Glycolytic gene expression

in

Saccharomyces cerevisiae.

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by

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This thesis is dedicated to

mum and dad for their

constant help, love and encouragement.

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

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ABBREVIATIONS

ATP	-	adenosine triphosphate
ADP	-	adenosine diphosphate
BSA	-	bovine serum albumin
cAMP	-	cyclic adenosine monophosphate
dATP	-	deoxyadenosine triphosphate
dCTP	-	deoxycytidine triphoshate
dGTP	-	deoxyguanosine triphosphate
dTTP	-	deoxythymidine triphosphate
DNA	-	deoxyribonucleic acid
cDNA	-	complementary DNA
ssDNA	-	single-stranded DNA
dsDNA	-	double-stranded DNA
RNA	-	ribonucleic acid
mRNA	-	messenger RNA
tRNA	-	transfer RNA
DEPC	-	diethylpyrocarbonate
DTT	-	dithiothreitol
EDTA	-	ethylenediaminetetra-acetic acid (disodium salt)
PAGE	-	polyacrylamide gel electrophoresis
PEG	-	polyethylene glycol
PEP	-	phosphoenolpyruvate
PMSF	-	phenylmethylsulphonyl fluoride
SDS	-	sodium dodecylsulphate
SSC	-	saline sodium citrate
TBE	-	tris-borate, EDTA
TE	-	tris, EDTA
TEMED	-	N,N,N',N'- tetramethylethylenediamine
Tris	-	tris (hydroxymethyl) amino ethane
uORF	-	upstream open reading frame
UAS	-	upstream activation sequence
DAS	-	downstream activation sequence
URS	-	upstream repression sequence
TF	-	transcription factor
pol	-	polymerase

V	-	volts
Kd	-	kilo dalton
bp	-	base pair
Kb	-	kilo base pair (s)
Mb	-	mega base pair (s)
nt	-	nucleotide
^{o}C	-	degrees centigrade
g	-	centrifugal force equal to gravitational acceleration
α	-	alpha
β	-	beta
τ	-	gamma
cm	-	centimetre (10^{-2} m)
mm	-	millimetre (10^{-3} m)
g	-	gramme
mg	-	milligramme (10^{-3} g)
µg	-	microgramme (10^{-6} g)
ng	-	nanogramme (10^{-9} g)
l	-	litre
ml	-	millilitre (10^{-3} l)
µl	-	microlitre (10^{-6} l)
kcal	-	kilo calories
mw	-	molecular weight
w/v	-	weight/volume
v/v	-	volume/volume
M	-	molar (moles per litre)
mM	-	millimolar (10^{-3} M)
µM	-	micromolar (10^{-6} M)
Ci	-	Curie
µCi	-	microCurie (10^{-6} Ci)
pH	-	acidity [negative $\log_{10}$ (molar concentration H^{+} ions)]
log	-	logarithim
UV	-	ultra violet light
Fig.	-	figure

cpm	-	counts per minute
rpm	-	revolutions per minute

Gene names

HXKI/II	-	hexokinase
GLK	-	galactokinase
PGI	-	phosphoglucose isomerase
PKK1/2	-	phosphofructokinase
TPI	-	triosephosphate isomerase
FBA1	-	fructose-bisphosphate aldolase
TDHI/II/III	-	triose-3-phosphate dehydrogenase
PGK	-	phosphoglycerate kinase
PGM	-	phosphoglycerate mutase
ENOI/II	-	enolase
PYK1	-	pyruvate kinase
PDC	-	pyruvate decarboxylase
ADHI/II	-	alchol dehydrogenase
PDH	-	pyruvate dehydrogenase
LDH	-	lactate dehydrogenase
CIT	-	citrate synthase

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SUMMARY

The regulation of glycolytic gene expression in *Saccharomyces cerevisiae* has been studied in this thesis.

It is demonstrated that the glycolytic genes are differentially regulated (at the level of mRNA abundance) in response to fermentative or non-fermentative growth. While the levels of certain glycolytic mRNAs are expressed at about the same level during growth on either glucose or lactate, others are expressed at a higher level when grown on glucose.

PYK1 shows the highest level of induction when yeast is grown on glucose rather than lactate, with a 3.9-fold increase in the mRNA level. This increase in the mRNA level was not accompanied by a change in the transcriptional start site.

PYK1:lacZ fusions were integrated at the *ura3* locus of DBY746. Analyses of the resulting strains demonstrated that an element(s) within the first 1150 bp of the *PYK1* coding region is required for optimal transcription. The presence of this Downstream Activation Site (DAS) results in a 10-fold increase in mRNA levels. However, the DAS element is not required for carbon source regulation of the *PYK1:lacZ* fusions.

In addition to carbon source regulation at the mRNA level, the expression of the *PYK1:lacZ* gene fusions was also induced at the level of translation when yeast was grown fermentatively.

Abnormally high levels of *PYK1* mRNA result in deleterious growth of *S.cerevisiae.* This was demonstrated when multiple copies of the *PYK1* gene were transformed into yeast on a multi-copy vector. Strong selective pressure exists to decrease the plasmid copy number to restore normal growth. This selection is disrupted when a premature translational stop codon is introduced at the beginning of the plasmid-borne *PYK1* coding region.

The deleterious effect on growth rate is not caused by high levels of pyruvate kinase. When the *PYK1* coding region is fused to the *PGK* promoter and untranslated leader on a multi-copy plasmid, high levels of pyruvate kinase are synthesised in yeast with no effect on the growth rate.

In contrast, the synthesis of high levels of pyruvate kinase, when the wild-type *PYK1* gene is present in multi-copy in yeast, is limited at both the level of mRNA abundance and at the translational level. An increase in the copy number of the *PYK1* gene does not cause a parallel elevation in the abundance of the *PYK1* mRNA. The effect on *PYK1* translation was demonstrated by comparing the *PYK1* mRNA level with pyruvate kinase activity and by measuring the ribosome loading on the *PYK1* mRNA. This effect on *PYK1* translation is not caused by the plasmid encoded *PYK1* mRNA having an aberrant 5' untranslated region.

The translational repression of *PYK1 mRNA* is mediated at the level of translational initiation. This is apparent from the requirement of the 5' untranslated leader for regulation and the observed decrease in ribosome loading as the level of *PYK1* mRNA is increased.

While it was established that the *PYK1* 5' untranslated region is required for translational regulation, it was not established if it is sufficient. Sequences within the coding region or 3' untranslated region may also be required.

The translational regulation of *PYK1* is not mediated by feed-back inhibition either directly or indirectly by excess pyruvate kinase. This is evident from the finding that in strains which contain abnormally high levels of pyruvate kinase, there is no translational inhibition of the chromosomally-encoded *PYK1* mRNA. This suggests that the translational repression of *PYK1* may occur through the saturation of a positive translational factor required specifically for *PYK1* mRNA translation.

The translation of at least one other glycolytic mRNA (*PFK2*) is affected by the presence of elevated *PYK1* mRNA levels. A dramatic reduction in the ribosome loading of the *PFK2* mRNA was observed when the abundance of the *PYK1* mRNA was increased. Conversely, there was no change in the ribosome loading upon the *PFK1, PGK* and *TDH* mRNAs, in the presence of high *PYK1* mRNA levels. This suggests that the *PYK1* and *PFK2* mRNAs are translationally co-regulated, requiring the same specific translational factor which becomes saturated in the presence of high *PYK1* mRNA levels.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND.

The last 37 years have witnessed the science of molecular biology progressing from the elucidation of the structure of DNA, to a stage where the technology and expertise have been developed to determine the entire DNA sequence of the human genome. With this sequence it is hoped to characterise and cure many genetically inherited diseases. Also, the analyses of genes uncovered by this approach should unravel many unanswered questions of biology.

However, the DNA sequence of the human genome, or any organism's genome, is of limited use without an understanding of how the expression of the genes encoded by that sequence are regulated. Regulated gene expression is critical for cell differentiation, cell development, and for a cell to respond and adapt to a changing environment.

Much of the information gained in understanding the regulation of eukaryotic genes has been gleaned from studies using model organisms which have proved to be amenable to genetic manipulations and are accessible to study in the laboratory. *Saccharomyces cerevisiae* has proved to be an ideal model eukaryotic system in which to study the fundamental aspects of gene expression.

This micro-organism, commonly known as bakers yeast, is a simple unicellular eukaryote, being approximately 3μm in diameter, and generally of spherical shape. It has a genome size of about 14 Mb, distributed among 16 chromosomes, which encodes an estimated 5500 genes (Watson *et al.*, 1987). Unlike the case with higher eukaryotes, *S.cerevisiae* carries very little "redundant" DNA, which is reflected in it's small genome size: it is only 3.5 times larger than the size of the *E.coli* genome. This characteristic has proved beneficial for the creation of gene libraries and for the isolation of genetic mutants.

S.cerevisiae is a facultative anaerobe, having a generation time of 90 mins when growing exponentially on a fermentative carbon source (under laboratory conditions), and can propagate both asexually and sexually. The organism is stable both in the diploid state and in the haploid state (Roman, 1981).

As haploid, *S.cerevisiae* is divided into two populations depending on the mating type. Haploid cells are either of the *a*-mating type or of the *α*-mating type. Diploids are generated by the fusion of an *a*-cell with an *α*-cell, to give a cell with an *a/α* genotype. Both haploids and diploids generate asexually by the process of budding to form daughter cells. Under the appropriate sporulation conditions diploid yeast cells will undergo a sexual division. Essentially this involves a meiotic division to produce four ascospores which together are known as a tetrad. When these ascospores germinate they give rise to two *a*-cells and two *α*-cells. These haploid cells then divide asexually by budding (Herskowitz and Oshima, 1981).

This simple life cycle of *Saccharomyces cerevisiae* has been exploited by geneticists to map and characterise genes uncovered by mutation analysis.

Mutations (often conditional lethals) are conveniently generated in the haploid state. In order to characterise mutants, they are crossed with a haploid of opposite mating type to form a diploid. In the diploid state it can be determined if the mutation is dominant or recessive. Often more importantly, after meiotic division, analyses of tetrads can allow the mutation to be mapped with respect to other mutants and allow complementation studies between different mutations to be carried out (Mortimer and Schild, 1981).

Along with powerful genetic methodology, molecular biology has introduced further techniques for the analysis of *S.cerevisiae* at the genetic level (Struhl, 1983). Yeast cells can be transformed efficiently using plasmids (which replicate in *E.coli* and *S.cerevisiae*) generated by recombinant DNA technology. These plasmids propagate autonomously either at high or low copy number, or they can integrate by homologous recombination into the yeast chromosome. Depending on the approach taken, integrative transformation can result in either gene duplication or gene replacement. Gene replacement has allowed genes cloned from yeast and altered *in vitro*, to be re-introduced into yeast and the phenotype determined (Struhl, 1983). The advent of yeast transformation methods has also allowed many genes to be cloned through complementation of a mutant by transformation with a yeast plasmid gene library (Watson *et al.*, 1987).

It must be emphasised that while *S.cerevisiae* can be manipulated at a genetic level in a similar way to bacteria, they are essentially similar to higher eukaryotic cells. They display all the characteristic internal compartments of eukaryotic cells: nucleus, endoplasmic reticulum, golgi apparatus, mitochondria, cytosol, vacuole and peroxisomes (Struhl, 1983). Yeast also contains ubiquitin, calmodulin, clathirin, actin and tubulin (Struhl, 1983) and in addition, macromolecular synthesis, chromosome replication and segregation are similar in *S.cerevisiae* and higher eukaryotes (Watson *et al.*, 1987).

The organism has proved to be an excellent model system in which to study the regulation of cell cycle, secretion and protein targeting both at the genetic level and at the physical level. The organism also shows a gene regulated response to environmental stimuli, both through the RAS and cAMP signal transduction pathways in a manner analogous to higher eukaryotes (Matsumoto *et al.*, 1987). Finally the organism also displays a developmental pathway through its sexual cycle (Herskowitz, 1989).

This study has involved the use of *S.cerevisiae* to study the regulation of glycolytic gene expression, with particular emphasis on the regulation of the pyruvate kinase (*PYK1*) gene in *S.cerevisiae*.

In this chapter, I will first review what is known about the levels at which gene expression is controlled in *S.cerevisiae*, drawing on comparisons with examples in higher eukaryotes. Particular emphasis has been placed on the processes of translation and transcription. This is due to the fact that much of the work carried out in this study explored the translational and transcriptional regulation of both the *PYK1* gene itself and also glycolytic gene expression in general. The chapter then specifically describes the regulation of glycolytic gene expression in *S.cerevisiae*, and is followed by a discussion of what is known about the *PYK1* gene, and its gene product from yeast and other organisms.

1.2 REGULATION OF GENE EXPRESSION IN *S.CEREVISIAE.*

The level at which any particular protein is synthesized in a cell can be controlled at any stage between the DNA which contains the protein coding gene, to the final synthesis of the complete protein. As more genes are being analysed, it is becoming apparent that it is common for regulation to be exerted at more than one level. While it appears that the primary level at which gene expression is controlled is at the level of transcription, there are many example of genes both in *S.cerevisae* and in other organisms which are subject to pre- and post-transcriptional control mechanisms.

1.2.1 DNA REARRANGEMENTS.

Perhaps the best understood example of DNA rearrangements controlling gene expression, involves the phenomenon of mating type in *S.cerevisiae* (Herskowitz, 1989).

In *S.cerevisae*, mating involves the fusion of an *a*-type haploid with an *α*-type haploid to generate an *a/α* diploid. The mating type of the haploid is dictated by whether the *MATa* or *MATα* allele resides at the *MAT* locus on chromosome III. However, as well as this locus, there are also silent copies of both the *MATα* and *MATa* sequences residing at two other loci on chromosome III: *HML* and *HMR* respectively. These silent copies serve as the genetic material to allow mating-type switching to occur. Depending on the allele residing at the *MAT* locus, the opposite silent copy allele is transposed to the *MAT* locus replacing the existing *MAT* allele, thus switching the mating type. This transposition event is catalysed by a double stranded endonuclease encoded by the *HO* gene. Homothallic strains of yeast carry a wild-type copy of this gene, whereas heterothallic strains are *ho*, and are therefore not capable of switching sex (Herskowitz and Oshima, 1981). The mating-type switch is under tight regulation: while mother cells can switch, freshly budded cells must divide once before they have the capacity to do so (Herskowitz, 1989). This phenomenon of sex switching is not limited to *S.cerevisiae*, as it is exhibited by most yeasts, as well as by many fungi (Herskowitz, 1989).

DNA rearrangements are also important to allow antigenic variation in trypanosomes, *Neisseria* and *Salmonella*. It is also important during lymphocyte development in mammals, as it allows antibody encoding genes to rearrange in such a way as to allow a wide variety of antibody proteins to be synthesized (Alberts *et al.*, 1987).

