pBEJ45 (HSE and TATA)
Lane 3 = pBEJ44 (HSE)
Lane 4 = pBEJ48 (partial ABFl, RAP1, HSE, TATA)
Lane 5 = pBEJ47 (partial ABFl, RAP1, HSE, partial TATA)
Lane 6 = pBEJ40 (whole promoter)
Lane 7 = pCH215
Lane 8 = Untransformed S150-2B
Lane 9 = Protein molecular weight marker (sizes shown)

Panel B:

Lane 1 = Protein molecular weight marker (sizes shown)
Lane 2 = pBEJ40 (whole promoter)
Lane 3 = pBEJ41 (ABFl)
Lane 4 = pBEJ44 (HSE)
Lane 5 = pBEJ46 (ABFl, RAP1, HSE)
deletion. Again, this corresponded with the documented work with the leu2-d construct. On the basis of this data, however, it was difficult to state if the expression from these deletion constructions, corresponded to relative plasmid performance characteristics.

Deletion of known protein binding sites in the PGK1 promoter, did not relieve plasmid inhibition and in fact loss of some sites actually decreased plasmid copy number. The data suggests that the absence of some components could cause further inhibition of the partitioning functions.

6.3.2 Effects of promoter deletions on plasmids containing heterologous genes.

In order to further examine the effects of removal of promoter binding sites on plasmids, heterologous APT1 gene plasmids containing other promoter deletions were constructed.

6.3.2.1 Construction of heterologous promoter deletions.

The strategy adopted for the production of the other promoter deletions was to create invitro deletions on promoter sequences. The deletions could be made using a number of restriction sites present within the promoter sequence of interest, and the subsequent promoter deletion fragment could be cloned into the promoterless APT1 plasmid, pCJ18. The deletion could then be compared in terms of expression and copy number to the plasmid lacking a promoter. Time constraints limited the number of promoter deletions which could be analysed.

Promoters which were considered were the ADH1 promoter and in addition the TRP1 promoter. The latter was chosen, since transcript analysis of the constructions described in Chapters 4 and 5, revealed that the amount of transcription obtained from the TRP1 marker on the plasmid, was in excess of that obtained from the promoter-driven APT transcript. As a consequence, it was considered that the TRP1 promoter could be useful in the expression of APT1 protein. The TRP1 promoter fragment employed, was identical to that on the TRP1 marker present on a large number of the previously described plasmids.
6.3.2.1.1 ADH1 promoter deletion construction

The ADH1 promoter deletion was constructed by isolating the complete promoter fragment from a pYcDE-2 vector (Appendix), and restricting with a number of enzymes to give a deletion fragment (Fig 6.3.2.1.1). The ADH1 promoter fragment employed here and in the constructions described in Chapter 4, was already truncated, derived by restriction with SphI at position -410, which contained a RAPl binding site between -635 and -615 (Tornow and Santangelo, 1990). This promoter fragment was found to exhibit a stronger activity in glucose and ethanol media (Brier and Young, 1982), in comparison to the wild-type promoter. The promoter deletion fragment, lacking the TATA box (at position -128 upstream of the translation initiation site of the gene) and the UAS, was created by restriction with MnlI (Fig 6.3.2.1.1).

This deletion fragment, which consisted of little more than the RNA initiation site, was then cloned into a polylinker vector to provide more convenient restriction sites and a fragment with compatible ends to sites upstream of the APT gene in plasmid pCJ18 isolated. The fragment was then cloned into pCJ18 to give plasmid pBEJ38 (Fig 6.3.2.1.2). Construction of the deletion is described in more detail in the figure legends.

6.3.2.1.2 Construction of a TRP1 promoter deletion

The TRP1 promoter employed in this analysis was in fact already a truncated promoter lacking the UAS and a TATA sequence (Fig 6.3.2.1.1). The promoter fragment was isolated and cloned into a polylinker vector to provide more convenient restriction sites. A fragment with compatible ends to plasmid pCJ18 was subsequently isolated and cloned upstream of the APT1 gene in plasmid pCJ18, to give pBEJ39 (Fig 6.3.2.1.2).

The TRP1 gene encodes the enzyme N-phosphoribosyl-1-anthranilate, which catalyses the third step in tryptophan biosynthesis. It is the only tryptophan biosynthetic gene which is not derepressible under the general control system (Miozzari et al, 1978). The TRP1 EcoRI fragment which was used in this work as a selective marker and other yeast vectors, (Parent et al, 1985), contains only a short 102 bp 5' region in which only one putative TATA element is present. The whole TRP1 promoter contains two such elements. The amount of transcription from the TRP1 fragment is similar to that of the whole promoter, but specific
The ADH1 promoter deletion was constructed as follows:
Plasmid pCH97 (Appendix) was restricted with AccI to isolate a 232bp fragment containing
the ADH1 promoter (181bp) and an additional 51bp of upstream DNA. This fragment was
subsequently restricted with BamHI and MnlI then cloned into plasmid pIC19R (Appendix)
at the NruI (blunt-ended) and BamHI sites. The UAS and TATA box were therefore
eliminated.

The TRPI promoter deletion required no construction and was the fragment employed in
most vectors. The fragment was isolated from plasmid YRp7 (Appendix) by EcoRI
restriction then subsequently restricted with AulI. The fragment was then isolated and
cloned into plasmid pIC19H (Appendix) at the EcoRI and SmaI sites, since AulI restriction
generated blunt-ended fragments.
a ADH1

UAS TATAA

Sall Sphi 222 Accl 106 MnII 75bp BamHI

b TRP1

UAS TATA TATA

EcoRI 94bp Alul

promoter fragment

promoter fragment
The promoter deletion plasmids were constructed by restriction of the ADH1 and TRP1 polylinker plasmids with XhoI and BamHI and ligating the resultant fragments into the Sall and BamHI sites in plasmid pCJ18. Both promoter fragments would be upstream of the APT1 coding sequence. Correct orientation was determined by restriction and separation on high percentage acrylamide gels.
enzyme activity is much reduced, which is considered a consequence of the full length TRP1 5' region containing a transcription termination sequence, allowing more accurate expression (Braus et al., 1988).

6.3.2 2 Performance of the promoter deletion constructions.

To determine if the use of the promoter deletions in the heterologous gene system relieved the effects of plasmid inhibition, transformability and plasmid copy number were investigated. In addition, the efficiency of the promoters was examined by determining APT expression and the amount of transcription.

6.3.2.2.1 Plasmid transformability and APT expression

Plasmids pBEJ38 and pBEJ39 were transformed into S. cerevisiae S150-2B and transformants selected for the APT1 gene or TRP1 marker on the plasmid, including the promoterless plasmid pCJ18 as a control.

APT expression from both constructs was also determined in YPD/G418 and minimal selective media. Results are shown in Table 3.

Data from the control plasmid (pCJ18) indicated that the competence of the transforming E. coli cells was reduced approximately 5-fold and thus the numbers of auxotrophic transformants obtained with the deletion plasmids would be 5-fold lower than expected. In both instances, auxotrophic transformability was reduced in comparison to the promoterless pCJ18 control, with the greatest reduction in the ADH1 truncated promoter plasmid. Although data from the full-length promoter ADH1 promoter was obtained in a different transformation and thus could not be directly compared, it seemed that the deleted promoter was less efficient in transformation, which was in agreement with a similar observation with the deleted PGK1 promoter, in section 6.3.1.1.

Unlike the promoterless plasmid pCJ18, both plasmids provided G418' transformants following a 90 m post-transformation incubation in YPD, being of a comparable number to full-length promoter-containing constructions previously investigated (Chapter 4).
### TABLE 3  Number of transformants and APT expression obtained with plasmids containing truncated TRP1 and ADH1 promoters in comparison to the promoterless plasmid

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>PROMOTER</th>
<th>NUMBER OF TRANSFORMANTS/μg DNA</th>
<th>APT UNITS/mg CELL PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G418</td>
<td>Minimal Selection</td>
</tr>
<tr>
<td>pBEJ38</td>
<td>truncated ADH1</td>
<td>4</td>
<td>47</td>
</tr>
<tr>
<td>pBEJ11a</td>
<td>ADH1</td>
<td>3</td>
<td>470</td>
</tr>
<tr>
<td>pBEJ39</td>
<td>truncated TRP1</td>
<td>6</td>
<td>114</td>
</tr>
<tr>
<td>pCJ18</td>
<td>-</td>
<td>0</td>
<td>235</td>
</tr>
</tbody>
</table>

* : All values shown are the average of three repetitions. Variation is approximately ±20 for minimal transformations and ± 40 for APT expression

*: Data obtained from different batch of *S. cerevisiae* competent cells
Both truncated promoter plasmids expressed greater amounts of APT protein than plasmid pCJ18, although less than that expressed from a number of full-length promoter containing APT1 constructions. Plasmid pBEJ38 (truncated ADH promoter) expressed greater amounts of APT than the truncated TRP1 promoter, which was surprising since the latter contained a TATA sequence, which was absent in the ADH1 promoter fragment. Unlike the full-length ADH1 promoter, expression from plasmid pBEJ38 was greatest in YPD/G418 medium and not minimal selective medium. One possibility for this effect was that the deletion removed a regulatory element which in some way increased expression in the minimal medium.

Initial data had indicated that a reduced transformability in minimal media was still evident and it was therefore expected that the copy number would be less than that for plasmid pCJ18. For this reason plasmid copy number was determined for the plasmids and compared to that obtained with the promoterless plasmid pCJ18. Data obtained is shown in Table 4 and Fig 6.3.2.2.

6.3.2.2 Plasmid copy number

The copy number of plasmid pBEJ39, containing the truncated TRP1 promoter, in YPD/G418 and minimal medium was found to be improved on comparison to other promoter containing plasmids. In comparison to plasmid pCJ18 in YPD/G418 medium, however, the copy number remained lower than that of plasmid pCJ18, but was comparable in minimal selective medium. Conversely the copy number of plasmid pBEJ39 (truncated ADH1 promoter) was only very slightly but not significantly improved. This suggests, that removal of certain protein binding sites in some promoters may have some effect on plasmid copy number. Although APT expression from the truncated ADH1 promoter, was 2-fold greater than the truncated TRP1 promoter, both plasmids were able to yield G418® transformants following only a 90m post-transformation incubation in YPD. In addition, although the expression levels differed, the number of G418 transformants obtained was very similar. This tended to suggest, that the lower expression level was not responsible for the increased copy number. Although the copy number was increased with the deleted promoter, the amount of APT protein produced per gene copy was very small, showing that promoter activity was greatly reduced. Thus, it would appear that an improving effect of the promoter deletions was evident in
### TABLE 4  Copy number of plasmids containing the truncated TRP1 and ADH1 promoters

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>PROMOTER</th>
<th>YPD/G418 Medium</th>
<th>Minimal Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBEJ38</td>
<td>truncated ADH1</td>
<td>20 (± 3)</td>
<td>20 (± 4)</td>
</tr>
<tr>
<td>pBEJ11</td>
<td>ADH1</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>pBEJ39</td>
<td>truncated TRP1</td>
<td>32 (± 5)</td>
<td>24 (± 5)</td>
</tr>
<tr>
<td>pCJ18</td>
<td>-</td>
<td>54 (± 2)</td>
<td>25 (± 3)</td>
</tr>
</tbody>
</table>

Plasmid copy number shown in bold was the mean of at least 6 determinations with the standard deviation shown in brackets. The value shown represents the average copy number per cell.
Tracks shown are from a number of different filters, hybridised and probed at different times.