Potentially, DNA rearrangements can also affect gene expression in a less specialised manner in *S.cerevisiae*. The retrotransposon Ty which randomly inserts itself at different sites in the yeast genome, contains flanking sequences which have strong promoter sequences (Rathgen *et al.*, 1987). Thus, if a Ty element is transposed into the promoter region of a dormant gene, it is capable of switching this gene on, and expression of the gene product would follow. Analogous retrotransposons to Ty are found in other organisms eg. Copia and P-elements are transposable elements found in *D.melongaster* (Watson *et al.*, 1987).

1.2.2 TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION.

Undoubtedly, the step where gene expression appears to be most often controlled is at the level of transcription. In all eukaryotes, the transcription of genes is mediated by three different RNA polymerases (Watson *et al.*, 1987). This contrasts with the situation in *E.coli*, where transcription is conducted by a single RNA polymerase.

In yeast, RNA polymerase I transcribes 3 of the 4 ribosomal genes into precursor rRNA which is processed to give two large rRNAs (25S and 17S) and one of the two small rRNAs (5.8S). RNA transcribed by this RNA polymerase contributes approximately 70% of total cellular RNA. RNA polymerase II transcribes all of the protein encoding genes to give mRNA, which contributes to about 5% of total cellular RNA. Finally, RNA polymerase III transcribes all of the tRNA genes and the 5S rRNA genes. These RNAs make up approximately 25% of the total level of RNA.

The three RNA polymerases can be separated by their sensitivity to the fungal toxin α-ammanatin (Watson *et al.*, 1987). For the remainder of this section I have focussed attention on transcription by RNA polymerase II, firstly describing the components of the transcription

process and then discussing examples of gene regulation, which highlight many important facets of transcription in *S.cerevisiae*.

1.2.2.1 Promoter elements.

Both the site of transcription initiation and the level of transcription hinge on the co-ordinated interaction of several protein "factors" including RNA polymerase, with DNA "promoter elements". These "promoter elements" signal the sequences of DNA to be transcribed into mRNA, and are predominantly located 5' to the coding region.

There are at least four *cis*-acting promoter elements which characterise eukaryotic genes: the initiation (I) site, the TATA box, activator elements, and operator elements.

Initiation site: This is the site where the RNA polymerase initiates transcription. In higher eukaryotes, transcriptional initiation occurs 30-35 bp downstream of the TATA box (Struhl, 1989a). However, in yeast, the distance between the TATA box and the I-site is more flexible, and can be between 40-120 bp (Struhl, 1989a). It appears that many sequences can fulfil this function. In highly expressed genes the sequences surrounding the I-site, have a loosely conserved structure of a CT rich stretch followed by the tetranucleotide CAAG (Rathgen and Mellor, 1990). Studies with the *PGK1* promoter have shown that sequences located 5' of the CAAG sequence motif direct initiation at discrete sites when placed at the correct distance from the TATA box (Rathgen and Mellor, 1990). It will be interesting if these sequences, termed "Determinator sequences", are found to be characteristic of more genes. Certainly some genes, such as *CYC1*, appear not to as they have multiple I-sites (Guarente and Mason, 1983).

Although the I-site is not considered to be important in dictating the level of transcription (Struhl, 1989a), it has been shown that alternative I-sites are used in the regulation of at least one gene (*SUC2*) to synthesize different proteins from the one cistron in response to different environmental conditions. *SUC2* encodes invertase and has two alternative transcription start sites which results in the synthesis of two proteins which differ in their amino-terminus (Carlson

and Botstein, 1982). While the upstream I-site is used to direct synthesis of a secreted form of invertase and is subject to carbon source regulation, transcription from the downstream I-site is constitutive, and directs synthesis of an mRNA which encodes an intracellular form of invertase (Carlson and Botstein, 1982).

TATA box: TATA elements are necessary for the transcriptional initiation of most yeast genes, and as mentioned above normally reside 30-120 bp upstream of the I-site. The TATA sequence is believed to have a general role in transcription. Recent observations have argued the existence of functionally distinct classes of TATA elements (Struhl, 1989a). In the case of the *HIS3* gene there are two functionally different TATA boxes which have been identified: a constitutive TATA T_C and an induced TATA T_r (Hope and Struhl, 1986). Interestingly when T_r is replaced by the binding site for the positive *trans*-acting factor *GCN4* in a *GAL:HIS3* fusion, transcription is still achieved under induced conditions, suggesting that the TATA box binding factor can be substituted for by an alternative sequence (Chen and Struhl, 1989). This may account for the transcription of *TRP3* which lacks a TATA element, but has a *GCN4* binding site 28 bp upstream from the mRNA start site (Struhl, 1989a). However, it must be emphasised that in the vast majority of genes, the TATA element is integral to transcription.

Activator sequences: These are usually short DNA sequences (10-30bp) which direct the activation of transcription upon the interaction and activation by *trans*-activator factors. These sequences are known as Upstream Activating Sequences (UAS), and it is these sequences which usually determine the particular regulatory properties of a given promoter. Genes which are co-ordinately expressed usually share similar activator sequences. Many different activator sequences have been described in yeast (Verdier, 1990), which direct transcriptional activation in response to different stimuli. These sequences are located relatively far from the I-site (100-1500bp away), and can act in either orientation (Struhl, 1989a). Unlike enhancer sequences in higher eukaryotes, these sequences do not operate downstream of the I-site. However, there are several genes which have been found to have sequences residing in the coding region which activate transcription.

These include *PGK1* (Mellor *et al.*, 1987), *TyA* (Rathgen *et al.*, 1987), and *PYK1* (Purvis *et al.*, 1987).

Operator sequences: These are similar to activator sequences in their structure, orientation and position. However, they function to repress transcription as opposed to activating. They generally repress more efficiently when located between the TATA box and the UAS (Struhl, 1989a). Operator sequences are not common to all promoter regions. For example, while a sequence which blocks transcription resides in the *ENO1* gene, it is absent from the *ENO2* gene (Cohen *et al.*, 1987).

1.2.2.2 General Transcription factors.

Transcription factors, which interact with DNA to regulate and direct transcription can be divided into two classes: those which are involved generally with transcription and those which interact specifically with a group of coordinately expressed genes. This section describes the general factors involved in transcription.

In any model for transcriptional activation, the ultimate target for modulation is RNA polymerase II. RNA polymerase II initiates mRNA synthesis at discrete sites on the chromosome when it recognises a transcription complex composed of upstream activator proteins, the TATA binding protein and the DNA itself. The yeast RNA polymerase II is composed of ten polypeptides with apparent molecular weights of 10-220 KDa (Woychik and Young, 1990). The largest subunit of RNA polymerase II from yeast to humans, RPB1, contains a highly conserved acidic seven amino acid sequence, which is repeated several times at the C terminus and is required for transcription *in vivo* and *in vitro* (Allison *et al*, 1985). In yeast, the sequence naturally consists of 26 repeats, of which it has been established that at least 10 are required for cell viability. Recent experiments in yeast have shown that the number of Carboxy-Terminal-Domains (CTD) are important for transcriptional regulation by specific *trans*-acting proteins. As the number of CTDs are decreased in deletion mutants of *RPB1*, the ability of *trans*-acting factors to modulate transcription is impeded (Scafe *et al.*, 1990). The extent of the block on transcriptional regulation depends

on both the *trans*-acting factor mediating regulation and the number of CTDs (Scafe *et al.*, 1990). This suggests that the CTD of *RPB1* is an important site for transcriptional regulation.

The development of *in vitro* transcription systems has greatly aided the characterisation of the protein interactions which mediate the transcription initiation complex. The earliest system was developed using cytoplasmic extracts from human cells (Weil *et al.*, 1979). Since then, several other systems have been developed, including a yeast system (Lue and Kornberg, 1987). These *in vitro* transcription reactions depend on recognition of a TATA element on a DNA template to allow initiation, thus paralleling the importance of the TATA element *in vivo*. These systems have allowed the sequence requirements within the DNA template to be dissected and have allowed the purification of some transcription factors. These factors have been separated and are classified as TFIIA, TFIIB, TFIID, TFIIE, TFIIF (Buratowski *et al.*, 1989). Studies with these general transcription factors suggest that TFIID binds DNA first along with TFIIA. After this TFIIB, RNA polII, and TFIIE/F are incorporated into the initiation complex (Buratowski *et al.*, 1989). TFIIF binds to RNA polymerase and probably catalyses the transition from a closed to open complex at the promoter (Sopta *et al.*, 1989).

Much work is being done to purify these fractions to homogeneity to allow biochemical analysis and cloning of genes by reverse genetics. This was aided by the fact that yeast TFIID can replace the mammalian TFIID in mammalian *in vitro* systems (Buratowski *et al.*, 1988). The *S.cerevisiae* TFIID protein was subsequently purified, the gene cloned (Horikoshi *et al.*, 1989; Hahn *et al.*, 1989), and the protein established as the TATA box binding factor. Using the sequence, it has been possible to clone the human (Hoffmann *et al.*, 1990) and *Arabidopsis* genes (Gasch *et al.*, 1990) and in addition, the gene has also been cloned from *Schizosaccharomyces pombe* by complementation of the *Spt15* mutation in *S.cerevisiae*, the gene which codes for TFIID (Fikes *et al.*, 1990). Comparison of the TFIID sequences from these different organisms (Hoffmann *et al.*, 1990) demonstrates that the C-terminus is highly conserved. This underscores the importance of the C-terminus which contains several motifs believed to

be important in DNA binding (Ptashne and Gann, 1990). This area contains similarities to the bacterial σ-factor, as well as a direct repeat sequence and a central basic core/lysine repeat (Hoffmann *et al.*, 1990). Mutagenesis in this region results in loss of activity emphasising its importance, (Hoffmann *et al.*, 1990). The N-terminal, by contrast, is not conserved at all, and has been proposed to be a target for species-specific regulatory factor interactions (Hoffmann *et al.*, 1990). *In vitro* studies have shown that purified TFIID can bind directly with the acidic activation domain of the transcriptional activator VP16 (Stringer *et al.*, 1990), suggesting that TFIID is indeed the principal target for transcriptional activation by sequence specific *trans*-acting factors. However, as pointed out by Ptashne and Gann (1990), there is some evidence to suggest that activators mediate their effect on TFIID through an intermediate. Indeed, recent evidence described below, using a GAL4:VP16 fusion protein, have uncovered the existence of "adaptor" molecules which mediate the interaction between the activator proteins and the general transcription machinery.

1.2.2.3 Regulation of transcription by specific *trans*-acting factors.

Many *trans*-acting factors of transcription have been identified in yeast through mutation analysis (Verdier *et al.*, 1990). These sequence-specific factors activate (or repress) transcription by interacting with the general transcription machinery. Studies involving these factors have shed light on the transcription process, and have also served as models for examples of genes which are transcriptionally regulated. Three examples of these well-characterised factors from *S.cerevisiae* are described below.

GAL4: This 881 amino-acid *trans*-activating factor functions to activate the transcription of galactose-inducible genes in the presence of galactose (Johnston, 1987a). There are three enzymes involved in the metabolism of galactose by *S.cerevisiae*, all three of which are only synthesized in the presence of galactose (Johnston, 1987a). *GAL1* which encodes galactokinase and *GAL7* which encodes galactose transferase are divergently transcribed from the same "promoter

elements", while the immediately adjacent *GAL10* encodes galactose epimerase. Upstream of these three genes there are four GAL4 binding sites, each being a 17bp sequence (Johnston, 1987a).

Interestingly, the GAL4 molecule binds to its DNA binding site resident upstream of galactose inducible genes even in the absence of galactose (Lohr and Hopper, 1985). However, activation by GAL4 is blocked by the binding of a repressor GAL80 (Johnston *et al.*, 1987a). This molecule does not bind to DNA, but rather it binds to the GAL4 molecule itself to block transcription. This protein interaction has been demonstrated *in vitro* by Lue and co-workers (1987). In the presence of galactose, the GAL80 dissociates from GAL4 and transcription proceeds.

GAL4 activator proteins act in synergy to activate transcription of galactose-inducible genes. The level of expression achieved by the binding of one GAL4 molecule is more than doubled by the binding of a second (Ginger and Ptashne, 1987). GAL4 has also been shown to act as a transcriptional activator in higher eukaryotes (Fischer *et al.*, 1988; Ma *et al.*, 1988) on genes which have been tailored to carry GAL4 binding sites in their promoter regions. The conservation of the transcription process is remarkable and recently it has been demonstrated that the synergistic effect achieved by GAL4 in yeast also occurs in a mammalian *in vitro* transcription system where a mammalian *trans*-activator, ATF, has been shown to act synergistically with GAL4 to activate transcription of a mammalian reporter gene (Carey *et al.*, 1990; Lin *et al.*, 1990).

When GAL4 is over-expressed in *S.cerevisiae* the transcription of genes possessing no GAL4 binding sites is decreased. This is presumably due to the saturation of a transcription factor (by GAL4) which is needed for the transcriptional activation of many genes, not only those bearing a GAL4 binding site. This phenomenon is known as "squelching" (Gill and Ptashne, 1988) and indicates that *trans*-activating factors interact with the general transcriptional machinery. Recent experiments exploiting this phenomenon have shown that while a GAL4:VP16 activator inhibits the transcription of other templates *in vitro*, only activated transcription is affected. Basal level transcription of templates without activator sequences remains unaffected thus

implying the factor being squelched is not a factor needed for basal level transcription but an "adaptor" which is needed to mediate the activation of transcription by *trans*-acting proteins (Berger *et al.*, 1990). Thus, it appears that TFIID is not contacted directly by *trans*-activators, but indirectly by an adaptor (Berger *et al.*, 1990).

Like other eukaryotic activator proteins, GAL4 has two separable properties: an ability to bind DNA and an ability to modulate transcription. The DNA binding region of GAL4 is localised to the N-terminal 74 amino acids, while regions of acidic amino acids at positions 148-196 and 761-881 mediate activation (Johnston, 1987b).

Several motifs have been found to be characteristic of DNA binding proteins and are conserved from prokaryotes through yeast to humans. In the case of GAL4, the motif that mediates DNA binding is similar to the "Zinc-finger motif" which was first proposed by its appearance in TFIIIA (Struhl, 1989b), a *Xenopus* protein which is required for transcription of 5S rRNA by RNA polymerase III. Mutations in the DNA binding region of GAL4 which disrupt the zinc finger structure disrupt function (Johnston, 1987b). An important role for zinc ions has been established genetically: certain *gal4* mutants will only function in the presence of elevated levels of zinc ions (Johnston, 1987b).

Studies with GAL4 suggest that at least some yeast transcriptional activator domains are defined by short acidic regions (Struhl, 1989a). When GAL4 responds to the presence of galactose, the acidic regions probably interact with some part of the general transcription machinery, possibly the TATA box factor. The importance of acidic residues in activating transcription is reflected by the fact that single amino acid changes in a GAL4 derivative that increase or decrease the level of activation are almost always associated with an increased or decreased negative charge respectively (Gill and Ptashne, 1987).

GCN4: This *trans*-activator which induces a large number of amino acid biosynthetic genes in response to amino acid starvation, shows certain similarities and differences to GAL4. Unlike GAL4, the GCN4 protein does not bind to its DNA binding site until induced by amino acid starvation. Upon induction, the synthesis of the GCN4

protein is increased at the translational level (1.2.5.3) and the protein is then transported to the nucleus where it activates the transcription of a large number of the amino acid biosynthetic genes by binding as a dimer to its DNA target, a 9 nt sequence of dyad symmetry (Hinnebusch and Fink, 1983).

GCN4 is 281 amino acids long and like GAL4 has distinct regions involved with DNA binding or activation. The DNA binding region of GCN4 is localised to the 60 amino acids at the C-terminus, and is dependent on a leucine zipper motif analogous to those found in several mammalian oncogenes: *jun*, *fos*, and *myc* (Struhl, 1989b). Within their amino acid sequence each of these proteins contains four or five leucine residues which are spaced exactly seven residues apart. It has been proposed that the leucine residues are important in allowing two *a*-helixes from each monomer to interdigitate (Struhl, 1989b). Thus the zipper motif is thought not to be the structure which binds to DNA, but is important for dimerisation which is a prerequisite for DNA binding. Interestingly, the GCN4 DNA binding domain shows 45% aa sequence homology with the *jun* oncoprotein, and experiments have shown that the GCN4 DNA binding domain can be functionally replaced by the homologous *jun* region in yeast (Struhl, 1989a).

The region of GCN4 protein which activates transcription has been mapped to an acidic region at amino acids 107 - 125 (Hope and Struhl, 1986). The importance of the acidity of this region for transcriptional activation is exemplified by the fact that different portions of the GCN4 acidic region are equally capable of activating transcription even though their primary sequences are different (Hope and Struhl, 1987).

MATα2: Unlike GAL4 and GCN4, this *trans*-acting factor acts as a repressor of transcription. The protein contributes to the specification of cell type, binding specifically to the *a*-specific genes to repress their transcription. The DNA binding domain of MATα2 shows strong homology to the homeobox motif of some *Drosophila* DNA binding proteins and also to the helix-turn-helix motif common to bacterial repressors and activators. This structure is composed of two *a*-helixes separated by a ß-turn (Struhl, 1989b). Mutations within the helix-turn-helix structure of MATα2 abolish function (Porter and Smith, 1986).

turn-helix structure of MATα2 abolish function (Porter and Smith, 1986).

There are many other *trans*-acting transcription factors, which have been identified in yeast (Verdier, 1990). These share many of the characteristics described for GAL4, GCN4, and MATα2, including strong homologies to factors from higher eukaryotic factors. Through detailed analysis of these factors, much has been gleaned on the mechanisms of transcriptional regulation in *S.cerevisiae*, and the transcriptional process itself.

1.2.3 mRNA PROCESSING.

After transcription there are several stages at which the level or the structure of the primary mRNA transcript can be modified in eukaryotes. These include alternative 3' end formation and mRNA splicing which are described below.

1.2.3.1 Alternative 3'end formation.

In higher eukaryotes there are an increasing number of examples of genes whose expression is regulated through differential use of polyadenylation sites at their 3' end which result in the synthesis of mRNAs which differ at their 3' end. For example, the adenovirus major late transcription unit which encodes five families of mRNA, uses alternative 3' polyadenylation sites to regulate expression of its mRNAs during infection (Friedman *et al.*, 1987). Differential usage of poly(A) sites also determines whether the membrane-bound or secreted form of the immunoglobulin μ-heavy chain is synthesized (Friedman *et al*, 1987).

However, unlike the situation with higher eukaryotes, the mechanism of yeast mRNA 3' end formation and poly-adenylation is poorly understood, with no general consensus sequence for 3' end formation having been deduced.

The expression of the cytochrome B processing gene *CBP1* is the only *S.cerevisiae* gene known to be regulated at this level (Mayer and Dieckman, 1989). This gene produces two mRNA transcripts (1.3kb and 2.2kb) with the same 5' start site, but different 3' ends. While the

combined steady-state levels of the two transcripts is constant during a switch from a non-fermentable carbon source to a fermentable carbon source, the levels of each of the two transcripts are inversely regulated (Mayer and Dieckmann, 1989). The different *CBP1* transcripts must be synthesized either by a choice between two transcription termination sites, which in turn dictates the site of polyadenylation, or by differential cleavage and polyadenylation of a long precursor mRNA. It has been proposed that *trans*-acting factors which respond to carbon source interact with *CBP1* in a way that affects the choice of polyadenylation or transcriptional termination sites in order to regulate the levels of the two different transcripts (Mayer and Dieckmann, 1989).

1.2.3.2 mRNA splicing.

Many eukaryotic mRNAs contain introns which have to be removed before an mRNA can be translated into the desired protein. The presence of introns permits the control of gene expression by alternative splicing of an mRNA.

The process of nuclear pre-mRNA splicing is conserved between yeast and higher eukaryotes and involves the recognition of conserved splice sites (at the 5' and 3' ends of the intron) by the spliceosome (Green, 1986). The spliceosome is a large complex which is composed of snRNPs, which themselves are made up of snRNAs (U1 - U6) and nuclear proteins. The first step of splicing involves cleavage at the 5' junction of the intron. The free 5' end of the intron then loops back to form a lariat structure by binding to an A residue, 18-40 nt 5' to the 3' splice site (Green, 1986). In yeast this A residue is found in a conserved sequence known as the UACUAAC box, but in higher eukaryotes the sequence requirements are less stringent. The splicing reaction is completed by cleavage at the 3' splice site, followed by ligation of exon 1 with exon 2 (Cheng and Abelson, 1987).

S.cerevisae has been an excellent system in which to study the process of splicing, with many splicing mutants isolated. However, the presence of introns in a yeast mRNA transcript is more the exception than the rule. Hence, it is not surprising that the regulation of gene expression based upon differential splicing has not been observed. It

has however been well characterised in other eukaryotes as a form of gene control. Perhaps the most elegant and detailed example involves sex determination in *Drosophila* which is controlled by a cascade of genes, whose expression are controlled by alternative splicing (Baker, 1989). The sex-lethal (*SXL*) gene is alternatively spliced in males and females with an incomplete protein being produced as a result in males. In females the *SXL* gene product then controls the splicing of the transformer (*TRA*) and transformer-2 (*TRA-2*) transcripts to generate the desired female mode of expression (Baker, 1989).

A limited number of yeast genes do contain introns and these include several of the ribosomal protein genes (Warner, 1989). The expression of at least two of these genes appears to be regulated at the level of splicing. When either the *RPL32* or *RPL29* genes are transformed into yeast on a multicopy vector, over-expression is limited by the accumulation of unspliced mRNA (Warner *et al.*, 1985). In the case of *RPL32*, accumulation of unspliced mRNA is dependant on the presence of excess RPL32 protein (Warner, 1989).

1.2.4 mRNA STABILITY.

After transcription and processing, an mRNA is transported to the cytoplasm where it is available to be translated. The level of an mRNA available for translation depends not only on the rate of arrival from the nucleus, but also on the stability of the mRNA once in the cytoplasm. There is now strong evidence to show that the expression of several eukaryotic genes is controlled at the level of mRNA stability (Brown, 1989).

Very little is known about the biochemistry of mRNA degradation, though the development of a mammalian cell free degradation system by Brewer and Ross (1988), will hopefully unravel the processes involved. An analogous system from yeast cells is not available. Several models have been proposed to describe the process of mRNA degradation (Brown, 1989), all of which require testing. It is possible that different degradation pathways are used for specific mRNAs.

In yeast there are several observations which any putative degradation pathway must account for. Firstly, yeast mRNAs are

separable into stable and unstable classes (Santiago *et al.*, 1986). It is believed that unstable mRNAs contain instability elements which render the mRNA susceptible to nucleolytic attack. In one case, an instability element has been mapped to a sequence of 42 nt within the coding region of the *MATa1* gene (Parker and Jacobsen, 1990a). Interestingly this sequence contains a high level of poor codons and to act as an instability element must be translated (Parker and Jacobsen, 1990a). Instability elements have also been identified in the *URA3* gene, and in the *STE3* gene (Brown, 1989; Parker and Jacobsen, 1990b).

Other experiments in yeast have shown that within each population of mRNA, there is a crude inverse relationship between length and stability (Santiago *et al.*, 1986).

The best characterised example where mRNA stability controls the level of expression of a yeast gene involves the histone genes (Lycan *et al.*, 1987). The level of histone mRNA fluctuates during the cell cycle with a 20-fold higher level during S phase than at any other phase of the cell cycle. This decrease in mRNA level is co-ordinated by a combination of both decreased transcription and increased degradation (Lycan *et al.*, 1987). Also when extra copies of the histone *H2A* and *H2B* genes are introduced into yeast, mRNA levels remain normal in these strains despite increased levels of primary histone mRNA transcripts (Osley and Hereford, 1981). Increased rates of histone mRNA degradation ensure that there is no build up excess histone mRNA (Osley and Hereford, 1981).

In higher eukaryotes, examples of regulated mRNA stability include a series of lymphokine, cytokine, and proto-oncogenic mRNAs which contain within their 3' untranslated region a 50 base pair AU rich sequence which destabilises the RNA (Yen *et al.*, 1989). However, perhaps the best defined example involves the autoregulation of the β-tubulin mRNA (Gay *et al.*, 1988). As excess free β-tubulin subunits accumulate they bind to the amino-terminal sequence of the nascent β-tubulin polypeptide chain as it emerges from the translating ribosome (Yen *et al.*, 1988). This results in the activation of a ribonuclease which degrades the β-tubulin mRNA. It has been suggested that the RNases which activate cleavages actually reside on the ribosome (Yen *et al.*, 1988).

1.2.5 mRNA TRANSLATION.

Eukaryotic mRNA translation involves the co-ordinated interaction of ribosomes, mRNA, tRNAs, aminoacyl tRNA synthetases and co-factors, and can be divided into three stages.

Initiation involves the binding of the small ribosomal sub-unit with a series of initiation factors and the initiator $tRNA_{met}$ to the 5' end of the mRNA, followed by scanning of the pre-initiation complex to the initiation codon where the large ribosomal sub-unit associates. Elongation involves translocation of the ribosome along the mRNA and peptide bond formation. Finally, termination involves the recognition of a stop codon by the ribosome and the subsequent release of the ribosome and the translated polypeptide.

What follows in this section is an outline of the process of translation initiation, a brief description of the components involved in translation and finally some examples of translational control. Emphasis is placed on the initiation of translation, because of its key role in the regulation of gene expression. The similarities between *S.cerevisiae* and other eukaryotes is discussed throughout.

1.2.5.1 Translational initiation.

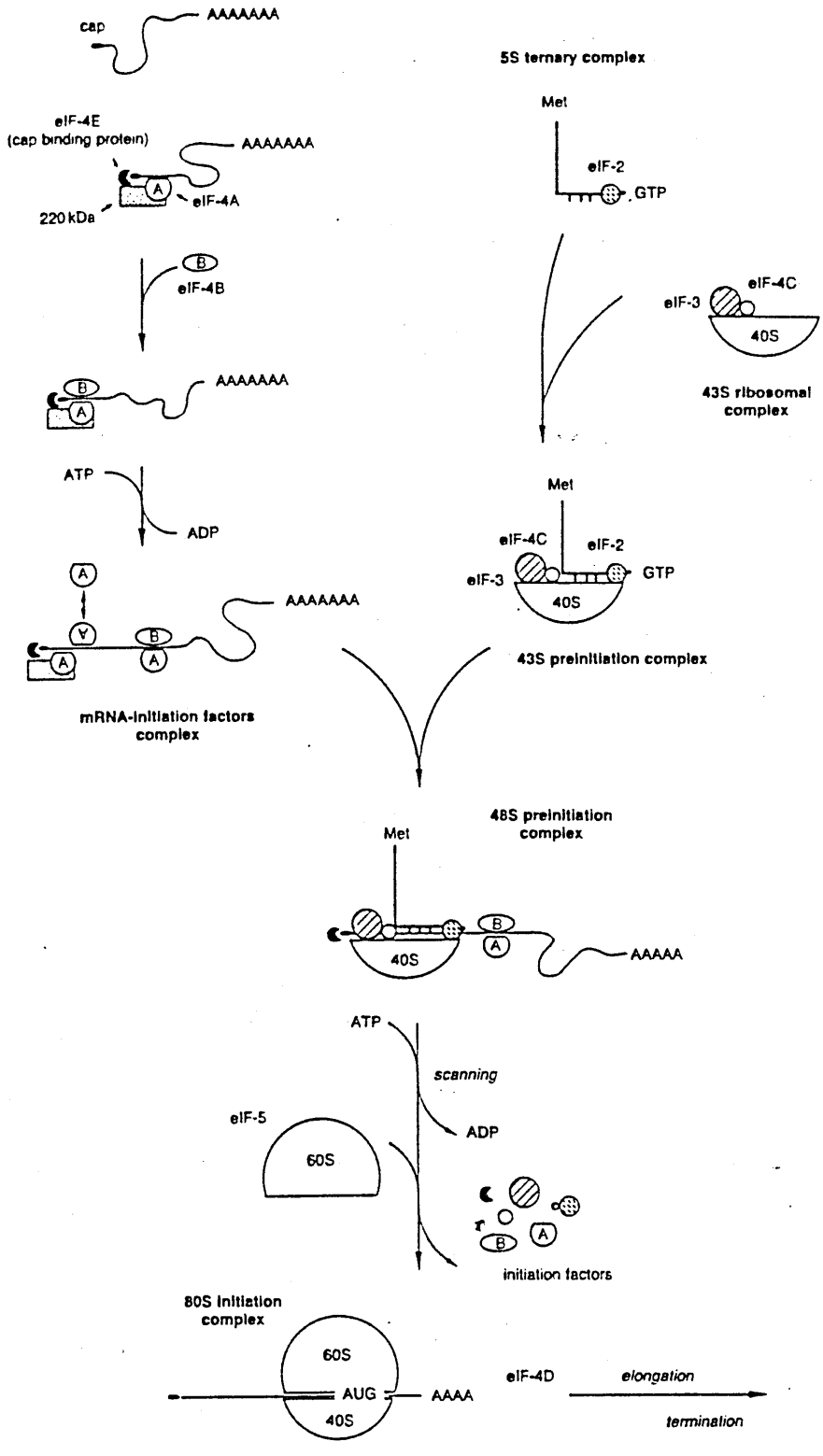
There are two models for translational initiation but the model which appears to account for the majority of mRNAs is the "scanning" hypothesis first suggested by Kozak (Kozak, 1983; Fig. 1.1). In this model the 40S ribosomal subunit, together with the $tRNA_{met}$ and cap-binding translation factors binds to the 5' end of the mRNA at the CAP structure. This CAP structure, which is characteristic of the majority of eukaryotic mRNAs, consists of a methylated guanosine residue (m^7G) linked to the 5' end by an unusual 5'-5' triphosphate linkage (Brown, 1987). With the aid of other translation factors, the pre-initiation complex then scans the 5' leader in a 5' to 3' direction until it reaches the first AUG, where it is joined by the 60S ribosomal subunit and proceeds to translate the AUG codon. The scanning process requires both ATP and GTP.