In all instances, genomic DNA was isolated from *S. cerevisiae* S150-2B transformed cells grown in either YPD/G418 medium (G418) or minimal selective medium (HUL) and restricted with EcoR1, SalI and BamHI. DNA was separated on 0.8% TAE/EtBr agarose gels and blotted using the LKB Vacugene vacublot system onto Hybond-N nylon membranes. The filters were then hybridised at 65°C in Church Gilbert solution (Section 2.8.7) using a TRP1 labelled fragment probe as described previously.

Arrows indicate the position of weaker chromosomal TRP1 bands.
certain heterologous constructions, unlike the homologous constructions, indicating that the relief of plasmid inhibition could be due to loss of promoter protein binding sites.

6.3.2.2.3 Transcript analysis of plasmids containing the truncated promoters

Previous transcript analysis of plasmid pCJ18 had shown a large amount of background transcription and thus the question was raised as to whether this was similar with plasmids containing the truncated promoters. Additionally since the copy number of plasmid pBEJ39 was increased, it would be interesting to determine if the amount of transcription correlated to the effect.

RNA was extracted (section 2.9.1) and Northern blotted (section 2.9.3) from transformed cells containing the promoter deletion plasmids and also the promoterless pCJ18 plasmid, for comparison purposes. RNA was then probed with both a TRP1 probe and an APT probe to determine the amount of transcription emanating from the constructions (Fig 6.3.2.2.3a and b).

On using the APT probe (Fig 6.3.2.2.3a) it was evident that a greater amount of background transcription was evident in plasmid pCJ18, in comparison to that previously observed in Chapter 5. Since the pattern of transcription obtained was very similar to that obtained with the whole plasmid probe in Chapter 5, it was considered that this may be a result of pUC contamination of the APT probe. This could also be the origin of the larger transcript of 2.4kb apparent in the truncated promoter plasmids. APT transcript sizes expected with plasmid pCJ18 were 1.5kb and 1.6kb and that from a plasmid containing a promoter and terminator 1.65kb. The APT transcript identified from both truncated promoters was approximately 1.2kb, since both promoters differed in size by only 19bp.

The transcripts therefore appeared to be initiating in the promoter deletion region of the plasmid and terminating at the CYC1 terminator. A proportion of the transcript must be initiated correctly since greater APT expression than plasmid pCJ18 is occurring. This would seem to be the case with plasmid pBEJ38 (truncated ADH promoter), since although less transcription is observed greater APT expression is obtained than with the truncated TRP1 promoter. In both cases, however, less transcription was observed in minimal media, in agreement with previous observations.
Figure 6.3.2.2.3a  Northern blot analysis of yeast cells transformed with plasmids pBEJ38 and pBEJ39

Panel A: Autoradiograph of filter containing 7µg of total RNA from cells transformed with plasmids pBEJ38 and pBEJ39 and grown in either minimal selective medium (HUL) or in YPD/G418 medium. RNA was separated on a 1% agarose/formaldehyde gel and blotted using the LKB Vacugene blotting system (section 2.8.5) onto a Hybond-N nylon membrane. The filter was hybridised for 48h at 42°C in Northern hybridisation solution with a labelled APT fragment. RNA from cells transformed with plasmid pCJ18 was included as a control. The filter was exposed to X-ray film at -70°C for 24h.

Panel B: Identical to Panel A but exposure was for 4 hours at -70°C

Panel C: Photo of RNA gel used for Northern Blotting showing RNA loading.

Lane 1 = BRL RNA ladder (sizes shown in bp)
Lane 2 = Untransformed S150-2B
Lane 3 = pCJ18 YPD/G418
Lane 4 = pBEJ39 YPD/G418
Lane 5 = pBEJ39 HUL
Lane 6 = pBEJ38 HUL
Lane 7 = pBEJ38 YPD/G418
Figure 6.3.2.3b. Northern blot analysis of yeast cells transformed with plasmids pBEJ38 and pBEJ39

As figure 6.4.2.3a, but the filter was hybridised with a labelled TRP1 fragment.

**Panel A**: Filter exposed to X-ray film at -70°C for 24h.

**Panel B**: Exposure at -70°C for 2h.

**Panel C**: Photo of RNA gel used for Northern Blotting showing RNA loading.

Lane 1 = BRL RNA ladder (sizes shown in bp)
Lane 2 = Untransformed S150-2B
Lane 3 = pCJ18 YPD/G418
Lane 4 = pBEJ39 YPD/G418
Lane 5 = pBEJ39 HUL
Lane 6 = pBEJ38 HUL
Lane 7 = pBEJ38 YPD/G418
A second RNA gel to be probed with a TRPI probe (6.3.2.2.3b), was not completely satisfactory, owing to some degradation of the RNA. Although some smearing was evident on the RNA gel, it was apparent that as in pCJ18, both heterogenous and discrete transcripts were evident. This was in contrast to the other promoter-containing plasmids in which two transcripts were only evident. The discrete transcripts were found to be approximately 2.3kb and 1.4kb, which were slightly smaller than those previously observed in Chapter 5. This could be accounted for by the fact that the gel did not run completely straight.

The transcript data described, provided evidence that a large amount of transcription was produced from the promoter deletion containing plasmids. As in plasmid pCJ18, however, not all transcripts are initiating in the correct region, and would therefore be less efficiently translated. The data did indicate however, that the amount of background transcription could correlate with the increased plasmid copy number observed in plasmid pCJ18.

6.4 DISCUSSION

The performance of plasmids containing homologous ADH1 or PGK1 genes and plasmids containing the same promoters directing expression of a heterologous gene, namely APT1, indicated some differences, particularly with the PGK1 promoter. The heterologous PGK1 construction was found to have a slightly lower plasmid copy number compared to the homologous plasmids, although transformability was very similar. This was in contrast to the ADH1 promoter constructions, in which plasmid copy number was comparable between the homologous and heterologous plasmids, but the heterologous ADH1 promoter construction was found to have a reduced transformability.

The data therefore indicated that the homologous gene plasmids were less inhibited by the promoters than the heterologous gene constructions.

Deletion a number of binding sites from a PGK1 promoter directing expression of the homologous gene, was found to impair plasmid performance. This resulted in reduced plasmid transformability, although no correlation with promoter strength was evident. Deletion of a HSE, had no effect on transformation efficiency, but more marked effects
were evident on deletion of the RAP1 and ABF1 binding sites. The promoter deletions were found to have little effect on plasmid copy number, although deletion of the RAP1 and ABF1 sites showed some increase. Again however, no correlation with deletion and copy number increase could be observed.

Transcript data which was obtained for the deletions, indicated that in all cases, PGK1 transcription occurred in all the promoters regardless of the deletion which they contained. Deletion of the ABF1 site has been shown merely to reduce transcription, since the ABF1 protein acts synergistically with other weak transcriptional activators to activate transcription to a high level (Buchman and Kornberg 1990). Variable transcript levels were evident and were found to be in agreement with results obtained by Ogden et al, (1986) with some of the deletions. No large amount of background transcription was evident, again contrasting to plasmid pCJ18. Transcription was not abolished when the deletion eliminated 0.4 kb of PGK1 promoter sequence, leaving only the Initiation site. Thus some other sequence outside the limits of the deletion, was responsible for directing transcription and was bound by the TFIID factor (Singer et al, 1990), which normally binds to the TATA box (Davison, et al, 1983). As a result, basal transcription observed in deletions lacking the TATA sequence could occur (pBEJ45).

Deletion of the Activator core sequence (-473 to -458) did not abolish transcription in plasmids pBEJ42, pBEJ43 and pBEJ47, (the latter two deletions also removed a number of other elements), with only a 2-fold reduction in comparison to a full promoter fragment, although functional protein could not be detected. This confirmed the work of Chambers et al, (1988), in which deletion of this site was found to almost completely abolished PGK expression. On analysis of protein expression from cells containing the deleted promoter plasmids, PGK1 protein was only evident in plasmids pBEJ40 (the full PGK1 promoter), pBEJ44, (heat shock element deletion) and pBEJ41 (ABF1 deletion).

The TRP1 and ADH1 truncated promoters employed to direct expression of the heterologous APT gene both retained the transcription initiation sites, but lacked the UAS, and UAS and TATA box respectively. Transformation data for the plasmids, indicated a reduced auxotrophic transformability, but G418R transformants were obtained with only a 90 minute post-transformation expression time, in contrast to plasmid pCJ18. The truncated ADH1 promoter plasmid (pBEJ38), gave an equivalent number of G418R transformants as
the undeleted promoter containing plasmid (pBEJ11) although fewer than pBEJ39 (truncated TRP1 promoter). Both truncated promoters were able to express APT protein, with greatest expression from the ADH1 truncated promoter than with TRP1. This was surprising since the truncated ADH1 promoter lacked TATA sequences which were present in the TRP1 deletion. Sequence analysis revealed no comparable TATA sequence, indicating again, that some other sequence unrelated to the consensus TATA was able to functionally replace it (Singer et al, 1990). APT expression from the truncated ADH1 promoter in both YPD/G418 and minimal media was found to be less than plasmid pBEJ11 (the undeleted ADH1 promoter) particularly in minimal medium where expression was 10-fold less. Additionally, in contrast to pBEJ11 and other ADH1 containing plasmids, expression in YPD/G418 medium was greater than in minimal medium. This indicated that the upstream promoter region contained an important element involved in this regulation. The RAP1 binding site, which was responsible for carbon source regulation in the PGK1 promoter (Stanway et al, 1987), is also present in the ADH1 promoter (Chambers et al, 1989), but in the truncated promoter fragment it is deleted. This could indicate some involvement, although the carbon source in both media is the same. Thus the effect may be related to the presence of differing amino-acids in the media. In partial TRP1 promoters, like the EcoRI/TRP fragment from which the truncated promoter was isolated, plasmid-encoded transcription is initiated predominantly in adjacent vector regions, resulting mainly in large, poorly translated transcripts (Braus et al, 1988). This was indicated from the transcript analysis performed and thus could explain the poor expression obtained with the TRP1 promoter.

Plasmid copy number of pBEJ38 (truncated ADH1 promoter) was found to be less than that of plasmid pCJ18 in both minimal and YPD/G418 selective media. Compared to plasmid pBEJ11 (full-length promoter), copy number in YPD/G418 was slightly higher, although not significantly. Copy number of pBEJ39 (truncated TRP1 promoter), however, was only 2-fold lower than plasmid pCJ18 in YPD/G418 medium and slightly higher in minimal medium. This indicated that deletion of some of the protein binding sites in the heterologous promoter constructions, could cause an effect.