This scanning hypothesis satisfies several of the criteria associated with translation initiation:

Figure 1.1 **The Scanning Model for Translational Initiation.**

40S: 40S, small ribosomal subunit
60S: 60S, large ribosomal subunit
eIF: eukaryotic Initiation Factor
see Table 1.1 for description of each factor
cap: mRNA cap structure
Met-tRNA$_i$: Initiator methionyl transfer RNA.

Adapted from Linder and Prat (1990).


cap
AAAAAAA
eIF-4E
(cap binding protein)
AAAAAAA
A
eIF-4A
220 kDa
B
eIF-4B
B
A
AAAAAAA
ATP
ADP
A
A
B
A
AAAAAAA
mRNA-initiation factors complex
5S ternary complex
Met
eIF-2
GTP
eIF-4C
eIF-3
40S
43S ribosomal complex
Met
eIF-4C
eIF-2
eIF-3
GTP
40S
43S preinitiation complex
48S preinitiation complex
Met
B
A
40S
AAAAA
ATP
scanning
ADP
eIF-5
60S
r
B
A
initiation factors
80S initiation complex
60S
AUG
AAAA
40S
eIF-4D
elongation
termination

a) In 95% of yeast and mammalian mRNAs the 5' proximal AUG is used as the initiation codon. Also, if an AUG is placed upstream of the natural AUG then translation will begin at the new AUG (Kozak, 1983; Cigan and Donahue, 1987).
b) Eukaryotic ribosomes are unable to bind to a circular mRNA implying the functional requirement for a 5' end (Kozak, 1983).
c) Translation is inhibited by secondary structure (Baim and Sherman, 1988; Kozak, 1983).
d) The initiation of translation at the next AUG when the first AUG has been mutated (Kozak, 1989).

For a limited number of viral mRNAs, translation can initiate by direct binding of the ribosome to a internal "ribosome landing pad" situated 5' to the start codon. This was first demonstrated for the poliovirus mRNA and subsequently for the encephalomyacarditis mRNA, (Herman, 1989)

The poliovirus mRNA is unusual in structure in that it lacks a 5' CAP structure, with instead a small peptide binding to its 5' end (Sonnenberg, 1988). Addition of a CAP structure does not increase its translatability and instead translation proceeds in a CAP-independent manner (Herman, 1989). Recently it has been shown that a cellular factor present in Hela cells mediates ribosome binding to the ribosome landing pad (Meerovitch *et al.*, 1989). Presumably once the ribosome has bound, it then scans towards the AUG.

Unpublished data also suggests that internal translational initiation also occurs in yeast (Mueller and Traschel, 1990), though this remains to be confirmed.

1.2.5.2 Components of the Translation machinery.

The Ribosome: Eukaryotic ribosomes are made up of a small subunit (40S) and a large subunit (60S). In yeast the small subunit contains one RNA molecule (18S) and about 30 ribosomal proteins, whereas the large subunit is comprised of three RNA molecules (25S, 5.8S, and 5S) and about 40 proteins (Warner, 1982).

mRNA:

a) Length of 5' Untranslated region: The length of the 5' untranslated leader sequence varies both in yeast and in higher eukaryotes. A comparison of leader lengths in *S.cerevisiae* shows that the longest leader so far uncovered belongs to the *GCN4* mRNA and is 591 nt long (Cigan and Donahue, 1987). In contrast, the shortest leader is 11 nt and belongs to the *MATa1* gene (Cigan and Donahue, 1987)

Studies have shown that in vertebrate genes, long 5' untranslated regions have no inhibiting effect on translation, as long as they are devoid of secondary structure (Kozak, 1983). However, short leader lengths can have a detrimental effect. When preproinsulin mRNAs with decreasing lengths of 5' leader were tested for their translatability in cultured monkey cells, short leaders were shown to be less efficiently translated (Kozak, 1988). Interestingly the inhibitory effect of short leaders were shown more clearly when the monkey cells had been subjected to an abrupt change in tonicity through an osmotic shock (Kozak, 1988). This infers that under conditions of stress, mRNAs with short leader sequences may be less efficiently translated.

Similar studies have been conducted in *S.cerevisiae*. As the 5' leader of the *HIS4* gene was shortened from 115 nt to 39 nt, no inhibition of translation was observed (Cigan *et al.*, 1988). A similar study with the *PGK1* gene also showed that reduction of the length of the 5'-untranslated region from 45nt to 27nt had no inhibitory effect on translation (van den Heuvul *et al.*, 1989). However, a further decrease from 27nt to 21nt resulted in a two fold decrease in translation. Hence, the length of the leader can affect translation if it is reduced to below 27nt in *S.cerevisiae*. No studies have been carried to test if the translation of mRNAs with short leaders are selectively inhibited in an analogous manner to higher eukaryotes under conditions of stress. This inefficient initiation of translation by short leader lengths is perhaps due to some steric block to 40S subunit binding.

b) The primary sequence of the 5' untranslated region: This has certain general and specific features which are important in dictating the level of translation. Generally, it is found that highly expressed yeast genes have mRNA 5' untranslated regions with a high proportion

of A residues. For example, the glycolytic genes which are highly expressed in yeast contain a high proportions of A nt in their 5' untranslated regions (Cigan and Donahue, 1987). Conversely, untranslated regions with a high number of G or C residues have been shown to be characteristic of regulatory proteins which are expressed at relatively low levels (Cigan and Donahue, 1987). This correlation may not be due to the effect of the primary sequence directly, but indirectly through the formation of secondary structure: an increased proportion of G and C residues promotes secondary structure formation.

The only apparent stringent sequence requirement in yeast 5' untranslated regions is at the initiation codon, where AUG is the only codon of physiological relevance (Cigan *et al.*, 1988). Studies which have replaced the AUG of the *HIS4* gene with the other eight permutations of codon which differ by only one nt from AUG, have shown that initiation of translation only occurs significantly from AUG (Cigan *et al.*, 1988). This has also been demonstrated for a *CYC7:lacZ* gene fusion in yeast (Clements *et al.*, 1989), in which negligible ß-galactosidase activity was achieved when the AUG was replaced. However, it has been shown in mammalian genes, that the codons CUG, GUG and ACG can initiate translation efficiently (Medhi *et al.*, 1990).

Surprisingly, it has also been shown that a stop codon can function as a start codon (Ghersa *et al.*, 1990). There are three stop codons used in eukaryotic cells - UAA, UGA, and UAG - which have no corresponding tRNA. Instead a termination factor binds to the ribosome which signals the termination of translation. A study of the aldolase gene from *Plasmodium falciparum* has shown that translational initiation occurs at the codon UAG, both *in vivo* and *in vitro* (Ghersa *et al.*, 1990). In this system, the UAG is found in a favourable sequence context for translation (discussed below) and also appears to be in a positive position for translation initiation as a consequence of secondary structure in the leader sequence.

Comparison of a large number of yeast 5' untranslated sequences has led to the derivation of a consensus sequence surrounding the AUG in highly expressed genes (Cigan and Donahue, 1987). This led to the suggestion that sequence context of the AUG is important in

dictating the efficiency of translation, a phenomenon previously shown in higher eukaryotes (Kozak, 1989). However, the consensus sequence found in *S.cerevisiae* (taken from Cigan and Donahue, 1987) differs from its mammalian counterpart (taken from Kozak, 1987) :

Consensus Sequence around AUG

S.cerevisiae	5'- A/Y A A/U A **A U G** U C U - 3'
Higher eukaryotes	5'- C A C A **A U G** G - 3'

This consensus sequence has been shown to be important in the level of initiation at the start codon in mammalian cells. Alteration of the AUG sequence context in the rat preproinsulin mRNA can result in a 20-fold decrease in translation initiation (Kozak, 1989). This effect supports the concept of "leaky scanning" which proposes that in mammalian cells, translation initiation occurs at the 5' proximal AUG on the mRNA only if that codon lies in a suitable sequence context for initiation. However, studies in *S.cerevisiae* have shown that alterations to the consensus sequence surrounding the AUG have very little effect on translation (Donahue and Cigan, 1988; Baim and Sherman, 1988). Thus, the more stringent requirement for an AUG as initiation codon in *S.cerevisiae* may be due to the fact that unlike translation in mammalian cells, there is no sequence context effect to direct translation initiation in yeast.

c) The poly(A) tail: This is added post-transcriptionally to an mRNA at its 3' end. It ranges in length depending on the organism and age of the mRNA; in yeast it ranges from 50-70 nt (Munroe and Jacobsen, 1990). While the poly(A) tail has an important role in mRNA stability, there is also now strong evidence showing that it is pivotal to translation initiation (Munroe and Jacobsen, 1990). Direct evidence for the involvement of the poly(A) tail in translation initiation came from genetic studies using *S.cerevisiae*. In this study Sachs and Davis (1989), analysed a strain of yeast which carried a conditionally-lethal mutation in the gene encoding the poly(A) binding protein, *PAB1*. When the poly(A) binding protein was depleted from the cell, a marked decrease in the number of polysomes was observed, and this was

paralleled by an increase in the number of non-translating ribosomal subunits. Moreover the link between translation and the poly(A) tail was strengthened with the observation that two extragenic suppressors of the *pab1*$_{ts}$ mutation mapped to genes involved in ribosomal biogenesis (Sachs and Davis, 1989; Sachs and Davis, 1990). These mutations overcame the depletion of poly(A) binding protein, allowing the initiation of translation to take place by affecting the amount of 60S subunit. This suggests that the poly(A) binding protein interacts with the 60S subunit to elicit its effect. Experiments in other systems have also demonstrated a link between polyadenylation and translation. The mouse tissue plasminogen activator mRNA requires the presence of a long poly(A) tail for translation (Vassali *et al.*, 1989). Also in an *in vitro* study using a cell-free rabbit reticulocyte extract, it was shown that mRNAs without a poly(A) tail are translated poorly, while mRNAs with a poly(A) tail are translated more efficiently (Munroe and Jacobsen, 1990). From all of these studies it appears that the formation of the 80S initiation complex is inhibited in the absence of a poly(A) tail.

While the participation of the Poly(A) tail in translation seems no longer in doubt, there is doubt as to its role. It has been postulated that its interaction with the poly(A) binding protein is needed for translation (Munroe and Jacobsen, 1990). This would block the translation of partially degraded mRNAs which cannot form such a complex. Presumably the translation of the small subset of mRNAs without a poly(A) tail is initiated in a different manner. It has also been proposed that the poly(A) tail acts as a translational enhancer (Munroe and Jacobsen, 1990). Perhaps further characterisation of suppressors of poly(A) binding protein mutations will throw light on the role of the poly(A) tail.

d) Secondary structure in 5' untranslated region: While most yeast mRNAs are devoid of strong secondary structure in their 5' untranslated regions, there have been several reports which have shown that the formation of secondary structure within the 5' untranslated region can inhibit translational initiation both in yeast (Bettany *et al.*, 1989; Baim and Sherman, 1988; Cigan *et al.*, 1988a) and in higher eukaryotes (Kozak, 1989b). Interestingly, yeast mRNA

translation appears to be more susceptible to inhibition by mRNA secondary structure than higher eukaryotes. For example, while in mammalian cells a hairpin of -30 kcal has no effect on the rate of translational initiation (Kozak, 1989a), a hairpin of -20.0 kcal inhibits translation completely in *S.cerevisiae* (Cigan *et al.*, 1987). Only when a secondary structure of -60 kcal is introduced is the translation of a mammalian transcript inhibited (Kozak, 1989a). As well as the strength of the hairpin, its position is also important. Experiments using a mammalian *in vitro* translation system have shown that a hairpin positioned 12 nt from the 5' CAP inhibited translation, but when it was positioned 52 nt from the CAP there was no inhibition of translation (Kozak, 1989b). Therefore, it appears that the 40S subunit and its associated factors are more efficient at denaturing secondary structure once they have bound to the mRNA. Recently it has been shown that secondary structure can increase the rate of translation of mammalian transcripts *in vitro* (Kozak, 1989c). In this case, a hairpin of modest secondary structure (dG = -19 kcal) was positioned after the AUG start codon of the preproinsulin mRNA. The AUG codon was in a sub-optimal context for initiation (Kozak, 1989c). It is believed that the secondary structure slowed ribosome scanning at the AUG, resulting in a greater frequency of translational initiation events (Kozak, 1989c). Further experiments have since shown that the position of the hairpin downstream is also important. The strongest facilitation was observed when the hairpin preceded the AUG by 14 nt (Kozak, 1990).

tRNAs and aminoacyl tRNA synthetases: Transfer RNAs are the "adaptor" molecules which transport the appropriate amino acid to the site of protein synthesis and provide the interface between the codon and the amino acid which it encodes. The initiator tRNA is always $tRNA_{met}$ which is formylated to distinguish it from the tRNA used to decode methionine codons translated during elongation. The initiator tRNA plays a pivotal role in translation initiation. It has been shown genetically in yeast to direct the scanning 40S subunit to the translational start site. When the anticodon of $tRNA_{met}$ was mutated from UAC to UCC, the mutant tRNA could direct scanning to initiate at an AGG codon (Cigan *et al.*, 1988b).

Specific amino acids are covalently linked to the appropriate tRNA molecules by aminoacyl tRNA synthetases. The recognition of amino acids by the synthetase is a precise process. Recent experiments have shown that for some tRNAs, sequences within the tRNA molecule allow it to be recognised only by the pertinent synthetase (Hou and Schimmel, 1988).

Interestingly, the level of any particular tRNA in yeast and *E.coli* correlates with the frequency of use of the codon for which the tRNA binds. Thus, preferred codons are generally translated with relatively abundant species of tRNAs, while non-preferred codons are translated by low abundant tRNAs. Strings of non-preferred codons could thus result in the pausing of the ribosome as it is translating. It has been suggested that these pauses are important in allowing the proper folding of the protein product (Purvis *et al.*, 1987b). Furthermore, highly expressed genes such as the glycolytic genes have a strong bias towards preferred codons, while poorly expressed genes contain a higher number of non-preferred codons.

Translation factors: A myriad of translation factors are known to be involved in eukaryotic translation. These factors are predominantly involved in initiation (Table 1.1). How these factors contribute to the process of translation initiation is summarised in Fig. 1.1 which demonstrates the scanning model for translational initiation. It is through these factors that modulation of translation initiation is achieved.

These factors have been characterised predominantly by biochemical methods. To date eIF-2 is the only factor for which a role in translation has been unambiguously, demonstrated genetically. Three yeast mutations (*sui1, sui2, SUI3*) were isolated on the basis that they each allowed translation at a UUG codon, in the absence of an AUG, in the yeast *HIS4* gene. *sui2* and *SUI3* have since been identified as the α and ß subunits of the eIF-2 respectively. Analysis of the *sui2* gene has uncovered the presence of a zinc finger in the ß-subunit, which may be involved in RNA binding. Mutations in the zinc finger of *SUI1* disrupt translation (Donahue *et al.*, 1988). Preliminary evidence suggests that the *sui1* gene encodes a component other

TABLE 1.1: Translational Initiation factors

Name	Function
eIF-1	Stimulates/stabilises 43S and 48S preinitiation complexes.
eIF-2	Mediates GTP-dependant met-tRNA binding to 40Ssubunits. The α-subunit is encoded by *sui2* and the β-subunit by *SUI3*
eIF-2B	GTP:GDP exchange on eIF-2.
eIF-3	Ribosome antiassociation activity, stabilises met-tRNA binding to 40S subunit.
eIF-4A	Required for mRNA binding to ribosomes and unfolds mRNA in an ATP driven manner.
eIF-4B	Unfolds mRNA in an ATP driven manner.
eIF-4C	Involved in ribosome dissociation and stabilises met-tRNA binding to 40S subunits.
eIF-4D	Enhances 80S initiation complex reactivity with puromycin.
eIF-4E	Cap recognition protein which cross links to cap.
eIF-4F	Multi-subunit complex of eIF-4A, eIF-4E, and p220, which is the cap binding complex.
eIF-5	Stimulates the junction of 48S preinitiation complex with 60S subunit.
eIF-6	Involved in dissociation by association with 60S subunit.

Data taken from Mueller and Traschel, 1990; Nygaard and Nilsson, 1990.

than eIF-2 that functions in establishing recognition by the pre-initiation complex of the AUG initiation codon (Yoon *et al.*, 1990).

A role for the initiation factor eIF-4E in the regulation of cell cycle in *S.cerevisiae* is apparent from the finding that it is the *CDC33* gene (Altmann and Traschel, 1989). This initiation factor is one of the three proteins which comprises the CAP binding complex. It is believed that eIF-4E is important in modulating mRNA translation during the cell cycle at G_0. Also, over-expression of eIF-4E in mammalian cell lines results in cellular transformation (Lazarius-Karatzus *et al.*, 1990) suggesting that eIF-4E is proto-oncogenic. Interestingly, the mouse eIF-4E can substitute for the yeast eIF-4E *in vivo* (Altmann *et al.*, 1989) thus showing the similarities between yeast and higher eukaryotes with respect to the translation process. However, mammalian eIF-4A cannot replace its yeast counterpart (Muller and Trashel, 1990). eIF-4A along with eIF-4B are important factors which melt secondary structure in the 5' untranslated region during scanning towards the initiation codon (Nygaard and Nilsson, 1990).

There are also three factors involved in translational elongation of *S.cerevisiae*. Elongation factor eEF1 and eEF2 are common to all eukaryotes. eEF1 is involved in the binding of the cognate aminoacyl-tRNA to the ribosome and requires the presence of GTP. eEF2 catalyses the translocation of the ribosome along the mRNA. The presence of a third elongation factor sets *S.cerevisiae* (eEF-3) apart from most other eukaryotes which only have two factors (Tuite, 1989). Interestingly, the factor is needed only for protein synthesis catalysed by yeast ribosomes. eEF1 and eEF2 from yeast are active with rat liver ribosomes even in the absence of eEF3, suggesting that the activity of eEF3 is provided by the ribosome itself in higher eukaryotes, (Nygaard and Nilsson, 1990).

How these translation factors interact and function should become clearer, now that a yeast *in vitro* translation system has been created (Tuite, 1989).

1.2.5.3 Regulation of translation initiation.

Several genes are regulated with regard to their expression in *S.cerevisiae* at the level of translation initiation, and exploration of these control mechanisms have helped in our understanding of the translation process itself. Translational regulation of *GCN4* and *CPA1* are described in detail as they represent the best characterised examples of such regulation in *S.cerevisiae*.

GCN4: The *GCN4* gene encodes a positive transcriptional *trans*-acting factor of amino acid biosynthetic genes in *S.cerevisiae* (Hinnebusch, 1990). Expression of *GCN4* is regulated at the level of translation, with translational derepression occurring under conditions of amino acid starvation (Hinnebusch, 1990).

The *GCN4* mRNA has a 5' untranslated leader sequence of almost 600 nt, which contains four small upstream open reading frames (uORFs). This configuration is pivotal to the rate at which the GCN4 mRNA is translated.

Translational derepression is dependent on the four uORFs and on the function of the GCD1, GCN1, GCN2, GCN3 *trans*-acting proteins. The way in which these factors interact to regulate *GCN4* translation is summarised in Fig. 1.2.

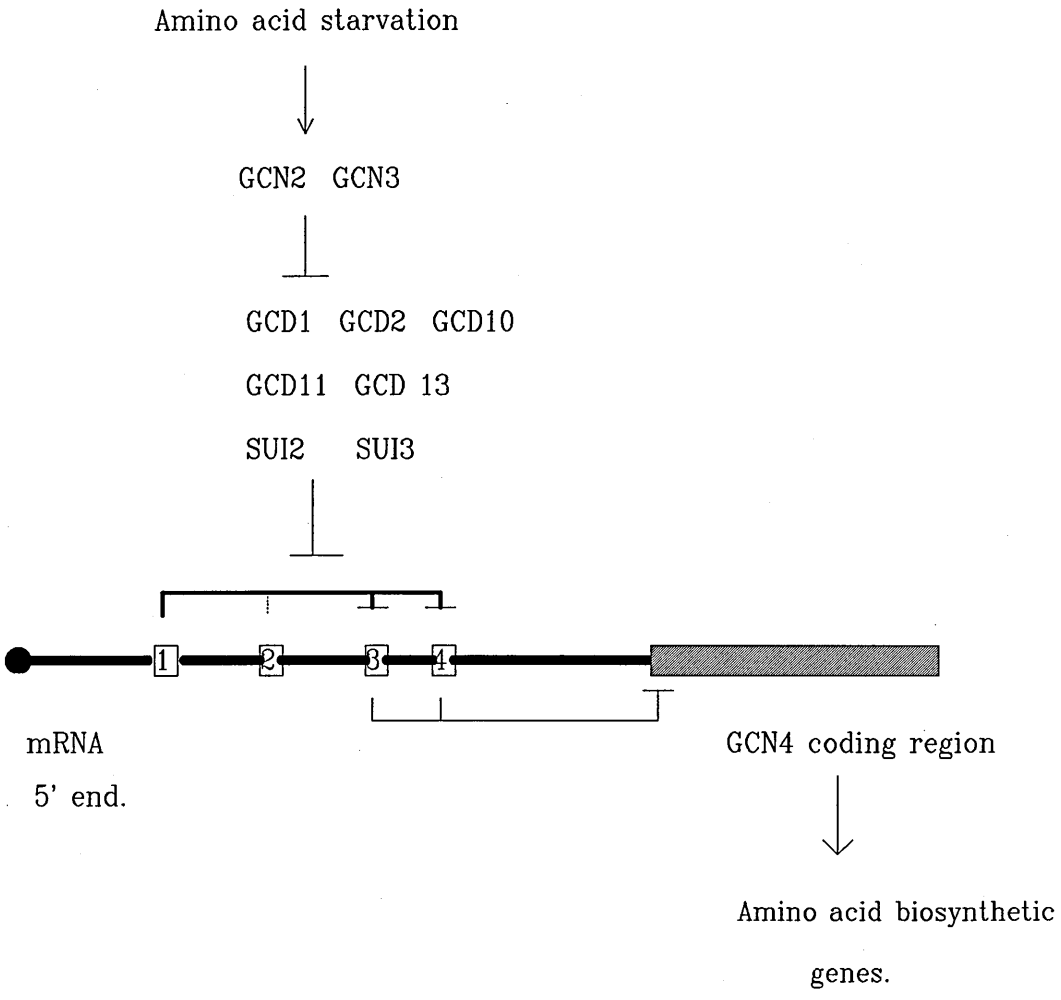
The importance of the 5' untranslated region in mediating the translational regulation is reflected by the fact that if the *GCN4* 5' untranslated leader is fused to a reporter gene, then that gene becomes translationally controlled in the same manner as *GCN4* (Mueller *et al.*, 1987). However, not all 4 of the uORFs nor all 600 nt are required for this regulation. Deletion analysis has shown that the presence of only uORF1 and uORF4 is required for regulation (Mueller *et al.*, 1988).

uORF1, and to a lesser extent uORF2, function as positive elements under conditions of amino acid starvation to allow ribosomes to by-pass uORF3 and uORF4 and to re-initiate translation at the structural open reading frame of *GCN4* (Mueller *et al.*, 1987). The uORF4 is a very efficient barrier to translation of the *GCN4* mRNA under conditions of translational repression (Mueller *et al.*, 1987). A plethora of site-directed mutants in the 5' untranslated region of *GCN4* have

Figure 1.2 Pathway of Regulatory Factors Involved in Translational control of *GCN4* expression.

Arrows: indicate stimulatory interactions.
Bars: depict inhibition or repression.
The four uORFs in the *GCN4* mRNA are shown as numbered boxes.
Under non-starvation conditions, uORFs 3 and 4 function to block translation of *GCN4*. Under starvation conditions the inhibitory effect of these sequences is reduced by uORFs 1 and 2.
Trans-acting factors act positively (GCN) and negatively (GCD and SUI) to regulate expression of GCN4.

Adapted from Hinnebusch (1990).


Amino acid starvation
GCN2 GCN3
GCD1 GCD2 GCD10
GCD11 GCD 13
SUI2 SUI3
1
2
3
4
mRNA
5' end.
GCN4 coding region
Amino acid biosynthetic
genes.

been tested in an attempt to unravel the mechanism of regulation (Hinnebusch, 1990).

These studies have shown that the nt sequences of the uORF1 or uORF4 can be changed without an effect on the translational regulation (Williams *et al.*, 1988). Also, when uORF1 or uORF4 are fused to a reporter gene, both are equally efficient at initiating translation (Hinnebusch, 1990). This implies that the differences in the function of uORF1 and uORF4 do not lie in differences in their ability to initiate translation, or in their translation products.

However, point mutations of uORF1 which lengthen or shorten the open reading frame significantly lower the level of the GCN4 protein under amino acid starvation, apparently disrupting the positive effect of uORF1 (Hinnebusch, 1990). Moreover, when 10 nt from the 3' end of uORF 4 were placed in the 3' end of uORF1 then the derepression was again abolished. This has led to the hypothesis that re-initiation at the major AUG is more probable when ribosomes have translated uORF1 than when they translate through uORF4. Under conditions of amino acid starvation it is presumed that ribosomes translate through uORF1, but not uORF4, and then re-initiate at the major AUG. Obviously this hypothesis awaits verification, but the mechanism by which it occurs has been made clearer through studies of the *trans*-acting factors involved.

GCN2 and *GCN3* encode positive factors, mutations in which result in high levels of unregulated *GCN4* expression (Hannig and Hinnebusch, 1988). Interestingly, *GCN2* itself is regulated transcriptionally by GCN4 (Tzamarias and Thireos, 1988). In contrast, mutations in *GCD1* and *GCD2* cause high levels of unregulated *GCN4* expression (Hinnebusch, 1990). The *gcd* mutants overcome the low *GCN4* expression observed in *gcn2* and *gcn3* mutants, implying that GCN2 and GCN3 proteins act to repress the translation of *GCN4* when amino acids are present. Studies with various uORF mutants have shown that the *GCD* genes possibly mediate their effects on uORF1 by preventing ribosomes from reinitiating at the coding region of *GCN4* (Hinnebusch, 1990). Studies with a *gcd1* mutant reveals that binding of $tRNA_{met}$ to the small subunit is impaired suggesting that GCD1 has a general

function in translation initiation, (Hinnebusch, 1990). It appears therefore, that under starvation conditions, the repression of *GCD1* and *2* by *GCN2* and *3* results in a reduction in activity of an initiation factor (Hinnebusch, 1990). This then perhaps allows ribosomes emerging from uORF1 to pass through uORF4 and initiate at the *GCN4* start codon.

Possible modes of activation of the GCN2 protein have been proposed following the sequencing of the gene. It was found to contain some homology to protein kinases as well as a 520 amino acid domain which is homologous to the $tRNA_{his}$ aminoacyl synthetase (Wek *et al.*, 1990). GCN2 has since been shown to act as a protein kinase *in vitro* (Hinnebusch, 1990). Point mutations in this part of the gene result in loss of activity. As amino acid tRNA synthetases bind uncharged tRNAs, the uncharged tRNAs which accumulate under conditions of amino acid starvation, could in theory bind to the region homologous to $tRNA_{his}$ aminoacyl synthetase and thence activate the GCN2 protein kinase (Wek *et al.*, 1989). GCN2 could then act on the GCD proteins which regulate translation reinitiation after uORF1. A summary of the steps involved in this complex regulation is presented in Fig 1.2.

CPA1: Translational control of the *CPA1* gene is also mediated by the presence of an uORF in the 5' leader (Hinnebusch, 1988). *CPA1* encodes the glutaminase subunit of the arginine pathway carbomyl phosphate synthetase, and its expression is repressed translationally by arginine: in the presence of arginine, protein levels drop 5-fold but there is no change in the mRNA levels (Messenguy *et al.*, 1983). Disruption of the 25 amino acid uORF results in abolishment of the translational control. Moreover, missense mutations in the uORF disrupt function, implying a role for the polypeptide encoded by the uORF in the regulation (Werner *et al.*, 1987). It is believed that under conditions of arginine repression the leader peptide inhibits the scanning of ribosomes further than the uORF thereby reducing translation at the major ORF. A complex formed between the leader peptide and the product of the *CPAR* gene is thought to prevent further scanning. The *CPAR* gene is switched on in the presence of arginine, but off in its absence. In the absence of arginine, ribosomes

are able to translate the uORF and re-initiate at the major initiation codon for the *CPA1* gene (Werner *et al.*, 1982).

While *CPA1* and *GCN4* are the best studied examples of translational control, there are several other examples. The levels of catalase are known to be translationally controlled, with translation of catalase mRNA only occuring in the presence of haem (Hamilton *et al.*, 1982). Recent studies have also shown that the *RAS2* gene is partially regulated at the level of translation (Brevario *et al.*, 1988). Also, data from this thesis demonstrates that over-expression of the *PYK1* gene is limited at the level of translation (Moore *et al.*, 1990a; Moore *et al.*, 1990c).