Transcript analysis of the deleted promoters indicated that particularly in rich selective medium, a large number of heterogenous TRP transcripts were evident. This observation correlated with that observed in plasmid pCJ18. Although an additional transcript of 2.4kb
was evident with an APT probe, comparison with previous transcript analysis obtained on using a whole plasmid probe in Chapter 5, indicated that this may be due to probe contamination. However, the pattern of transcription observed with the truncated promoter plasmids, was very similar to that of pCJ18, and not the other promoter-containing plasmids. This indicated a similar level of background transcription to the promoterless plasmid. Shorter autoradiographic exposure revealed two transcripts of 2.4kb (described earlier) and 1.2kb. The size of the latter transcript was in agreement with initiation in the deleted promoter region. The level of transcription from the truncated ADH1 promoter was less than that of the truncated TRP promoter, although since expression levels were greater with the former promoter, it would seem that these transcripts must be initiated correctly and are therefore more accurately translated. This is surprising, since the truncated ADH promoter lacks TATA sequences which are present in the TRP promoter, although the latter is known to be a weaker promoter.

In summary, the analysis of the heterologous ADH1 and TRP1 promoter deletions, indicated that protein binding sites may have some role on the copy number reduction observed in full length promoter constructions, although it was not a significant effect. Interestingly, the amount of APT protein expressed from the plasmids indicated that even with the deletion of a large portion of the promoter, they are still functional. In deleting parts of the promoter, however, spatial differences will occur which can result in problems of translation and thus this would reduce the amount of expression.

In all, deletion of important binding sites from the promoter, had some, but not significant effect on the copy number, but was dependent on the promoter. Ultimately, however, the deletion of protein binding sites resulted in the production of less efficient promoters, expressing less protein. If the copy number had been substantially increased, however, this reduction in expression would have been overcome.
CHAPTER SEVEN

STUDIES WITH AN ADH REPORTER GENE AND THE
DISCOVERY AND INITIAL CHARACTERISATION OF POSSIBLE
TRANSACTING EFFECTS BETWEEN PLASMID AND
CHROMOSOMAL GENES
7.1 INTRODUCTION

Placing a gene under the control of various yeast gene promoters, does not guarantee good expression nor the highest yields of the gene product. This was illustrated in Chapters 4 and 5, when on using an efficient glycolytic promoter and a 2µm multi-copy plasmid, maximum yields of APT protein were only in the range of 0.2% of total cell protein.

This effect has been observed by a number of workers. Mellor, et al (1985) found that expression of human interferon-α-2 using the PGK1 promoter, was much reduced in comparison to that obtained from the whole PGK1 gene on a similar plasmid. This was considered to be a result of low mRNA levels and additionally to the more rapid turnover of the interferon protein. Differences observed on expressing interferon on various heterologous cartridges, was also attributed to low mRNA (Chen, 1985). Investigation of the low expression of the APT gene in Chapters 3 and 4, showed that it was attributable in part to slightly lower mRNA, but was considered to be mainly a consequence of poor translation efficiency. However, this was found not to be due to either the AUG context nor secondary structure and it was therefore considered that the low codon bias index of the APT gene could be responsible. This concurs with the observations of Hoekema et al (1987) that an increasing number of minor codons on the PGK1 gene, caused a corresponding decrease in both the mRNA and protein levels of the gene. Also, in nature, a strong correlation exists between the level of gene expression and the degree of biased codon usage (Sharp et al, 1986; Hoekema et al, 1987).

The codon bias index is a measure of codon choices for each amino-acid that are used (Bennetzen and Hall, 1982). A value of 1 indicates that for all of the triplets in the mRNA, only codons of the preferred variety are used. A value of zero on the other hand, indicates totally random choice. There is a strong positive correlation between the extent of codon bias towards these preferred codons and the level of a particular protein and its mRNA in yeast cells. This also correlates with the relative levels of tRNA's for particular codons. The glyceraldehyde-3-phosphate dehydrogenase gene and the enolase gene, both known to encode abundant cellular proteins, have a codon bias index of 0.99 and 0.96 respectively. On the other hand, the codon bias of iso-2 cytochrome c is 0.15, (Bennetzen and Hall, 1982) which reflects the low abundance of its mRNA, of 0.003%.
The codon bias index of APT1 is very low at 0.07 and that of APT2 is only marginally better with a codon bias of 0.1 (Hadfield et al., 1990) and thus the low expression could be a result of the high content of minor codons present in the APT gene. To investigate the role of codon bias in heterologous gene expression levels, a gene with a high codon bias index was constructed and its expression in yeast analysed. It would be expected that a gene with a high codon bias should permit high efficiency translation of the mRNA and reflect more accurately the level of transcription. In addition, it would be of interest to determine if the copy number effects could be reduced if codon bias was improved.

7.2 Expression studies with an ADH reporter gene.

7.2.1 Selection of a high codon bias reporter gene.

Since different organisms show different bias in the choice of codons in their genes (Grantham et al., 1986), it is very difficult to find a foreign gene that has a high codon bias for yeast. Short of completely resynthesising a foreign coding sequence with preferred yeast codons, which was impractical, the alternative was to use a coding sequence from a highly expressed natural yeast gene.

Genes with a high codon bias index which could be considered for use in this context, were the PGK1, GPD1 and ADH1 genes, with an index of 0.95 (Sharp et al., 1986), 0.99 and 0.92 (Bennetzen and Hall, 1982) respectively. The alcohol dehydrogenase gene was chosen, since its activity could be easily monitored spectrophotometrically, with a sensitive assay, and the amount of ADH protein expressed could be determined. This is based on the fact that ADH protein catalyses the reduction of acetaldehyde to ethanol by NADH, which also proceeds in the opposite direction. On the addition of NAD and ethanol to crude protein extracts (section 2.10.5), the conversion of ethanol to acetaldehyde can be followed between 335-340 nm.

7.2.1.1 Construction of a promoterless and promoter driven reporter gene plasmids

In order to perform a similar investigation to that performed with the APT gene it was necessary to produce an ADH1 reporter gene to which other promoters could be easily added. This required the isolation of the ADH1 coding region without its promoter, which could be
achieved by endonuclease restriction digestion and was performed as described in the figure legend (Fig 7.2.1.1).

**ADH** promoterless and promoter-driven reporter genes were constructed on 2μm based plasmids analogous to those employed in the initial study of **APT** expression. The promoter-free **ADH** construction, **CYC1** promoter-containing construction and homologous **ADH1** promoter-containing gene constructions (pBEJ34, pBEJ35 and pBEJ32 respectively; Fig 7.2.1.2) were based on the **TRP1** containing plasmid, pCJ17. For the **PGK1** containing construction, the **LEU2** plasmid, pBEJ35 (described in chapter 3) was employed (pBEJ33; Fig 7.2.1.3). Construction of all plasmids is described in the relevant figure legend. These constructions enabled a direct comparison of **APT** and **ADH1** gene expression.

### 7.2.2 Investigation of the constructions.

#### 7.2.2.1 Transformation efficiencies.

As in previous transformations all plasmids were transformed into *S. cerevisiae* S150-2B. Transformant cells containing pBEJ32, pBEJ34 and pBEJ35 were selected for the **TRP1** marker in minimal medium, with transformants containing plasmids pBEJ15 and pBEJ33 selected using the **LEU2** marker. Plasmids pCJ17 and pBEJ15, lacking the **ADH1** reporter gene, were employed as controls. Transformant efficiencies are shown in Table 1.

None of the plasmids transformed with a particularly high frequency, with the **ADH** reporter without a promoter (pBEJ34) giving the greatest number of transformants. In comparison to the promoterless **APT** plasmid, plasmid pCJ18 (Table 4) demonstrated a notably higher transformability than pBEJ34. In the presence of the **CYC1** promoter (pBEJ35), or the whole **ADH** gene (pBEJ32), a reduction in the number of transformants was observed, which was consistent with similar effects observed when a promoter was added to the **APT** gene. The **PGK1** promoter-driven **ADH1** reporter gene plasmid pBEJ33, also transformed less efficiently than the analogous **APT** plasmid pBEJ16 (Table 4). However, in the absence of the **ADH** coding sequence in plasmid pBEJ15, transformation was very slightly worse.
The reporter fragment was obtained as follows; Plasmid pAC2 (supplied by Dr. A. Carter), was restricted with BamHI to isolate the ADH1 gene. The gene fragment was then restricted with SspI (yielding blunt ends) and a 1.4Kb SspI-BamHI fragment isolated. This was subsequently cloned into the BamHI and Smal sites of pIC19H (Appendix 1).

Arrows indicate the site of mRNA initiation.
pADH

TATAA

ADH

1.8kb

638 148

33 0 1268 26

reporter gene fragment

1.4kb

tADH
Figure 7.2.1.2 Construction and structure of ADH1 reporter gene plasmids.

Plasmid pBEJ34 was constructed by restricting the reporter polylinker plasmid with BglII and BamHI and ligating this into the BamHI site of plasmid pCJ17. Very careful restriction analysis was required to ensure the fragment was in the correct orientation.

Plasmid pBEJ35 was constructed by isolation of a CYC1 promoter fragment by Sall and BamHI restriction of plasmid pCH83. The fragment was then cloned into the Sall site of plasmid pBEJ34.

Plasmid pBEJ32 contains the whole ADH1 gene and was constructed by isolating the ADH1 gene by BamHI restriction of plasmid pAC2 and cloning the fragment into the BamHI site of plasmid pCJ17.
This plasmid was constructed by isolating the ADH1 reporter gene fragment on a BamHI - BgIII fragment and cloning this into the BgIII site of plasmid pBEJ15 (Fig 3.3.2.1b), between the PGK1 promoter and terminator.
**TABLE 1** Number of transformants obtained with plasmids containing varying promoter and ADH reporter cartridges in minimal selective medium

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Marker</th>
<th>Promoter</th>
<th>Reporter cartridge</th>
<th>Number of transformants/µg DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBEJ15</td>
<td>LEU2</td>
<td>PGK1</td>
<td>-</td>
<td>223</td>
</tr>
<tr>
<td>pBEJ33</td>
<td>LEU2</td>
<td>PGK1</td>
<td>ADH1</td>
<td>296</td>
</tr>
<tr>
<td>pCJ17</td>
<td>TRP1</td>
<td>-</td>
<td>-</td>
<td>321</td>
</tr>
<tr>
<td>pBEJ34</td>
<td>TRP1</td>
<td>-</td>
<td>ADH1</td>
<td>384</td>
</tr>
<tr>
<td>pBEJ32</td>
<td>TRP1</td>
<td>ADH1</td>
<td>ADH1</td>
<td>176</td>
</tr>
<tr>
<td>pBEJ35</td>
<td>TRP1</td>
<td>CYC1</td>
<td>ADH1</td>
<td>164</td>
</tr>
</tbody>
</table>

* : All values shown are the average of three repetitions

Variation = ± 10
7.2.2.1 Stability and copy number of the transformants

Stability and copy number were determined in the ADH1 containing plasmids, in order to further investigate any similarities between the expression of a high codon bias gene and the APT gene on similar plasmids. In addition, these determinations would indicate if previous observations with the APT containing constructions was a consequence of the APT gene.