Several mitochondrial mRNAs in yeast are dependent on nuclear gene products for their specific translation, and have been identified as *PET* mutants. One of these nuclear factors, *PET 494* which is required for the translation of the *COXIII* mRNA, has itself been shown to be regulated at the translation level in response to oxygen (Marykwas and Fox, 1989). Furthermore, *PET111* which is needed for the translation of the *COXII* gene, has a 5' untranslated leader which is long and contains AUG codons upstream of the major AUG initiation codon suggesting the potential for translational regulation (Strick and Fox, 1987). It has been proposed that *S.cerevisiae* uses translational control to regulate expression of mitochondrial genes in response to carbon source (Fox, 1986).

Translational regulation may be a common feature of organellular genetic systems. Induction of the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase by light is mediated by translational activation in both *Euglena* and *Armanath* (Fox, 1986).

Many mRNAs have been shown to be regulated translationally in other systems. The bacteriophage T4 regulates the translation of *gene32* and *regA* mRNAs by autoregulation (Stormo, 1987). *E.coli* itself regulates transcriptional termination (Wesley-Hatfield and Sharp, 1987), amino-acyl tRNA synthetase expression (Springer and Grunberg-Manago, 1987), and ribosomal protein synthesis (Draper, 1987) at the level of translation. The expression of ribosomal proteins are also known to be translationally controlled during development in *Drosophila* and *Xenopus* (Jacobs-Lorena and Fried, 1987). In mammalian systems, the best

defined example of translational control involves the regulation of ferritin mRNA translation by ferrous ions (Munro *et al.*, 1985). The ferritin mRNAs are translationally repressed in the absence of iron by the binding of a protein to a specific and conserved stem-loop structure in the 5' untranslated leader (Aziz and Munro, 1986). This repression is released in the presence of iron.

As well as translational regulation of specific mRNAs, it is well documented that general translational rates are subject to translational control. For example during the development of sea-urchins and *Xenopus*, certain mRNAs are translationally repressed in mRNP complexes in oocytes, eggs, and early embryos (Rosenthal and Wilt, 1987; Richter, 1987). Dramatic changes in protein synthesis occur during oogenesis and fertilisation, with mRNAs previously translationally repressed now available to be translated.

Also upon infection, poliovirus uses its unique translational mechanism to overcome its host's translational machinery (Sonnenberg, 1988). Poliovirus protease 2A indirectly causes the proteolysis of the large subunit (p22) of eIF-4F. This results in inactivation of CAP-dependent mRNA translation thus rendering cellular mRNAs unavailable for translation as they depend on the CAP-binding protein (Sonnenberg, 1988). As poliovirus mRNA is translated in a CAP-independent manner its translation continues.

Heat shock mRNAs are translationally activated upon heat shock in yeast (Tuite, 1989) and in *Drosophila* (Lindquist, 1987). This demonstrates that the translational machinery can adapt to preferentially translate groups of mRNAs in response to differing environmental conditions. In *S.cerevisiae* it has also been reported that there is a differential translational of glucose-repressed and glucose-derepressed mRNAs in a rabbit *in vitro* translation system. If true, this infers that there is a translational control mechanism involved in the regulation of gene expression on different carbon sources (Parents-Soler *et al.*, 1987).

1.2.6 POST-TRANSLATIONAL CONTROL.

Even once an mRNA has been translated into protein, there is still the potential for post-translational regulation of gene expression. For example, yeast ribosomal proteins are known to be regulated at the level of protein stability when not incorporated into the ribosome. These proteins are naturally unstable having a half-life of 2-3 mins and this is believed to be important to avoid the accumulation in the nucleus of proteins with an affinity for RNA (Warner, 1989). The stability of certain ribosomal proteins has also been shown to be decreased further upon increasing the level of its corresponding mRNA (Warner, 1989).

The stability of four yeast ribosomal proteins has recently been shown to be increased through a transient association with ubiquitin (Finley *et al.*, 1989). These ribosomal proteins are transcribed and translated together with ubiquitin in four separate cistrons (Finley *et al.*, 1989). The association has been proposed to protect the ribosomal proteins from degradation, allowing an increased rate of transport of the ribosomal proteins to the nucleus (Finley *et al.*, 1989). Conversely, it is possible that the association with ubiquitin may increase the assembly of these particular proteins into the nascent ribosome. The reason why these four ribosomal proteins are protected against degradation should become clearer when their function is deduced.

Finally, in the case of metabolic enzymes, it is well documented that these proteins are under biochemical control. Common modes of biochemical regulation include post-translational modification of proteins (eg. by covalent addition of phosphates) and allosteric regulation. These are very important mechanisms of dictating an enzyme's metabolic activity *in vivo*. However, it is outside the scope of this thesis to discuss these modes of control.

1.3 REGULATION OF GLYCOLYTIC GENE EXPRESSION.

1.3.1 CARBOHYDRATE METABOLISM IN *S.CEREVISIAE.*

When *S.cerevisiae* is grown on a fermentative carbon source such as glucose, fermentative metabolism dominates. This involves the breakdown of the sugar by the glycolytic pathway to ethanol with the concomitant production of two ATP molecules (Fig. 1.3). When the fermentative carbon source becomes exhausted, or when *S.cerevisiae* is grown on a non-fermentable carbon source, respirative metabolism predominates (Wills, 1990; Fig. 1.3). This is achieved by the derepression of TCA cycle enzymes and the mitochondrial respiration machinery. Thus, the concentration of glucose is important in repressing the TCA cycle. This phenomenon is known as catabolite repression.

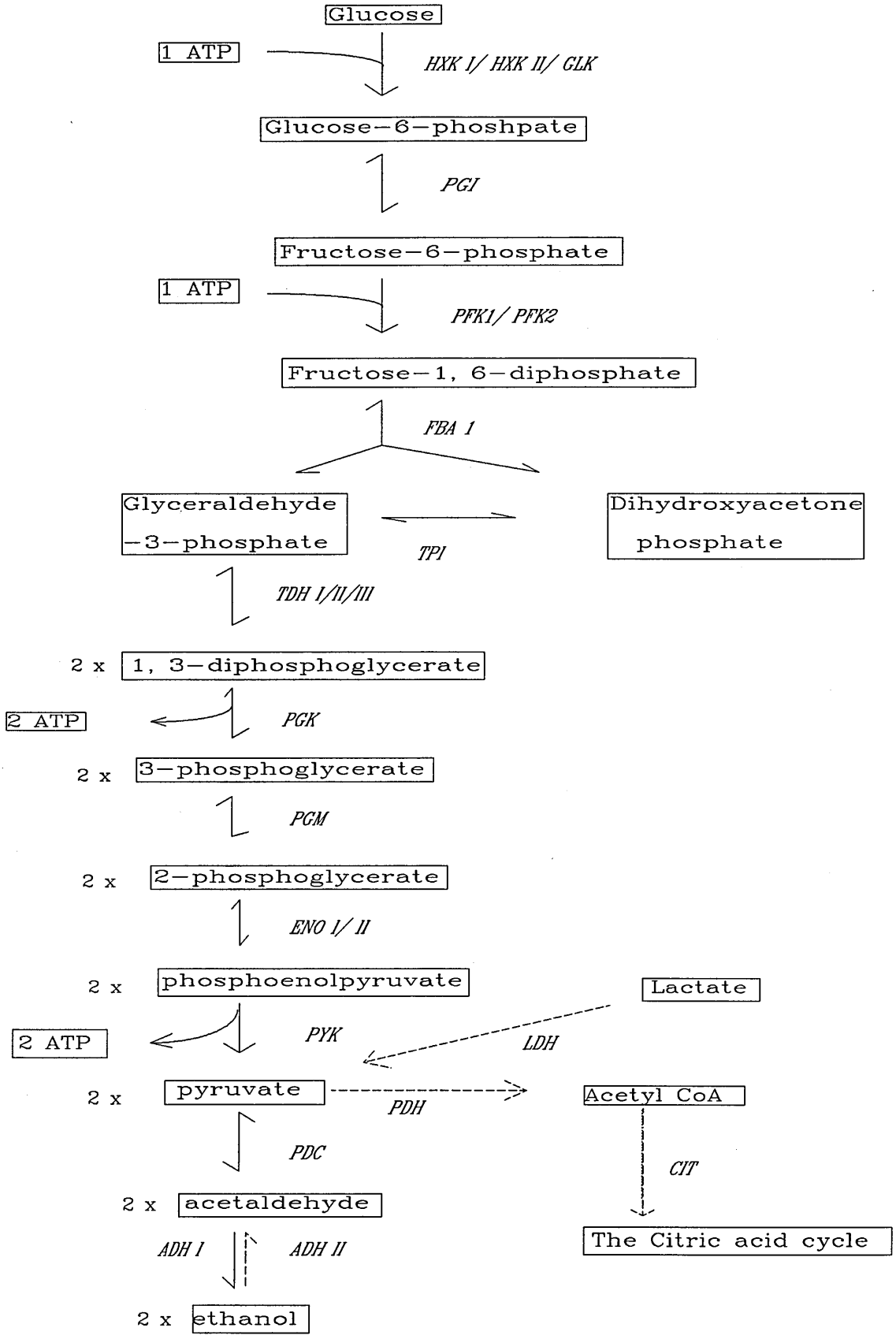
It is postulated that the cell responds to changes in glucose concentration through changes in the concentration of cAMP (Wills, 1990). Addition of glucose or a related fermentable sugar to *Saccharomyces cerevisiae* grown on non-fermentable carbon sources induces a transient increase in cAMP levels which lasts for 1-2 mins (Mbonyi *et al.*, 1988; Wills, 1990). This brief pulse is sufficient to begin a cascade effect leading to a number of protein phosphorylations, which in turn result in activation or inactivation of enzymes of several different metabolic pathways, including the inactivation of gluconeogenesis and the stimulation of glycolysis (Mbonyi *et al.*, 1988). The mechanism by which glucose triggers the cAMP response is mediated by the RAS signal transduction proteins and the CDC 25 protein (Mbonyi et al., 1988; van Aelst, L. *et al.*, 1991).

Interestingly, it appears that the messenger function carried out by cAMP can also be carried out by alternative messengers as cells which are deficient in cAMP can still respond to glucose activation (Wills, 1990).

The levels of the enzymes involved in carbohydrate metabolism are also known to be under genetic control, with the expression of TCA cycle genes and mitochondrial genes repressed in the presence of glucose (Wills, 1990). There is debate as to whether genes involved in

Figure 1.3 The Yeast Glycolytic Pathway.

Yeast ferments glucose to ethanol through the glycolytic pathway. The intermediate metabolites (boxed) and steps (complete arrows) involved in this pathway are presented. Also shown are the ATP requiring and ATP generating steps. The genes encoding the enzymes which catalyse each step are shown in italics (see list of abbreviations for complete names). The irreversible steps of this pathway are catalysed by pyruvate kinase and phosphofructokinase (one-ended arrows). During respirative growth (eg. during growth on ethanol or lactate), pyruvate is not converted to ethanol but is diverted to the TCA cycle via acetyl CoA (broken line).


Glucose
1 ATP
HXK I/ HXK II/ GLK
Glucose−6−phoshpate
PGI
Fructose−6−phosphate
1 ATP
PFK1/ PFK2
Fructose−1, 6−diphosphate
FBA 1
Glyceraldehyde −3−phosphate
TPI
Dihydroxyacetone phosphate
TDH I/II/III
2 x 1, 3−diphosphoglycerate
2 ATP
PGK
2 x 3−phosphoglycerate
PGM
2 x 2−phosphoglycerate
ENO I/ II
2 x phosphoenolpyruvate
Lactate
2 ATP
PYK
LDH
2 x pyruvate
PDH
Acetyl CoA
PDC
CIT
2 x acetaldehyde
The Citric acid cycle
ADH I
ADH II
2 x ethanol

glycolysis are transcriptionally activated in the presence of glucose. This question is addressed in Chapter 3 of this thesis.

1.3.2 GLYCOLYTIC GENE EXPRESSION IN *S.CEREVISIAE.*

The isolation of glycolytic mutants which grow on non-fermentative carbon sources but are unable to do so on glucose has facilitated the cloning of glycolytic genes (Kawasaki and Fraenkel, 1981). All the glycolytic genes have now been cloned by functional complementation and sequenced (Holland and Holland, 1979; Holland *et al.*, 1981; Alber and Kawasaki., 1982; Bennetzen and Hall, 1982; Hitzeman *et al.*, 1982; Watson *et al.*, 1982; Burke *et al.*, 1983; Kopetzki *et al.*, 1985; Kellerman *et al.*, 1986; Stachelek *et al.*, 1986; Tekamp-Olson *et al.*, 1988; White and Fothergill-Gilmore, 1988; Heinisch *et al.*, 1989; Schwelberger *et al.*, 1989). Deletion analysis of the promoters of many of these genes has identified the *cis*-acting sequences both in the 5' untranslated region and coding region which direct transcription.

It has been shown that the expression of some glycolytic genes can be regulated in response to heat shock, entry into stationary phase, and to changes in the carbon source (Brindle *et al.*, 1990). There is debate as to whether all glycolytic genes are regulated at the level of transcription in response to carbon source. This question is addressed in Chapter 3 of this thesis, where I discuss the results generated from this study in the context of those in the literature. Therefore, the aspect of carbon source regulation of glycolytic gene expression is not dealt with here.

Co-ordinate transcriptional regulation of glycolytic enzyme genes was initially purported by the isolation of the *gcr1* mutation, which results in a reduction in glycolytic enzyme levels to 1-10% of those found in wild-type cells grown under gluconeogenic conditions (Clifton and Fraenkel, 1981). This mutation was subsequently shown to reduce the mRNA levels of all three *TDH* genes and *ENO1* and *ENO2* genes, and has been proposed to be a transcriptional activator of glycolytic genes (Holland *et al.*, 1987). The *GCR1* gene has been cloned and sequenced (Baker, 1986) and recently a second mutation *gcr2*, which displays a similar phenotype to *gcr1* has been isolated (Uemura and Fraenkel,

1990). The importance of *GCR1* in respect of carbon source regulation is discussed in more detail in chapter 3.

A second *trans*-acting protein which appears to be involved in the co-ordinated regulation of glycolytic genes is encoded by the *RAP1* gene. This protein (believed to be identical to TUF and GRF proteins; Buchmann *et al.*, 1988) binds a consensus sequence on DNA known as the RPG box. The RAP1 protein binds specifically upstream of several glycolytic genes thereby activating their transcription, presumably in conjunction with other DNA binding proteins (Buchmann *et al.* ,1988). Deletion of the RPG box has been shown to result in a twenty to thirty fold decrease in the level of *ADH1* transcription (Tornow and Santangelo, 1990). Thus its importance in transcriptional activation of glycolytic genes is clear. Interestingly, the RPG box is found upstream of many other genes: it was first identified upstream of genes encoding proteins involved in the translational apparatus (Huet and Sentenac, 1987) and also binds to telomeres (Conrad *et al.*, 1990) and to the silencer region of the silent mating type locus (Buchman *et al.*, 1988). Sequences flanking the RPG box determine whether the RAP1 protein acts as a transcriptional activator, (as in the case with glycolytic genes) or as a silencer (as in the case of the *HMRE* gene; Buchman *et al.*, 1988).