Stability in non-selective media over 10 generations was determined for three individual transformants and plasmid copy number determined for at least three transformants. Results are shown in Table 2 and copy numbers additionally in Fig 7.2.2.2.

As with the APT containing plasmids, the stability of the ADH1 containing plasmids was found to be relatively high, although stability was slightly reduced by addition of a CYC1 (pBEJ35) or ADH1 (pBEJ32) promoter. This mirrored the findings with APT.

On analysis of the copy number data, no significant differences between the ADH1 reporter plasmids was evident. However, the ADH reporter construct lacking a promoter, pBEJ34, had a slightly lower copy number in minimal medium than its APT counterpart, pCJ18. Likewise plasmid pCJ17, which lacks a reporter coding sequence. This suggests that the presence of the APT sequence may cause an increase in plasmid copy number. Copy number of the LEU2 based plasmids was slightly lower that those of the TRP1 based plasmids. Most notable was that in the absence of the ADH1 reporter coding sequence in the pBEJ15 plasmid, a distinct decrease in copy number was observed. This plasmid contains a promoter that is not "hooked up" to a coding sequence and so its activity will not give rise to a product. This observation has been reported earlier (Chapter 5) and may implicate viable translation in the plasmid inhibition phenomenon.

7.2.3 Transcript analysis

Transformants were grown in minimal selective medium to a density of 1x10^7 cells/ml and RNA isolated as described in section 2.9.1. Equal amounts of RNA from all constructions were separated on agarose gels (section 2.9.2) including RNA from cells transformed with pCJ17 and pBEJ15 as controls, in addition to an untransformed S150-2B control for the detection of background ADH activity. The gels were then Northern blotted (2.9.3) and both resultant filters
Table 2: Plasmid copy number and plasmid stability over 10 generations of plasmids containing the \( ADH1 \) reporter gene.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Coding sequence</th>
<th>Promoter</th>
<th>% Non-selective Stability over 10 generations</th>
<th>Plasmid copy number in minimal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCJ17</td>
<td>-</td>
<td>-</td>
<td>88.0</td>
<td>21 (± 3)</td>
</tr>
<tr>
<td>pBEJ34</td>
<td>( ADH1 )</td>
<td>-</td>
<td>89.0</td>
<td>20 (± 2)</td>
</tr>
<tr>
<td>pBEJ35</td>
<td>( ADH1 )</td>
<td>( CYC1 )</td>
<td>84.0</td>
<td>23 (± 4)</td>
</tr>
<tr>
<td>pBEJ32</td>
<td>( ADH1 )</td>
<td>( ADH1 )</td>
<td>79.5</td>
<td>20 (± 2)</td>
</tr>
<tr>
<td>pBEJ15</td>
<td>-</td>
<td>( PGK1 )</td>
<td>93.4</td>
<td>16 (± 2)</td>
</tr>
<tr>
<td>pBEJ33</td>
<td>( ADH1 )</td>
<td>( PGK1 )</td>
<td>nd</td>
<td>19 (± 2)</td>
</tr>
</tbody>
</table>

*: Stability values shown are the average of three repetitions with a variation of ± 5%

Plasmid copy number shown in bold is the mean of at least 4 determinations. The standard deviation is shown in brackets. The value shown represents the average copy number per cell.
Tracks shown are from a number of different filters, hybridised and probed at different times.

In all instances, genomic DNA was isolated from *S. cerevisiae* S150-2B transformed cells grown in minimal selective medium (HUL) and restricted with EcoRI, Sall and BamHI. DNA was separated on 0.8% TAE/EtBr agarose gels and blotted using the LKB VacuGene vacublotting system onto Hybond-N nylon membranes. The filters were then hybridised at 65°C in Church Gilbert solution using a TRP1 labelled fragment probe as described previously.
were then probed with a 3.5 kb ADH1 probe which contained the whole gene, isolated by BamHI restriction of plasmid pAC2 (A. Carter). Transcription obtained is shown in Fig 7.2.3. An ADH transcript of approximately 1.4 kb could be detected in all the samples, which would also reflect chromosomal ADH transcription. Taking into account that the loadings of the gel were not identical, it was evident that the amount of transcription obtained in the untransformed control and that transformed with plasmid pCJ17, were identical and very low (Panel A). An approximate 2-fold increase in transcription was evident in the presence of the ADH1 reporter gene (pBEJ34), which was further increased 3-fold in the presence of the CYC1 promoter (pBEJ35). Plasmid pBEJ32, containing the homologous ADH gene, gave the greatest amount of transcription, which was approximately 20-fold more than the untransformed control or the pCJ17 transformant.

Only one major transcript was observed with pBEJ34 (in selective minimal medium), with little background transcription. This was in contrast to pCJ18, which suggests that for some reason the high background transcription of pCJ18 (Chapter 5) results from the presence of the APT sequence, correlating with the higher copy number.

ADH transcription in the presence of plasmid pBEJ33 (panel B), containing the PGK promoter-driven ADH1 gene was found to be equivalent to that obtained with the homologous ADH plasmid (pBEJ32). However, an interesting observation was that ADH transcription from plasmid pBEJ15, (the LEU2 plasmid lacking the ADH reporter) was almost equivalent to plasmid pBEJ33 (containing the ADH reporter gene). This was very unexpected since plasmid pBEJ15 only contained a PGK1 promoter and terminator region, with no part of the ADH coding-region. This suggests that the chromosomal ADH1 gene is “transactivated” by plasmid pBEJ15 causing the production of the mRNA.

7.3 Determination of ADH activity in the constructions

In order to determine if the previous transcript data was reflected in actual protein expression and to determine if more protein was expressed from a higher codon bias gene in a similar plasmid, to that of a lower bias (APT1), ADH activity in the transformant cells was determined. ADH activity was assayed by following the conversion of ethanol to acetaldehyde (as described in section 2.10.5), in crude protein extracts, measuring the change in
Figure 7.2.3 Northern blot analysis of cells transformed with the ADH1 reporter containing plasmids

Autoradiograph of filter containing 7μg of total RNA isolated from cells grown in minimal selective medium and probed with a labelled 3.5kb ADH1 gene fragment. RNA was separated on a 1% agarose/formaldehyde gel and blotted using the LKB Vacugene blotting system (section 2.8.5) onto a Hybond-N nylon filter. The filter was hybridised overnight at 65°C in Church Gilbert solution lacking BSA with the ADH1 labelled gene fragment.

Panel A:

Lane 1 = BRL RNA marker
Lane 2 = Untransformed S150-2B
Lane 3 = pCJ17
Lane 4 = pBEJ32
Lane 5 = pBEJ34
Lane 6 = pBEJ35

Panel B:

Lane 1 = pBEJ34
Lane 3 = pBEJ32
Lane 4 = pBEJ33
Lane 4 = pBEJ15
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA ladder</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S150</td>
<td>CU17</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A**

- 2.4
- 1.4

**B**

- BEJ34
- BEJ32
- BEJ33
- BEJ15
absorbance per minute in the reaction mixture during the initial part of the reaction.

ADH units, representing ADH specific activity, could be determined by the use of the following equation:

\[
\text{E/minute} \times \text{Reaction volume} = E \times \text{path length} \times \text{sample volume}
\]

where;

- \( E/\text{min} \) = change in absorbance per minute
- \( E \) = Extinction coefficient (6.22 in this instance)
- path length = 1
- reaction volume = 3 ml
- sample volume = 0.1 ml

All transformants were grown in minimal selective medium to a density of 1 x 10^7 cells/ml, then harvested and crude protein extracts made (section 2.10.1).

Initial pilot assays, using identical samples with different amounts of protein, indicated that the assay results were more consistent if a constant amount of crude protein extract was included in each set of reactions. Owing to low ADH activity in some of the samples, 100 μg of protein was employed. In samples having large amounts of activity, the paper speed was increased, taking this into account when the E/min was calculated. Since all samples were grown in glucose containing medium, the ADH activity being assayed would be ADH1 activity.

### 7.3.1 Construction of a standard curve of ADH activity versus the amount of ADH

To relate the amount of ADH activity obtained in the assay to μg of ADH, a standard curve was constructed. Varying amounts of a stock solution of pure ADH enzyme isolated from Bakers yeast, was diluted in ADH protein extraction buffer, to a final volume of 100μl and the activity assayed as previously described. Since yeast protein extracts contained functional ADH, these could not be employed to provide background protein since aberrant ADH activity of an unknown amount would also be detected. The amount of ADH obtained from the graph was therefore an absolute amount and not a percentage of total cell protein. A graph of ADH units against amount of ADH protein, was plotted using the data, which could be used to equate units to the amount of ADH1 protein produced in any transformant cell (Fig 7.3.1).
Figure 7.3.1

ADH Standard curve

ADH Units/mg cell protein vs. µg ADH protein
In order to confirm that the activity being followed was a true enzyme reaction, an inhibitor, Pyrazole, was added to the reaction mixture which was known to inhibit ADH activity. It was not known, however, if the inhibitor also affected other enzyme activities.

7.3.2 Confirmation that activity determined was enzymatic

A 1M solution of Pyrazole was made in sterile water and 100μl (to give a 33mM final concentration) added to a 1/10th dilution of pure ADH enzyme which gave the greatest activity in the standard assay (Fig 7.3.2a). On addition of ethanol to the reaction, no change in absorbance was measured, indicating that the activity was inhibited (Fig 7.3.2b). A similar effect was also observed when a 1/10th dilution of Pyrazole was employed (Fig 7.3.2c). On addition of 100μl of Pyrazole to a reaction in which ethanol had already been added, it was observed that the reaction stopped immediately on Pyrazole addition, showing no further absorbance increase (Fig 7.3.2d).

It was thus concluded that the activity being measured was indeed enzymatic.

7.3.3 ADH activity of yeast transformants

ADH activity of cells transformed with the previously described plasmids was determined as described in section 2.10.5. 100ml of minimal selective cultures were grown to a density of 1x10^7 cells/ml and crude protein extracts produced (section 2.10.1). An untransformed S150-2B culture was grown to the same density in minimal non-selective medium, to determine the amount of background ADH activity. Background activity could therefore be subtracted from that obtained with the transformed cells, giving only the ADH activity produced by the plasmid. As all cultures were grown in glucose medium, the ADH activity determined would represent ADH1 activity, since the other ADH isoenzymes are not active in this medium (Ciriacy, 1979; Beier et al, 1985).

100μg of crude protein extract was employed in each assay, which was performed several times and data obtained shown in Table 3.

ADH activity in the untransformed S150-2B cells and those transformed with plasmid pCJ17, was not very high and very similar, reflecting previous transcription data and that pCJ17
The effect of the addition of Pyrazole to an ADH enzyme reaction.

The spectrophotometer traces shown were obtained when assaying for ADH activity. The traces shown show the change in absorbance (vertical direction) over time (horizontal direction) when following the conversion of ethanol to acetaldehyde, catalysed by the ADH enzyme. The reaction begins when ethanol (depicted as i on the figure) is added to the reaction mix containing the crude protein extract and NAD.

Figure a, shows the normal trace obtained as the ADH enzyme reaction proceeds on the addition of ethanol (i) to the reaction mixture.

Figure b and c show the effect of the addition of both pyrazole (reaction inhibitor) and ethanol (ii) to the reaction mixture. The reaction does not proceed and remains at baseline absorbance.

Figure c shows the effect of using a lower concentration of Pyrazole and the same effect observed in b is apparent.

Figure d shows the effect of adding pyrazole (ii) as a reaction is underway following the addition of ethanol. The reaction stops at the absorbance reached prior to Pyrazole addition.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Marker</th>
<th>Promoter</th>
<th>Reporter cartridge</th>
<th>E/min/100μg cell protein (x10⁴)</th>
<th>ADH Units/mg Cell protein</th>
<th>ADH Units activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransf S150-2B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19</td>
<td>0.92</td>
<td>-</td>
</tr>
<tr>
<td>pCJ17</td>
<td>TRP1</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>0.72</td>
<td>-</td>
</tr>
<tr>
<td>pBEJ34</td>
<td>TRP1</td>
<td>-</td>
<td>ADH1</td>
<td>41</td>
<td>1.97</td>
<td>1.25</td>
</tr>
<tr>
<td>pBEJ35</td>
<td>TRP1</td>
<td>CYC1</td>
<td>ADH1</td>
<td>55</td>
<td>2.64</td>
<td>1.92</td>
</tr>
<tr>
<td>pBEJ32</td>
<td>TRP1</td>
<td>ADH1</td>
<td>ADH1</td>
<td>48</td>
<td>2.32</td>
<td>1.60</td>
</tr>
<tr>
<td>pBEJ15</td>
<td>LEU2</td>
<td>PGK1</td>
<td>-</td>
<td>32</td>
<td>1.54</td>
<td>0.82</td>
</tr>
<tr>
<td>pBEJ33</td>
<td>LEU2</td>
<td>PGK1</td>
<td>ADH1</td>
<td>34</td>
<td>1.64</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Change in absorbance readings are the average of 3 different reactions, performed at different times, using different protein extracts. Variation was ± 0.002.
contains neither an ADH sequence nor a possible transacting promoter. By use of the ADH standard curve (section 7.3.1), this was equated to approximately 10µg of ADH protein in 1mg cell protein (i.e. 1% of total soluble cell protein). In the presence of pBEJ34, which contains the ADH reporter without an associated promoter, over double the amount of ADH activity was found in the cells, showing that the promoterless construct gave rise to over 1% of total cell protein. On comparison to plasmid pCJ18 containing the promoterless APT gene, which expressed approximately 0.0025% APT, expression was 400-fold greater, which was despite there being more APT mRNA detectable from pCJ18. This suggests that higher translation efficiency of the high codon bias mRNA is occurring as would be predicted. When the ADH1 promoter, (pBEJ34) or the CYC1 promoter, (pBEJ35) directed expression, only 20% and 40%, respectively, ADH activity was produced. Thus the effect on expression of the presence of the promoters was surprisingly slight.

In the minimal medium used, the PGK1 promoter expression construct in pBEJ33 gave rise to notably less ADH units than the CYC1 or ADH1 promoters. This concurs with relative levels of APT expression (Figure 4.2.4.1). However, with ADH1, the CYC1 promoter gave rise to the greatest expression, whereas with APT the ADH1 promoter gave the greatest yield in minimal selective medium. The most interesting observation was that the PGK1 promoter on pBEJ15, which lacks an associated ADH coding sequence, gives rise to almost as much ADH as pBEJ33. As indicated by the mRNA studies, this could result from a trans-activation effect of the chromosomal ADH gene.

7.3.4 ADH activity of cells containing other PGK1 promoter and whole gene plasmids.

In further investigating the increased ADH activity observed in cells containing plasmid pBEJ15 containing only the PGK1 promoter region, the following points were considered:

i) Does the presence of a heterologous coding sequence of non-yeast origin have any influence? Plasmids containing the PGK promoter region linked to a different heterologous coding region, such as APT1 and CAT on TRP1 based plasmids were therefore investigated. Data obtained are shown in Table 4.

ii) Do other glycolytic promoters cause a similar effect or is it limited to the PGK1 promoter? Does the ADH1 promoter also have a transacting influence on chromosomal ADH1 gene
expression? Plasmids containing the ADH1 promoter with an APT gene, the glyceraldehyde-3-phosphate dehydrogenase promoter and the pyruvate kinase promoter were examined. A plasmid containing the CYC1 promoter was included as a non-glycolytic promoter. ADH activities obtained is shown in Table 5.

The results in Table 4 show that the other PGK1 promoter containing plasmids investigated also induced elevated levels of ADH activity. These were not identical in all cases however. The APT1 construct pCH230, induced similar levels to pBEJ15. This construct was in fact a PGK1-CAT fusion and in previous studies had been observed to have a number of anomalous effects on expression (pers comm. C. Hadfield) and hence this increase may be due to some similar effect. It was interesting to note that slightly less activity was observed when the whole PGK1 coding region was present. This may indicate some transacting effect mediated via the PGK1 product. However, the increases in ADH activity observed occurred irrespective of the coding regions which the plasmids contained.

In the presence of the ADH1 or GAPD promoters (Table 5), negligible ADH activity, above background level was detected, and no activity was observed in the presence of the CYC1 promoter. A preliminary assay in the presence of the PYK1 promoter, also indicated little activity. Thus none of these promoters appeared to cause the observed transactivation of ADH expression, although further investigation could be warranted.

The results indicate that the increase in chromosomal ADH, was a consequence of the PGK1 promoter region, and not the presence of PGK1 protein, which, if anything had a dampening effect on expression level. Time constraints ruled out analysis of other glycolytic promoters, but clearly GAPD, ADH1 and probably PYK1 failed to exert the trans effect, as did the non-glycolytic promoter CYC1.

7.3.5 The effect of plasmids containing PGK1 promoter deletions on ADH activity.

The PGK1 promoter region was implicated as the reason for the increased ADH activity, although whether this was confined to a particular region within the promoter was not known. To determine if a particular sequence of the PGK promoter was responsible for the observed
TABLE 4  ADH Units obtained from cells containing plasmids with a PGK1 promoter and a different coding region

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter</th>
<th>Gene</th>
<th>E/min/100µg cell protein ($\times 10^3$)</th>
<th>ADH Units/mg cell protein$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCH230</td>
<td>PGK1</td>
<td>CAT</td>
<td>33$^a$</td>
<td>1.59</td>
</tr>
<tr>
<td>pCH210</td>
<td>PGK1</td>
<td>APT1</td>
<td>19$^a$</td>
<td>0.92</td>
</tr>
<tr>
<td>pBEJ40</td>
<td>PGK1</td>
<td>PGK1</td>
<td>13$^a$</td>
<td>0.63</td>
</tr>
</tbody>
</table>

TABLE 5  ADH units obtained from cells containing plasmids with different promoters

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter</th>
<th>Gene</th>
<th>E/min/100µg cell protein ($\times 10^3$)</th>
<th>ADH Units/mg cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCH108</td>
<td>ADH1</td>
<td>APT1</td>
<td>1$^a$</td>
<td>0.05</td>
</tr>
<tr>
<td>pPG1</td>
<td>GPD</td>
<td>PGK1</td>
<td>4$^a$</td>
<td>0.19</td>
</tr>
<tr>
<td>pPG3</td>
<td>GPD</td>
<td>PGK1</td>
<td>0$^a$</td>
<td>-</td>
</tr>
<tr>
<td>pBEJ1</td>
<td>CYC1</td>
<td>APT1</td>
<td>0$^a$</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Values shown are the average of 3 different reactions.

$^*$ Change in absorbance readings shown have had background activity subtracted
(pCI17: E/min/100µg protein = 0.015)
effect, ADH activity of cells containing plasmids with varying PGK1 promoter deletions were assayed.

The PGK deletion plasmids employed were all based on a TRPl plasmid and are described in Chapter 6, Fig 6.3b. They were:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Deletion coordinates (from mRNA initiation site)</th>
<th>Deleted Elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBEJ48</td>
<td>-511 to -91</td>
<td>Partial ABFl, RAPl, HSE, TATA</td>
</tr>
<tr>
<td>pBEJ43</td>
<td>-538 to -397</td>
<td>ABFl, RAPl</td>
</tr>
<tr>
<td>pBEJ41</td>
<td>-538 to -473</td>
<td>ABFl</td>
</tr>
<tr>
<td>pBEJ44</td>
<td>-402 to -256</td>
<td>HSE</td>
</tr>
<tr>
<td>pBEJ45</td>
<td>-402 to -91</td>
<td>HSE, TATA</td>
</tr>
</tbody>
</table>

The results obtained are shown in Table 6.

A lesser effect on chromosomally encoded ADH activity is observed with the PGK1 promoter deletion plasmids. However, the extent of the decrease does not bear any readily identifiable correlation with the extent of the deletion. Thus, the promoter containing the ABFl deletion (pBEJ41) is still able to affect chromosomal ADH, but lesser effects are apparent on deletion of the HSE and the TATA box (pBEJ45) and the ABFl and RAPl binding sites (pBEJ43). No effect on chromosomal ADH is evident with the large promoter deletion (pBEJ48) or on deletion of the HSE alone (pBEJ44). On deletion of both the ABFl and RAPl binding sites (pBEJ43), however, some effect is still observed. Thus, the transactivation effect cannot be consigned to a single site within the PGK1 promoter, which suggests the possibility that the effect may involve complex interactions, possibly involving a number of proteins.