Recently, it has been shown that the *GCR1* product acts through the RPG box present in the 5' untranslated region of *ADH1* (Santangelo and Tornow, 1990). When constructs were made which placed *ADH1* coding sequence under the transcriptional control of the RPG box, activation from the RPG box was shown to be dependant on functional GCR1 (Santangelo and Tornow, 1990). Transcription was reduced in a *gcr1* mutant background. Similar results were shown for *TEF1*, *TEF2*, and *RP59* (Santangelo and Tornow, 1990). However, in the case of *ENO2*, the site important for the *GCR1* mediated effect has been pin-pointed to a site bound either by the ABF1 or RAP1 protein (Holland *et al.*, 1990). The involvement of ABF1 in glycolytic gene regulation is discussed further below.

No studies have shown the GCR1 protein itself binding to the activator or operator elements of glycolytic genes. However, it has been shown that the chromatin structure within the 5' untranslated region

of *TDH3* is dependent on the *GCR1* gene (Pavlovic and Horz, 1988): in *GCR1* strains, the 5' untranslated region is free of nucleosomes, while in a *gcr1* strain, two phased nucleosomes were observed in the region. Therefore, it appears that the *GCR1* gene product plays an important role in modulating the chromatin structure of yeast genes. Perhaps, *GCR1* mediates its affect on the transcription of genes through DNA binding factors such as RAP1 and ABF1.

One of the most intensively studied glycolytic genes is *PGK1*, which has been shown to contain *cis*-acting sequences which are shared by several of the other glycolytic genes. The UAS of *PGK1* has been mapped to -538 and -402 nt upstream of the initiation codon (Chambers *et al.*, 1989). This UAS contains three regions important in directing transcription, including an activator core region which contains the RPG box (Chambers *et al.*, 1989). It also has three copies of the pentamer sequence CTTCC, known as the "CT" block (Stanway *et al.*, 1989). The "CT" block has also been identified in other glycolytic genes and deletion of one from the *PGK1* UAS results in a 50-75% drop in transcription (Stanway *et al.*, 1989). However CT blocks have not been shown to bind proteins *in vitro*. The third region of the *PGK1* UAS known to be important in transcription does bind proteins strongly *in vitro*. Recent studies have identified this protein as being the ARS Binding Factor ABF1 (Chambers *et al.*, 1990). This factor, like RAP1, has functions other than transcriptional activation. It also binds close to the ARS consensus sequence at *ARS1* (located near to the *TRP1* gene) and to other ARS regions including the origin of replication on the 2 micron plasmid ARS (Halter *et al.*, 1989). It has not been determined whether ABF1 activates transcription of *PGK1*. However, it has been shown to act as a weak transcriptional activator, in conjunction with other proteins, of non-glycolytic genes, (Buchman and Kornberg, 1990). Thus, perhaps RAP1 and ABF1 act synergistically with other *trans*-acting factors (eg GCR1) to activate the transcription of many genes including the glycolytic genes. Consensus sequences for the binding of ABF1 have been identified in the 5' untranslated regions of several other glycolytic genes (Chambers *et al.*, 1989). It has been shown to bind to *ENO2*, but not *ENO1* (Brindle *et al.*, 1990). Also, the *PYK1* gene has been shown to bind ABF1 *in vitro*, (Chambers *et al.*,

1990). *PYK1* appears in many respects to be activated in a similar manner to *PGK1* and this is discussed further below.

To summarise this section, it appears that while several *trans*-acting factors have been identified that control the expression of the glycolytic genes, the mechanism by which they interact is unclear. It is also unclear whether all of the glycolytic genes are regulated in the same manner; while some genes share common sequences in their 5' promoters, others do not. Obviously much remains to be unravelled.

1.3.3 PYRUVATE KINASE.

Pyruvate kinase catalyses the conversion of phosphoenolpyruvate to pyruvate with the concomitant conversion of ADP to ATP and requires K^+ and Mg^{2+} for activity (Murcott *et al.*, 1991). It is the final net energy producing reaction of glycolysis. The reaction is essentially irreversible *in vivo* and has been postulated to be a control point. The enzyme is controlled allosterically in yeast and lower organisms; it is positively regulated by fructose-1,6-diphosphate, and negatively regulated by ATP and citrate (Yoshima and Matsumoto, 1972). Structurally, the enzyme is a homotetramer, with a subunit of between 55,000 and 60,000 (Murcott *et al.*, 1991).

The *PYK1* gene has been isolated and sequenced from several organisms. Pyruvate kinase synthesis has been well characterised in mammalian cells, where it has been demonstrated that there are four isozymes (Vaulont *et al.*, 1986). Expression of each isozyme is tissue specific with M1 being the major form found in muscle, heart, and brain; M2 being present in foetal and in most adult tissue; L being the major form in the liver and R being the only form found in erythroid cells (Vaulont *et al.*, 1986).

The L and R isozymes are made from the same cistron but are generated by the use of a different promoters. M1 and M2 are generated by alternative splicing from one gene (Vaulont *et al.*, 1986).

The best characterised of these four gene forms is the L-form. Transcription of this gene is under the control of four proteins, all of which are known transcriptional activators (Vaulont *et al.*, 1989). These four proteins are the hepatocyte nuclear factor (binds between -95 and -66nt with respect to the initiation codon), ubiquitous nuclear factor

(binds between -114 and -97nt), liver factor A1 (binds between -144 and -126nt), and major late transcription factor (binds between -168 and -145nt) (Vaulont *et al.*, 1989). There also appears to be an element downstream of the initiation codon which is important in the regulation of transcription in liver (Tremp *et al.*, 1986). Regulation of transcription is under positive control mediated by carbohydrate level, and negative control mediated by glucagon (Vaulont *et al.*, 1986). The stability of L-type *PYK1* mRNA is also affected by changing nutrient environments. While the mRNA is stable when hepatocytes are treated with carbohydrate, the mRNA is unstable in glucagon treated hepatocytes (Vaulont *et al.*, 1986). Therefore the mammalian L-type *PYK1* is under both transcriptional and post-transcriptional control.

The yeast pyruvate kinase gene (*PYK1*) has been cloned and sequenced (Burke *et al.*, 1983). This was achieved by functional complementation of a *pyk1* mutant. *pyk1* mutants are the most commonly isolated glycolytic mutation on the basis of growth on non-fermentable carbon sources but not fermentable ones (Maitra and Lobo, 1977). This illustrates that there is no alternative pathway to glycolysis when the *PYK1* step is blocked.

Sequencing of the yeast *PYK1* gene has shown that the gene is homologous to the coding region of other sequenced *PYK1* genes from other organisms (Burke *et al.*, 1983). At the protein level it is highly homologous at the ATP binding domain to the bovine muscle PYK. In a 34 stretch of amino acids, 20 are identical, and only three show non-conserved changes.

Genetic regulation of yeast *PYK1* has been examined. Like other glycolytic genes, *PYK1* shows a reduced level in a *gcr1* background (Clifton and Fraenkel, 1981). Recently Nishizawa and co-workers (1989) mapped elements within the 5' untranslated region which regulate transcription of a reporter gene (*xylE*). They identified two UAS sequences, a URS sequence, and the functional TATA box. UAS1 resides 634 to 653 nt upstream of the initiation codon. This *cis*-acting sequence is needed for basal to intermediate levels for full activation. UAS1 has been mapped to a 20nt sequence which contains homology to the RPG box. It is known that the RAP1 protein will bind to *PYK1*, and activate its transcription *in vitro* (Buchman *et al.*, 1988). Recently, it has been

demonstrated that the transcriptional activation by RAP1 is mediated by the GAL11 protein (Nishizawa *et al.*, 1990). The GAL11 protein is important for the regulation of many genes and appears to be a general mediator between the RAP1 protein and the general transcriptional machinery. Interestingly when the distance between the RPG box and the TATA box is reduced transcriptional activation is observed in the absence of GAL11 protein (Nishizawa *et al.*, 1990). This infers that GAL11 transduces signals from elements upstream to the transcriptional initiation complex located close to the RNA initiation site.

There are other sequences close to the UAS1 which may be important in controlling transcriptional activation. There is a CT block and an ABF1 site analogous to sequences found in the *PGK1* promoter (Chambers *et al.*, 1990). While it is not known whether these sequences are involved in controlling *PYK1* expression, it has been shown that ABF1 will bind the *PYK1* 5' untranslated region *in vitro* (Chambers *et al.*, 1990). Also, the importance of the CT block has been shown *in vitro*: *in vitro* transcription from a DNA template containing the *PYK1* RPG box fused to the *lacZ* gene, was diminished 20-fold when the CT block was mutated (Buchman *et al.*, 1988).

UAS2 lies between 714 and 811 nt upstream from the initiation codon; its deletion reduces the level of *PYK1* mRNA though not as much as the deletion of UAS1 (Nishizawa *et al.*, 1989). Therefore it has been proposed that UAS2 functions to generate full activation. It contains direct and inverted repeat sequences which may prove to be sites for protein binding (Nishizawa *et al.*, 1989).

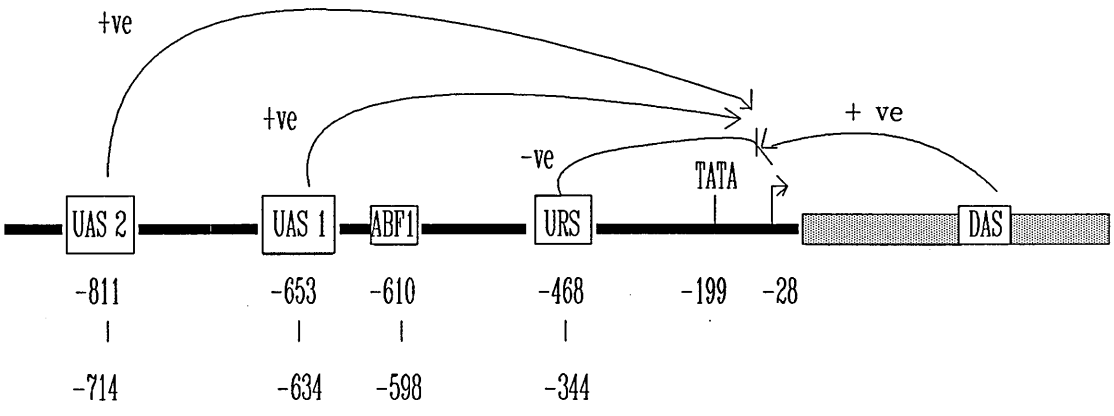
The URS sequence has been mapped between 344 and 468 nt upstream from the initiation codon (Nishizawa *et al.*, 1989). It is involved in the repression of transcription during growth on a non-fermentable carbon source. The sequence also acts to repress transcription on fermentable carbon sources but to a lesser extent (Nishizawa *et al.*, 1989).

Finally sequences within the coding region of *PYK1* itself are important in activating transcription (Purvis *et al.*, 1987a). This region has been termed DAS (Downstream Activation Site). When the *lacZ* gene is placed under the control of the *PYK1* promoter the abundance of the mRNA is 15-fold less than the wild type *PYK1* mRNA level. Part of this

Figure 1.4 ***cis*-acting Sequences Involved in the Regulation of *PYK1* transcription in *S.cerevisiae*.**

UAS 1, UAS 2, URS: mapped by Nishizawa and co-workers (1989). UAS1 includes the rpg box and the "CT" block.
DAS: mapped by Lithgow and co-workers (1990).
ABF1: identified by Chambers and co-workers (1990).
TATA: identified by Nishizawa and co-workers (1989).
Transcript start site maps at -28 (arrow).

The position of each element is numbered relative to the first nucleotide of the coding region.



PYK1 5'-promoter

PYK1 Coding region.

difference can be explained by a change in stability (2-fold) though it is partly (8-fold) due to reduced transcription (Purvis *et al.*, 1987a). The element which activates transcription from within the coding region has recently been mapped (Lithgow *et al.*, 1990). A similar activating sequence resides within the coding region of the *PGK1* gene (Mellor *et al.*, 1987). Further studies on this element were conducted in this study. Fig. 1.4 summarises the *cis*-acting sites on the *PYK1* gene which have been discussed here.

1.4. OUTLINE OF THESIS.

The central theme to the work conducted in this study is glycolytic gene expression. This has predominantly involved analysis of *PYK1* over-expression in yeast and its consequence on yeast cell growth. Interestingly, it was found that *PYK1* is subject to an unusual mode of translational control, and it was decided to extend analysis of this mechanism. In addition, the regulation of all the glycolytic genes in response to carbon source has been studied.

The results generated are presented in four chapters, each of which contains a brief introduction, followed by the results and discussion.

Chapter 3 describes the experiments conducted to determine the steady state levels of all the glycolytic mRNAs on both a fermentable and a non-fermentable carbon source. Furthermore, the regulation of the *PYK1* gene in response to carbon source was studied in detail.

The deleterious growth effects observed when *PYK1* is over-expressed in yeast, and the studies performed to characterise the strong selective pressure against *PYK1* over-expression are documented in Chapter 4.

Chapter 5 deals with the *PYK1* gene dosage limitation observed when *PYK1* is over-expressed. This occurs both at the level of mRNA abundance and at the translational level. Chapter 6 continues to dissect the sequences which are involved in mediating the translational regulation of *PYK1*. The conclusions drawn from each of these chapters are collated and discussed in Chapter 7.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS.

2.1.1 Suppliers of laboratory reagents.

2.1.1.1 Enzymes.

DNA Restriction and modification enzymes were obtained from BRL, Amersham and IBI. Proteinase K, lysozyme, β-glucouronidase and rabbit muscle pyruvate kinase were obtained from Sigma. Zymolyase was obtained from Miles laboratories.

2.1.1.2 Chemicals and Membranes.

Radiochemicals were obtained from Amersham. General laboratory chemicals were obtained from Sigma, BDH Chemicals, Koch-Light, Pharmacia, Bio-Rad laboratories, May and Baker and Aldrich.

Nitrocellulose and nylon membranes were obtained from Amersham. X-Ray film was obtained from Kodak.

PEG (3350 M.Wt.), Sephadex G-50 and hydroxyapatite, were obtained from Sigma.

Gene clean kits were obtained from Stratech Scientific.

2.1.1.3 Growth Media and Additives.

All media ingredients were obtained from Difco. Amino acid, bases and antibiotics were obtained from Sigma. X-Gal (5-Bromo-4-chloro-3-indolyl-β-galactoside) and IPTG (Isopropylthio-B-galactoside) were obtained from BRL.

2.1.2 STRAINS.

2.1.2.1 *E.coli.*

C1400: Ed8767, *supE*, *supF*, *hsd5*, *met-*, *recA*.

This strain of *E.coli* was used throughout as a recipient for plasmids, as it was found to have a high transformation frequency.