7.3.6 The effect of a single copy and multiple copies of the PGK1 promoter on ADH activity.

The transactivating effects on chromosomal ADH activity were apparent when the cells contained PGK1-promoter containing plasmids with a copy number of 15-25. One possibility this raises is that the effect may be a consequence of the promoter titrating out trans-acting
### TABLE 6  ADH Units obtained from plasmids containing various PGK promoter deletions

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter Deletion</th>
<th>E/min/100µg Cell protein (x10^3)°</th>
<th>ADH units/mg Cell protein°</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBEJ40</td>
<td>wt</td>
<td>13</td>
<td>0.63</td>
</tr>
<tr>
<td>pBEJ41</td>
<td>ABF1</td>
<td>7</td>
<td>0.34</td>
</tr>
<tr>
<td>pBEJ43</td>
<td>ABF1, RAP1</td>
<td>3</td>
<td>0.14</td>
</tr>
<tr>
<td>pBEJ44</td>
<td>HSE</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pBEJ45</td>
<td>HSE, TATA</td>
<td>4</td>
<td>0.19</td>
</tr>
<tr>
<td>pBEJ48</td>
<td>pABF1, RAP1, HSE, TATA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

° In all instances background ADH activity has been subtracted (pCJ17: E/min/100µg protein = 0.015). Data shown is the average of 2 different ADH reactions performed at different times. Variation = ± 0.001

---

### TABLE 7  ADH Units obtained from cells containing differing copy number plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Gene</th>
<th>E/min/100µg Cell protein (x10^3)°</th>
<th>ADH units/mg Cell protein°</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMA27/dleu2a</td>
<td>100+ copies PGK gene</td>
<td>13</td>
<td>0.63</td>
</tr>
<tr>
<td>pCH153</td>
<td>1 copy PGK gene</td>
<td>15</td>
<td>0.72</td>
</tr>
<tr>
<td>pBEJ40</td>
<td>20+ copies PGK gene</td>
<td>13</td>
<td>0.63</td>
</tr>
</tbody>
</table>

° In all instances background ADH activity has been subtracted (pCJ17: E/min/100µg protein = 0.015). Data shown is the average of 2 different ADH reactions performed at different times. Variation = ± 0.001
proteins. If so, increasing the number of plasmid copies in the cell may result in a more severe effect; Conversely fewer copies could exert a lesser effect.

To determine if this was the case, ADH activity was determined for cells containing either an integrated PGK1 promoter/APT1 cartridge, with a copy number of 1 or a plasmid containing the whole PGK1 gene on a leu2-d plasmid with a copy number of 100 (Chambers et al, 1988). The results are shown in Table 7.

ADH activity from cells containing these differing copy number plasmids, indicated that regardless of the copy number of the plasmids containing the PGK1 promoter or gene, no reduction or increase on ADH activity resulted. This effect was very surprising, since it showed that only one extra copy of the PGK1 promoter was required to cause a 2-fold increase in chromosomal ADH activity. Since further copies of the promoter did not cause further increases, it could be speculated that the system is very tightly regulated.

Time constraints ruled out a more extensive analysis, which may have revealed reasons for the observations.

7.4 DISCUSSION

The amount of mRNA and protein produced by a gene is considered to be governed to some extent by its codon bias. Sharp et al, (1986), determined that a gene having a pattern of codon usage optimal for expression in E. coli, would not be as highly expressed in yeast, due to be the presence of non-preferable or rare codons. This would also be due to the low abundance of rare tRNA species, which would produce a rate limiting step for translation, since the rare codons would be translated faster than the tRNA’s would be replenished. If the gene having a poor codon bias was on a multi-copy plasmid, the translational delay which the rare codons manifest, would be amplified. As described previously, the presence of an increasing number of rare codons in the PGK1 gene, (Hoekema et al, 1987) resulted in both a decline of PGK1 mRNA and PGK protein, indicating that the latter was controlled by the translation elongation rate of the mRNA.

The APT1 gene investigated in the previous chapters had a codon bias of 0.07, which was thought to explain lower expression of this gene. Use of an APT2 cartridge with a codon bias
of 0.1 (Chapter 3), did not result in increased expression, since this low increase in codon bias was considered not sufficient to manifest itself in an increased translation of APT protein.

An ADH reporter gene was constructed in order to study heterologous expression of a gene with a high codon bias index. The ADH gene has a codon bias of 0.92, which would indicate a near optimal codon sequence for optimal expression in yeast. Thus, its expression on equivalent plasmids could be compared to expression of the APT1 gene. ADH activity was measured by following the conversion of ethanol to acetaldehyde (as described in section 2.10.5), in crude protein extracts. A system was developed whereby ADH activity could be equated to the amount of ADH protein being expressed, enabling direct comparison between expression of APT and ADH. It was observed that in the presence of the CYC1 promoter expression of ADH protein was found to be approximately 3% of the total cell protein (subtracting background activity). The best expression of APT protein obtained with a PGK1 promoter, was only in the order of 0.2% of total cell protein, 15-fold less. On the basis of the results, it would seem that expression of APT was indeed limited by the poorer codon bias and thus confirms that translation efficiency is a major factor in determining expression levels.

It was apparent that the addition of a promoter to the ADH reporter gene resulted in a reduction in the transformation efficiency. This effect was observed in the APT promoter-driven constructions, in which the plasmid inhibition phenomenon was evident. However, plasmid copy number determination indicated that the copy number of plasmids containing the ADH reporter gene were very similar, regardless of the presence of a promoter. Thus it would seem that the effects are a consequence of the APT1 gene. As the APT protein had no detectable deleterious effect on the cell, this suggests that the plasmid inhibition observed, results from the very low codon bias index of the APT gene. The inhibition must therefore act at the level of translation, although it is unclear in what manner. Also, much more background transcription of the APT gene is observed than with the ADH gene, indicating the occurrence of greater aberrant transcription due to low codon bias.

In minimal selective media, the copy number of plasmids containing the ADH1 or APT1 genes with a promoter were very similar. Thus it would seem that the promoters are able to override the APT coding sequence copy number effect, possibly by reducing the amount of background transcription via the quenching effect which the promoters have. The copy number of plasmid pBEJ15 having a PGK1 promoter lacking a coupled coding sequence was found to be
decreased, which again implicated a role of translation in plasmid inhibition.

In the presence of plasmid pBEJ15, described above containing only the PGK1 promoter region, a transactivating effect on chromosomal ADH gene expression was evident. Investigation revealed that this effect was only induced by the PGK1 promoter and that the presence of PGK protein had no additional effect and in fact seemed to reduce the extent of activation. Transactivation was not apparent with the homologous ADH1 promoter or additionally by high expression of ADH protein by other promoters. Additionally, the presence of the GAPD, CYC1 or PYK1 promoters, did not exert an effect. One extra copy of the PGK1 promoter was sufficient to cause the effect, with no further activation in the presence of increasing numbers of copies.

The use of a number of plasmids containing various PGK promoter deletions could not tie down the regulatory effect to a particular region of the PGK promoter. This would suggest that the effect is a consequence of the action of a complex of proteins.

Expression of the ADH gene (Tomow and Santangelo 1990), is dependent upon the binding of the RAP1 protein to the sequence between -635 and -615 to the mRNA start site (Shore and Nasmyth, 1987). In addition it is thought that the product of the GCRI gene, a positive transcription factor, acts through the RAP binding site and in some way participates in or modifies a transcription activating complex (Santangelo and Tornow, 1990). If binding proteins were responsible for the observations, increasing the number of binding sites within the cell would titrate out the required binding protein and thus expression of the chromosomal gene would be reduced.

Similarly, if binding proteins were titrated away from the chromosomal ADH1 promoter, a situation similar to that described by Irani et al, (1987), could occur. The presence of excess copies of an ADH2 promoter on a plasmid, inhibits ADH2 expression, since positive activators which would normally bind to the chromosomal ADH2 promoter region are titrated away. In the case of the chromosomal ADH1 activation, the same amount of activity was apparent in the presence of either one or 100 copies of the PGK1 promoter region, indicating that the titration of proteins was not a contributing factor. However, it could be postulated that negative transcriptional factors could also be titrated away, resulting in an increase in activity. Santangelo and Tornow (1990), postulated that ADH1 transcription is activated by an as yet
unidentified transacting regulatory protein which binds to the promoter region and causes some type of titration effect, which could also be involved in these observations.

The ADH activity resulting from the transactivation detected in this work should be a result of ADH1 activity, since the yeast cells were grown in minimal medium containing glucose and thus ADH2 enzyme should not be expressed. The assay employed, however, was not specific for ADH1. In addition, the transcription detected on Northern analysis may be a consequence of both ADH1 and ADH2 transcription, since it was noted by Bennett and Hall (1982b), that the genes are 90% homologous in nucleotide sequence and differ in only 15 of 284 amino-acid positions (Wills and Jornvall, 1979). In addition Denis et al., (1982), found that it was difficult to hybridise the ADH1 gene solely to ADH1 mRNA. ADH2 expression occurs when glucose is removed from the growth medium (Ciriacy, 1979) and requires the binding of a positive regulatory protein ADR1, upstream of the ADH2 locus (Beier and Young, 1982; 1985).

ADH2 activity can occur in the presence of glucose (Denis and Young, 1985), if excess copies of ADR1 are also present, which is thought to override a regulatory mechanism which normally keeps ADR1 inactive in glucose medium and thus unable to bind to ADH2. Thus, ADR1 protein may be directly interacting with and saturating ADH2 control regions, or activating another regulator which interacts with ADH2 (Denis, 1987). In the presence of excess promoter regions, the regulatory mechanism may be overridden and ADH2 may be expressed. Binding proteins bound to the ADH2 gene to stop expression in glucose medium may be titrated away, allowing ADR1 protein to bind, resulting in activity. Thus, the increase in ADH activity that is observed in the presence of the PGK1 promoter, could be a result of expression of the ADH2 enzyme in the glucose medium.

In all cases, the cultures from which crude protein extracts were isolated were all grown to a mid-log density and thus glucose would still be present in the growth media. However, cultures containing the PGK1 promoter plasmids, may utilise glucose at a faster rate and thus ADH2 expression may be induced. Although it can be postulated that the effect apparent is a consequence of transactivation, the absence of ADH2, would need to be confirmed. ADH2 activity can be assayed by utilising the method outlined by Sealy-Lewis (1990), in which ADH2 activity can be measured by the production of formazan from 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride. Alternatively, ADH2 protein could be detected by staining activity on a Polyacrylamide gel as described by Sealy-Lewis and Lockington, (1984).
The observed effect of the PGK1 promoter on chromosomal ADH activity, could point to involvement of the glycolytic pathway. The PGK enzyme is known to catalyse an energy-producing step of glycolysis, in which 1,3 diphosphoglycerate is converted to 3-phosphoglycerate, resulting in the production of ATP, which would be tightly regulated. The ADH catalysed step on the other hand is much further down the pathway (see Fig 7.4.5) and results in the regeneration of NAD⁺ from NADH and thus direct regulation of both enzymes would seem unlikely. Since the PGK catalysed step is early in the glycolysis pathway and the ADH step much further down, a trans-regulator could monitor the incoming demands on the pathway and modify ADH expression accordingly. The glycolytic pathway also feeds into the TCA cycle, although metabolism through the latter is aerobic and thus the link with ADH is important when the glucose substrate is abundant and the cells are able to grow via the fermentative pathway. The GAPDH enzyme catalyses the reaction resulting in the production of 1,3-diphosphoglycerate, which is utilised by PGK enzyme, but in the presence of the GAPD promoter region, very little transactivation was evident. Another energy producing step in the glycolytic pathway (the third step from the PGK reaction) is catalysed by the PYK enzyme. This enzyme is the key enzyme in the control glycolysis, but preliminary data indicated that this promoter region again had no effect on ADH activity. Thus, it would seem that glycolytic pathway involvement is unlikely.

A comparison of the effect on the chromosomal ADH activity of a larger number of the glycolytic promoters may have found others that are able to cause transactivation. Additionally, it may also have been of interest to ascertain whether the effect was confined to ADH activity or whether other glycolytic genes were affected in a similar manner, for example; would the presence of the ADH1 promoter cause an increase in PGK activity?

Thus it would seem that the PGK promoter is involved in the regulation of ADH expression via a titration effect which can be detected. This also shows for the first time that a plasmid-borne gene construct can affect the regulation of expression of a chromosomal gene and most probably vice-versa. Such interactions may contribute to the promoter-induced plasmid inhibition effect previously observed.
Figure 7.4.5  The glycolytic pathway in the yeast *Saccharomyces cerevisiae*

Glucose → ATP
Hexokinase

Glucose 6-phosphate
Phosphoglucone isomerase

Fructose 6-phosphate → ATP
Phosphofructokinase

Fructose 1,6-diphosphate

Dihydroxyacetone phosphate
Glycerolaldehyde 3-phosphate
Phosphoglycerate kinase

Phosphoglycerate kinase

2-Phosphoglycerate → ATP
Enolase

Phosphoenolpyruvate
Pyruvate
CHAPTER EIGHT

GENERAL DISCUSSION
It is a fundamental requirement of yeast molecular biologists to be able to express homologous or heterologous genes in yeast, from a variety of plasmids and other vector molecules. As a result there is a requirement to know what factors influence gene expression in yeast. Over the last several years, some basic ground rules have been worked out, but continuing problems with low expression from heterologous gene constructions, indicate that not all influential factors have been accounted for. In some part, the difficulties may arise from the diverse nature of the heterologous genes being expressed and the gene products themselves, although a large number of problems, still result from unknown factors.

The previously described work was aimed at addressing the problems that influence heterologous gene expression in yeast. In order to identify any influencing factors, a heterologous gene was constructed such that its performance could be monitored accurately. The gene chosen for expression in yeast was that encoding the enzyme aminoglycoside phosphotransferase (APT), which was encoded by the bacterial transposon Tn903. This gene also had potential to be useful as an additional dominant marker in yeast, since it provided resistance to one of the few antibiotics which yeast is susceptible to, G418.

The gene coding sequence was isolated from the bacterial transposon and a number of coding sequence cartridges constructed. One of these cartridges, APT1, was inserted into a heterologous 2µm based gene construct utilising the PGK1 promoter and terminator. In this form the gene was found to function highly efficiently as a selectable marker, both in multiple and single copy (Hadfield et al., 1990). Cell growth was found to be normal and plasmid stability remained high, indicating that expression of the APT protein had no adverse effect on the cell.

Total soluble cell protein was extracted from transformed cells and separated on an SDS-polyacrylamide gel, in order to analyse expression levels. This revealed, however, that the APT protein could not be readily detected. As a consequence it was necessary to use a more sensitive method to detect activity and for this reason an APT assay was developed for use with yeast cell protein extracts. The APT enzyme inactivates the antibiotic by transferring a phosphate group from ATP to the 2' deoxystreptamine moiety at the 3' hydroxyl site of the antibiotic. This action could be utilised as a means of assaying activity, since if the phosphate groups of ATP are labelled with $\gamma^{32}$P, the transfer to G418 could be monitored. The assay incorporated a means of reducing background radioactivity to a very low level and this coupled
numbers were not restored. Thus, although plasmid configuration did have some effect on copy number, it did not affect the underlying promoter inhibition. The degree of the reduction of plasmid copy number observed with the investigated promoters was found to be variable, with CYC1 promoter addition causing the more profound effects. However, no correlation between expression and copy number was evident.

Gene expression was found to be affected by changes in plasmid configuration such as a change in the orientation of the APT1 gene cartridge or the use of an alternative auxotrophic marker and even the presence of different or two terminators. The effects on expression were also found to be dependent on the promoter being employed to direct expression, with varying degrees of change. Thus the investigation revealed a number of unexpected factors that could influence expression of a heterologous gene. However, the effects of the changes on expression, may be manifest to a greater extent with a low codon bias gene (APT1 has a low codon bias of 0.07) than with a gene having a more favourable codon usage. Incorporation of a higher codon bias gene into identical plasmids would reveal if this was the case. In addition to the effects on expression, the alterations in plasmid configuration also resulted in changes in transformation efficiency. Improved transformation was evident with plasmids containing the LEU2 auxotrophic marker, which was thought to be a result of LEU2 transcription on the plasmid being correctly terminated, unlike the TRP1 marker, although this could merely reflect that LEU2 was a more efficient plasmid marker. The differences in transformation efficiency of plasmids containing a promoter would seem to reflect the presence of APT protein in the cell, although the protein was not toxic to the cell, illustrated by normal growth rate and high stability in cells containing APT producing plasmids. Thus the observation may be due to the presence of additional sequence, which in some manner alters uptake by the yeast cell.

It was considered that the effects on plasmid copy number, could be a consequence of interference of 2μm origin function by through-transcription, causing an impairment of replication. This was discounted as an explanation, however, since transcript analysis revealed that they did not extend into this region. This was additionally confirmed by the investigation of other constructions with differing terminators and opposite orientation of the APT gene. Promoter addition also correlated with a disappearance of background transcription, which was thought to result since polymerase molecules that are scanning the plasmid DNA are drawn to the promoter. This could therefore implicate background transcription in an as yet
unknown manner, as a factor influencing the reduced plasmid copy number.

Analysis of ARS based plasmids containing the APT expression cartridges, showed differing effects to the 2μm based plasmids. Plasmid stability was, as expected, very variable, although it was evident that the addition of the PGK1 promoter had a more pronounced effect on stability. However, the analysis indicated that orientation of an ARS/TRP cartridge on the plasmid caused profound effects on plasmid performance, which tended to mask any promoter type inhibition effect. On the other hand, ARS plasmids are so highly unstable to begin with that any effect of promoter inhibition would be negligible. Thus, although the effect in ARS plasmids could not be totally discounted, it would seem that the effect was restricted to 2μm-based plasmids. This therefore indicated an influence of the 2μm partitioning system, although plasmid stability in all cases remained high. One possibility is that the promoter sequences cause some inappropriate adherence to the nuclear matrix and prevent efficient migration of the plasmid through the nucleus. A lower total number of plasmids in the population would then result in lower plasmid copy numbers, even though partitioning acts to distribute the plasmids to the progeny cells.

The plasmid copy number reduction was not considered to be related to the strength of the promoter and thus some other aspect of promoter function was deemed to be responsible. Since plasmid stability was unaffected, it would seem that only replication was affected and not partitioning. It was considered that proteins binding to the promoter, may in some way inhibit the progression of the replication fork, either by interference with the transcription complex or by altered chromatin structure diminishing the activity of the replication origin. Analysis of promoter window deletions of homologous PGK1 constructions, however, indicated that an improved plasmid copy number was not evident in the majority of deletions analysed. However, a heterologous construction containing the TRP1 truncated promoter, consisting of a UAS, TATA box and an initiation site, was found to have an improved copy number, although this was not evident with an ADH1 truncated promoter. This could indicate, however, that the complete TRP1 promoter, was less inhibitory to a plasmid than the stronger ADH1 promoter. This would need to be investigated.

Use of the truncated homologous and heterologous promoters, however, indicated that loss of the TATA box or portions of the UAS (PGK1 deletions), did not result in total loss of transcription or expression. This indicated that the Initiator site or a sequence present in the
pBR322 sequences upstream of the promoter could direct assembly of activators. In promoters lacking the TATA sequence some other sequence was able to replace it, providing more accurate transcription.

The copy number of a plasmid containing the APT1 gene without a promoter was high, even in the absence of APT selection in rich and minimal medium. It was therefore concluded that the initial high copy number was not a consequence of the plasmid artificially raising its copy number to enable growth in selective medium. Further evidence for this was provided when on increasing the concentration of G418 (up to 1mg/ml) in YPD medium, (i.e the selection pressure), the plasmid copy number of the plasmid containing the promoterless APT1 gene was not increased and neither was APT expression. In addition, expression from a promoter-driven APT cartridge (Chapter 5, pCH218) was also found to be low, but plasmid copy number was not increased in YPD/G418 medium. Copy number of a plasmid containing a PGK1 promoter, but lacking the APT coding sequence, was lower than that of plasmid pC1J8 containing the APT1 gene or a similar plasmid containing the PGK1 promoter and APT1 gene, indicating that the presence of a gene without a promoter, could also cause a decrease in plasmid copy number. This again implicates promoter presence in the copy number reduction and in addition could point to some involvement of transcription in the copy number reduction. Further investigation of other plasmids containing differing promoters could reveal if other promoters exhibit a similar effect.

The use of an ADH1 reporter gene, showed that to a large extent the reduction in heterologous gene expression level observed with the APT gene could be alleviated by a more favourable codon bias. Unlike the codon bias index of APT1 which was only 0.07, and that of APT2 was 0.1, the codon bias index of ADH was much more favourable, being 0.92. Expression from this gene on a similar plasmid to the heterologous APT1 gene, was almost 40-fold greater than that of the APT1 gene, indicating that poor codon bias did limit its expression.

However, failure of a multicopy PGK1-promoter driven ADH gene to produce as much protein as a multicopy homologous PGK1 gene, indicates some other influence on the expression level. It may be that other sequences normally present in the ADH promoter are lacking in the PGK promoter and hence expression is sub-optimal. Additionally, the ADH protein may be rapidly turned over, so that high intracellular levels cannot accumulate. Alternatively, since ADH is a natural yeast protein and its mRNA is expressed to as high a
level as multicopy homologous PGK, its translation may be regulated by a feedback mechanism. This type of effect has been previously observed by Moore et al., (1990), in that expression of the PYK gene on a plasmid is limited by a gene dosage effect that acts at the level of translation.

The observation that the promoterless ADH plasmid did not attain as high a copy number as the APT counterpart, nor give rise to as much transcription, suggests that the presence of the APT sequence itself may be responsible for elevating the copy number. APT has no evident effect on the cell in terms of growth or plasmid stability and copy number is unaffected by the presence or absence of selection. Despite giving rise to much more transcription, the APT promoterless plasmid gives rise to much less product than the ADH plasmid. Poor codon usage is known to give rise to low product yield (Hoekema et al., 1987), reduced mRNA (Hoekema et al., 1987; Hadfield et al., 1990) and reduced mRNA stability (Herrick et al., 1990). In reducing transcription and translation of a gene, poor codon usage may somehow favour enhanced replication. Yeast plasmid replication occurs once per cell cycle during the S phase (Zakian et al., 1979), but 2μm plasmids can increase their copy number through FLP-mediated amplification. This normally acts through the 2 cis FRT elements in the inverted repeats. In plasmids containing only one inverted repeat, amplification can be achieved by co-amplification with the endogenous 2μm plasmids following recombination. Plasmid pCJ18 and its derivatives contain one inverted repeat and it is possible that the defective transcription and translation associated with the poor codon bias gene could somehow facilitate a certain amount of FLP-amplification. Although an exact mechanism for this, cannot be elucidated, it can be postulated that the FRT element in the inverted repeat may interact with the APT region primed by transcription. In the presence of a promoter, the background transcription is greatly reduced so that the possibility of aberrant FLP amplification is removed. Alternatively, the presence of a promoter or an efficiently transcribed gene could alter the chromatin structure on the plasmid and in some manner diminish the activity of the replication origin. This would result in fewer copies of the promoter-containing plasmids.

A further effect observed in the course of this work, related to the effect of the presence of a gene on a plasmid to other processes occurring in the cell. Work by Birnbaum and Bailey (1991), indicated that the presence of increasing copies of a plasmid in E. coli, resulted in effects on levels of TCA cycle enzymes in the cell. In work presented in Chapter 7, a possible transacting effect was evident such that the expression of chromosomal ADH was
increased in the presence of the PGK1 promoter on a multi-copy plasmid. Investigation of a number of PGK1 window deletions, showed that the PGK1 promoter sequence responsible for the transactivating effect seemed to include the RAP1 site in the UAS and also a large part of the rest of the promoter. This implicated that other glycolytic promoters containing the RAP1 site may also affect ADH, although investigation with the ADH1, PYK (Chambers et al., 1990) and GAPD promoter indicated that this may not be the case. If the RAP1 binding site was in some way responsible for the observation, this implies an involvement of regulatory binding proteins. This would seem unlikely however, since the effect was evident even in the presence of only one copy of the PGK1 promoter, although it could be argued that the effect is tightly regulated. Although far from complete, the work suggested that there was a regulatory interaction between plasmid-borne and chromosomal genes. Further investigation would be warranted, particularly to observe if the presence of an ADH promoter had a similar influence on chromosomal PGK1 and also whether the effect is limited to chromosomal ADH or if other chromosomal genes are also affected. Although it is as yet unclear how this occurs, such an interaction could be one factor responsible for limiting the expression of heterologous genes, particularly in the case of PGK1. The plasmid inhibition effects of other promoters may be other manifestations of such trans-regulatory effects.

The work described provides an indication that the expression of heterologous genes on plasmids transformed into cells, is much more complex than one would expect. The various plasmid constructions described, indicate that the yield of protein expressed from a plasmid can be affected by the choice of terminator, autotrophic marker, orientation, promoter, and presence of bacterial sequences, all of which can have a much larger effect than initially thought. The extent of the effect of any of those parameters, can also be affected by the gene being expressed. A gene may be more affected the lower its codon bias.

The inhibition effect that was observed in the presence of a promoter was a complicated problem to analyse, since changes to the plasmid and deletion of protein binding sites at the promoter, resulted in effects on transcription and translation. A large number of constructions were made and analysed, but in order to provide an adequate explanation, differing constructions in terms of a differing heterologous gene would be required. On the basis of the investigations that were performed, the inhibition effects were only evident in 2μm based plasmids containing heterologous genes of non-yeast origin and having low codon bias index for yeast. Protein binding at the promoters would seem to have some effect on plasmid
partitioning or may interfere with the passage of replication forks. Both would result in a reduction in plasmid copy number, which must be manifest to a greater extent in a lower codon bias gene.

In the course of this work, it became apparent that the method employed for the determination of plasmid copy number, was very tedious and time consuming. In a large number of instances, genomic DNA which had been subjected to a number of procedures in order to eliminate contaminants, was still very difficult to restrict. This was not always apparent on running a small aliquot of the restricted DNA on a mini-gel and often was only evident after the "restricted" DNA had been blotted and probed with the relevant radiolabelled probe. This therefore resulted in wastage of expensive reagents and also required the whole process to be repeated. As a consequence of the problems which were encountered, an alternative method for plasmid copy number determination was considered.

One possibility which was contemplated, was the use of the polymerase chain reaction. It was considered that by using primers specific for a region of yeast chromosome and also primers specific for a region of the yeast plasmid and amplifying these for the same number of cycles, would produce amplified DNA in quantities relative to the amount of template DNA present within the cell. As a consequence, amplified chromosomal DNA should represent the single copy present in the cell, whilst the amplified plasmid DNA would represent the number of plasmid molecules in the yeast cell and thus their ratios should reflect the plasmid copy number. An advantage of the method is that the DNA would not have to be totally pure and thus the time-consuming purification steps required to produce genomic DNA sufficiently clean to restrict would not have to be performed.

Although this method in theory would seem feasible, a number of problems were envisaged which could result in inaccurate copy numbers. One problem considered was that the amplification of both sets of DNA molecules would not proceed at the same rate. This could be countered by using primers for both the chromosomal and plasmid DNA of approximately the same size. An additional problem was that the amplification of the DNA would reach a plateau at different times, which would result in differing amounts of amplified DNA being produced. In order to determine the relative amounts of plasmid and chromosomal DNA, it would be important to ensure that both sets of DNA were analysed within the exponential phase of the PCR reaction.
Further considerations which were raised was the way in which the ratio between the amplified DNA was to be measured. This was an important issue, since the method used should be quick and relatively simple and most importantly accurate. A number of methods were considered.

In considering the design of the primers, it was decided that in order to identify the amplified DNA, both primers should be slightly different in size. To ensure that the amplification reactions would still proceed in a uniform manner, this size difference must be minimal and as a consequence a difference of 100 bp was decided. The DNA could still be separated using a higher percentage agarose gel. The next question addressed was which regions of the chromosomal and plasmid DNA should be amplified. The chromosomal region employed should be a gene which would be present in most laboratory yeast strains and would not be employed as a marker on the yeast plasmids employed throughout this work, the TRP1, URA3 or LEU2 genes were therefore eliminated as prospective choices. The gene which was chosen was the PYK1 gene which fitted the previous criteria and 800bp of this DNA was chosen for the primer, ensuring that this region did not have any homology to any regions on the plasmid. A plasmid primer was then chosen using 900bp of pUC DNA, again ensuring that no regions of homology existed.

A number of standard primer and template concentrations and also of buffer and Taq polymerase have been described which are considered to be the optimal for amplification. Temperatures for denaturation, annealing and extension vary dependent upon the GC content of the primers and the size of the product. In all cases a control containing pUC19 DNA was included to act as both a positive and negative control. A problem encountered was that there was some variability observed between the same genomic DNA samples. This was considered to be a result of inaccurate DNA concentrations resulting from carbohydrate contamination. In some instances non-specific product was observed which may be absent in other samples again due to contamination. Unfortunately due to time constraints this method could not be refined, but initial indications were that if this could be done, a much better method could be produced.
CHAPTER NINE

REFERENCES


220


Birnbaum, S., and J.E. Bailey. 1991. Plasmid presence changes the relative levels of many host cell proteins and ribosome components in recombinant Escherichia coli. Biotech. and Bioeng. 37, 736-745

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7, 1513-1522

Gene 32, 263-274


Cantwell, B., G. Brazil, N. Murphy and D.J. McConnell. 1986. Comparison of the expression of the endo-1,3,1,4-glucanase gene from Bacillus subtilis in *Saccharomyces cerevisiae* from the CYCl and ADH1 promoters. *Curr. Genet.* 11, 65-70


Denis, C.L., J. Ferguson, and E.T. Young. 1982. mRNA levels for the fermentative Alcohol Dehydrogenase of Saccharomyces cerevisiae decrease upon growth on a non-fermentable carbon source. Journal of Biol. Chem. 258, 1165-1171

225


Dice, F. 1987. Molecular determinants of protein half-lives in eukaryotic cells. FASEB J. 1, 349-357


Erhart, E., and Hollenberg C.P. 1983. The presence of a defective LEU2 gene on 2 µm DNA recombinant plasmids of Saccharomyces cerevisiae is responsible for curing and high copy number. J. Bacteriol. 156, 625-635

Ernst J.P. 1986. Improved secretion of heterologous proteins by Saccharomyces cerevisiae, Effects of promoter substitution in alpha-factor fusions. DNA, 5, 483-491

Etchevery, T., W. Forrester and R. Hitzeman. 1986. Regulation of the chelatin promoter


Futcher, A.B. 1988. The 2μm circle plasmid of *Saccharomyces cerevisiae*. *Yeast* 4, 27-40


Gritz, L. and J. Davies. 1983. Plasmid encoded Hygromycin B resistance, the sequence of the hygromycin B phosphotransferase gene and its expression in *E. coli* and *S. cerevisiae*. *Gene* 25, 179-188


Guarente, L. and T. Mason. 1983. Heme regulates transcription of the CYC1 gene of *S.
cerevisiae via an upstream activation site. Cell 32, 1279-1286


Huez, G., C. Bruck and Y. Cleuter. 1981. Translation stability and deadenylated rabbit
globin mRNA injected into HeLa cells. Proc. Natl. Acad. Sci. USA. 78, 908-911


coli as a gene fusion marker. *Proc. Natl. acad. Sci.* 83, 8447-8451


linked glycosylation on biological activity. J Biol. Chem. 266, 15348-15355


activation sequence but does not require TATA sequences. Mol. Cell. Biol. 6, 4335-4343


Sealey-Lewis, H.M. 1990. The identification of mutations in *Aspergillus nidulans* that lead to increased levels of ADHII. *Curr. Genet.* 18, 65-70


1. Gene 83, 47-55

Sidhu, R.S., and A.P. Bollon. 1990. Bacterial plasmid pBR322 sequences serve as upstream Activating sequences in Saccharomyces cerevisiae. Yeast 6, 221-229


Tomow, J., and G.M. Santangelo. 1990. Efficient expression of the *Saccharomyces cerevisiae* glycolytic gene ADH1 is dependent upon a cis-acting regulatory element (UASre) found initially in genes encoding ribosomal proteins. *Gene* 90, 79-85


APPENDIX

ADDITIONAL PLASMID CONSTRUCTIONS
plC
2.68 Kb

Amp R

lac

ori

HaeII

BglII

TaqI

NarI

HaeII

BglII
**Figure**:  

The figure illustrates a map of the PYcDE-2 plasmid. The plasmid is encircled with various restriction enzyme sites indicated, including XbaI, HindIII, EcoRV, BamHI, BglII, and EcoRI. The map also includes markers for the TRP1, pADH1, and pBR322 sequences. The plasmid is described as 7.50 Kb in size.
pLGG669
7.30 Kb
pCH137

4.50 Kb

AmpR

plac

HindIII

tPGK1

BgIII

HindIII

PstI

EcoRI

pPGK1
pCH147
5.50 Kb

HindIII

BCII/BgIII

plac

tPGK1

APT1

ampR

pPGK1

EcoRI

PstI

BamHI

HindIII

XmnI
YRP7
5.80 Kb