2.1.2.2 *S.cerevisiae.*

DBY746: a, *his3, ura3-53, leu2-3, leu2-112, trp1-289.*

X4003-5B : *a, ade1, gal1, his4, leu2, met2, trp5, ura3.*

DBY746 and *X4003-5B* were used throughout.

2.1.3 GROWTH MEDIA.

All growth media were sterilised for 15 mins at 120°C, and % refers to w/v except where stated. Antibiotics and amino acids were sterilised by passage through a 0.45µm Minisart disposable filter, and were added, after media had been autoclaved.

2.1.3.1 *E.coli.*

LB: 1% Bactotryptone, 0.5% Bacto-yeast extract, 1% NaCl pH to 7.5 with NaOH.

L agar: As LB with addition of 1.5% Bactoagar.

Ampicillin was added to a final concentration of 100ug/ml, when plasmid-containing *E.coli* were being propagated.

2.1.3.2 *S.cerevisiae.*

Minimal media: This was used primarily when growing plasmid containing yeast, in order to select for plasmid containing cells.

GYNB: 2% Glucose, 0.65% Yeast Nitrogen base .

Amino acids and bases to satisfy auxotrophic markers of particular yeast strains were added to a final concentration of 0.05mg/ml.

GYNB agar: As GYNB with addition of 2% Bactoagar.

LYNB: 3% v/v lactic acid, 0.65% Yeast Nitrogen base.

pH to 7.0 with NaOH.

LYNB agar: As LYNB with addition of 2% Bactoagar.

ß-Galactosidase assay plates: 0.1M KH_2PO_4, 0.015M $(NH_4)_2SO_4$ 0.075M KOH, 0.8mM $MgSO_4$, 2µm $Fe_2(SO_4)_3$, 2% glucose, 40µg/ml thiamine, 40µg/ml pyridoxine, 40µg/ml pantothenic acid, 200µg/ml inositol, 2µg/ml biotin, 2% agar and X-gal to a final concentration of 40µg/ml.

Rich media: this was used for the propagation of untransformed yeast.

YPG: 2% Glucose, 1% Yeast extract, 2% Bactopeptone.

YPG agar: As YPG with addition of 2% Bactoagar.

YPL: 3% v/v Lactic acid, 1% Yeast extract,
2% Bactopeptone. pH to 7.0 with NaOH.

YPL agar: As YPL with addition of 2% Bactoagar.

2.2 METHODS.

2.2.1 PROPAGATION OF ORGANISMS.

2.2.1.1 *E.coli.*

Storage of strains: *E.coli* C1400 plasmid-containing strains were stored by firstly growing the culture in 2.5ml of LB containing antibiotic at 37°C with shaking overnight. 1.5ml of the culture was then added to 300µl of 100% (v/v) glycerol, mixed, and stored at -70°C indefinently.

Growth of strains: An inoculum from the stored culture was taken and streaked onto L agar (containing ampicillin if necessary) and incubated at 37°C overnight. These plates could be stored at 4°C for short periods of time. Liquid cultures were obtained by inoculating 5ml of selective LB with a single colony from a storage plate and shaking for at least 8 hours, at 37°C. Often 1ml of this culture was added to 100-400ml of prewarmed, selective LB and shaken overnight at 37°C.

2.2.1.2 *S.cerevisiae.*

Storage: A stationary culture of the yeast strain to be stored was routinely prepared by growing in 5ml of YPG with vigorous shaking (250 rpm), at 30°C for 24-36 hours. The strain was then stored in the same way as described for *E.coli* cultures above.

Growth of strains: Auxotrophic yeast strains were grown at 30°C with vigorous shaking (250 rpm). To obtain a mid-logarithmic culture, a small volume (usually 20-100µl) of a stationary rich or minimal culture was added to the appropriate volume of the required medium (prewarmed to 30°C) and grown at 30°C for 12-16 hours with shaking

(250 rpm). Growth rate was determined by measuring the optical density (at 600nm) of 1ml samples taken every hour during logarithmic growth phase.

2.2.2 TRANSFORMATIONS.

2.2.2.1 *E.coli.*

Transformation of *E.coli* was carried out using a variation of the method described by Mandel and Higa (1970).

Preparation of competent cells: 3ml of LB was inoculated with C1400 cells and grown overnight as described (2.2.1.1). 1ml of the overnight culture was then added to 100ml of prewarmed LB and grown for 1.5 hrs at 37^{o}C, until an absorbance at 650 nm of 0.3 was reached. The cells were then cooled on ice, and harvested at 12000g, 4^{o}C for 5 mins. The cell pellet was resuspended in 50ml of ice cold 0.1M $CaCl_2$, and left on ice for 30 mins. The cells were again harvested as above, resuspended in 1ml of 0.4M $CaCl_2$/20% v/v glycerol, and stored in 100µl aliquots until use.

Transformation: 50µl of competent cells (thawed on ice) were mixed with 10µl of DNA (up to 20ng), and held on ice for at least 30 mins. The mix was then heat-shocked for 5 mins, at 37^{o}C. 1ml of prewarmed LB was added, and the mix left at 37^{o}C for up to 45 mins. 100µl of the transformation mix was then spread out per LB (+ampicillin) plate and incubated overnight at 37^{o}C.

2.2.2.2 *S.cerevisiae.*

Transformation of yeast was carried out by the spheroplast method first described by Beggs (1978).

Solutions:

SED: 1M Sorbitol, 25mM EDTA (pH 8.0),

0.1 M DTT (added fresh and filter sterilised)

SEC: 1.2 M Sorbitol, 10mM EDTA (pH 8.0),

100mM NaCitrate (pH 5.8), 3% B-glucouronidase
(added fresh and filter sterilised).

SC: 1.2 M Sorbitol, 10 mM $CaCl_2$.

Bottom agar: 2% Glucose, 0.65% Yeast Nitrogen Base, 2% Bactoagar, 1.2 M Sorbitol.

Top agar: 2% Glucose, 0.65% Yeast Nitrogen Base, 3% Bactoagar, 1.2M Sorbitol.

Both top agar and bottom agar contained the amino acids and base requirements to satisfy the auxotrophic markers of the yeast strain transforming, at a final concentration of 50 µg/ml.

A 5ml YPG stationary culture of the yeast strain to be transformed was prepared, by growing as described (2.2.1.2). 100ml of YPG was then inoculated with an inoculum from the stationary culture and grown until an absorbance at 600nm of 0.6-0.8 was reached. The cells were harvested by centrifugation at 960g for 5mins at 20°C, resuspended in 50ml of SED, and placed in a shaking waterbath at 30°C, for 20 mins. Yeast cells were then harvested (as above) and resuspended in 50ml of SEC, and incubated for 30-40 mins at 30°C in a shaking waterbath. Spheroplast formation was monitored by light microscopy and was judged adequate when over 90% of cells lysed on addition of 35% (w/v)-lauryl sarcosine. After this treatment, cells were again harvested (as above), washed 3 times with 1.2M Sorbitol, and resuspended in 100µl of SC to give a thick suspension of spheroplasts. At this stage 15µl of plasmid DNA (1-2 µg) was mixed with 50µl of spheroplasts, and left at room temperature for 15 mins. 1ml of 20% (w/v) PEG 4000 (in 10mM $CaCl_2$, 10mM Tris pH 7.5, made up fresh and filter sterilised) was added to help uptake of DNA, and left for 30-45 secs. Cells were centrifuged at 12000g for 1 sec, at 4°C. The PEG was removed and the protoplasts resuspended in 100µl of YPG containing 1.2M Sorbitol, and incubated at 30°C for 30 mins. After this incubation, the cells were diluted by adding 950µl of 1.2M Sorbitol, and 100µl of the resulting dilution spotted onto bottom agar. 10ml of molten top agar were then poured on top, and when set the plates were incubated at 30°C for 3-7 days. The auxotrophic markers of presumptive transformants were checked before further analysis.

2.2.3 DNA ISOLATION AND ANALYSIS.

2.2.3.1 Mini-Plasmid preparation from *E.coli.*

Solutions:

STET buffer: 8% w/v Sucrose, 5% v/v Triton X-100, 50 mM EDTA, 50mM Tris-HCl (pH 8.0).
TE : 10mM Tris-HCl (pH 8.0), 1mM EDTA.

When a large number of *E.coli* transformants were being analysed to determine the plasmid they carried, the method of Holmes and Quigley (1981) was used. 2.5ml of LB containing ampicillin was inoculated with a single colony and grown as described (2.2.1.1). The cells were centrifuged at 12000g, for 3 mins at 20^{o}C and resuspended in 350µl of STET buffer. 25µl of lysozyme (10mg/ml) were then added and the resulting suspension placed in a boiling waterbath for 45 secs. The cell lysate was centrifuged at 12000g for 15 mins at 4^{o}C and the loose pellet. 40µl of 3M NaOAc were added, followed by 400µl of isopropanol to the supernatant, and the mix centrifuged at 12000g for 15 mins at 4^{o}C, to pellet precipatated material. The pellet was then washed with 70% (v/v) ethanol, dried, and resuspended in 50µl of TE. Prior to restriction analysis the DNA was treated with RNase by incubating the DNA with 1µl of 10 mg/ml RNase for 15 mins at 50^{o}C. Routinely a plasmid DNA yield of 100-500 ng/µl was obtained.

2.2.3.2 Maxi-Plasmid preparation from *E.coli.*

Solutions:

BD 1: 50mM Glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA. Lysozyme was added to 1 mg/ml immediately before use.
BD 2: 0.2 M NaOH, 1% w/v SDS.
BD 3: 3M KOAc (pH 4.8).

In order to prepare plasmid DNA for recombinant DNA manipulation, the method of Birnboin and Doly (1979) was used. 100ml of LB containing ampicillin was inoculated with a colony of the *E.coli*

strain carrying the plasmid to be amplified and grown as described previously (2.2.1.1). Cells were harvested by centrifugation at 12000g for 5 mins, at 4°C. The supernatant was discarded and the cells resuspended in 3ml of BD 1, vortexed and left at room temperature for 5 mins. 8ml of BD 2 were then added, and the mix left on ice for 5 mins. 6ml of precooled BD 3, were then added, and the mix left on ice for a further 5 mins. The resulting cell lysate was then centrifuged at 35000g for 30 mins at 4°C, with the supernatant being collected, and the cell pellet being discarded. 12mls of isopropanol were added and the mix left to precipitate DNA at room temperature for 15 mins.

Precipitated material was then pelleted by centrifugation at 39200g for 30 mins at 20°C, and dried under vacuum. The pellet was dissolved in 4.58ml of TE and 4.5g of CsCl added, along with 0.243ml of EtBr (10 mg/ml). The density of the mix was checked to ensure it was between 1.56-1.58 g/ml and was then transferred to a 5ml pollyallomer Beckman Vti 65 tube. The tubes were heat sealed and centrifuged in a Beckman VTi 65 rotor at 55000rpm for 4 hrs. After centrifugation, two bands were normally visible: a lower supercoiled plasmid band and an upper chromosomal and relaxed plasmid DNA band. The super-coiled DNA was removed, and the EtBr removed by 5 extractions with H_2O saturated butanol. The plasmid DNA was then precipitated by adding 2 volumes of H_2O, followed by the addition of 6 volumes of ethanol, and leaving at -20°C for at least 1hr. The DNA was pelleted by centrifugation at 35000g, 4°C, for 10 mins, and resuspended in an appropriate volume to give 1 µg/µl after the concentration had been determined by monitoring absorbance at 260nm. The DNA was stored under ethanol until use (2.2.3.5).

2.2.3.3 Total DNA preparation from *S.cerevisiae.*

The method used to prepare DNA from yeast was based on the method described by Livingston and Hahn (1979).

Solutions:

Buffer 1: 1M Sorbitol, 20mM EDTA, 20mM KH_2PO4, pH 7.5.

Buffer 2: 0.15 M NaCl, 5mM KCl, 1mM EDTA, 0.2% (v/v) Triton X-100, 1M Sorbitol, 20mM NaHepes, pH 7.5.

Buffer 3: 1mM EDTA, 5mM NaOAc, 40 mM Tris-HCl, pH 7.5.

100ml of the yeast strain from which total DNA was to be isolated from was grown to the late logarithmic phase of growth as described in 2.2.1.2. Cells were harvested by centrifugation at 960g for 5 mins at 20°C, resuspended in 5ml of buffer 1, and re-centrifuged as above. The cell pellet was resuspended in 0.2ml of buffer 2, and 50µl of 0.1M DTT added. 50µl of zymolyase (60 mg/ml) were then added, and the cell mix was incubated at 37°C for 30 mins to permit digestion of the cell wall.

After digestion the following was added: 0.5ml of 10% (w/v) SDS, 1ml of 0.5M EDTA, 0.125ml of 4M NaCl, 3.025ml of buffer 3, and 50µl of 10 mg/ml proteinase K (pre-digested at 37°C for 1 hr). The resulting mix was vortexed and incubated at 37°C for 90mins. 5 ml phenol pre-equilibrated to pH7 were added, followed by 5ml chloroform and the resulting mix vortexed. If necessary the mix was centrifuged at 1200g to separate phases. A further phenol/chloroform extraction was performed on the aqueous phase followed by two ether extractions. DNA was then ethanol precipitated (2.2.3.5) and left at -20°C until use.

2.2.3.4 Restriction of DNA.

Restriction of plasmid DNA was carried out at a concentration of 1µg of DNA per 10µl of digest mix, using 1µl of the appropriate restriction enzyme buffer (provided as a 10x buffer by manufacturer), and 1-10U of restriction enzyme per 10µl. The volume of reaction mix used depended on the amount of plasmid to be digested, with the final volume being made up with sterile H_2O. Reaction mixes were incubated at 37°C for at least 1hr to ensure complete digestion. *Sma*I digests were carried out at 30°C. For the restriction of total yeast genomic DNA, a similar digest mix was used, but the mix was incubated at 37°C overnight.

2.2.3.5 Ethanol precipitation.

To the DNA sample to be precipitated, 3M NaOAc pH 5.2 was added to a final concentration of 0.15 M, and 2 volumes of ethanol added. After mixing, the DNA was precipitated by placing at -20°C for at least 10 mins, and then pelleted by centrifugation (27000g, 4°C for

large volumes, or 12000g, 15 mins, 4°C, for small volumes in eppendorf tubes). The pellet was usually washed in 70% (v/v) ethanol, dried briefly in a vacuum drier, and resuspended in TE.

2.2.3.6 Agarose gel electrophoresis.

Solutions:

TBE buffer: (1x)	89mM Tris-HCl pH 8.3, 89mM Boric acid, 2.5mM EDTA.
Loading buffer: (10x)	0.025% (w/v) Bromophenol blue, 0.025% (w/v) xylene cyanol, 30% glycerol.

To the DNA sample to be separated by gel electrophoresis, loading buffer was added to give a 1x final concentration. The sample was loaded into a horizontal agarose gel which had been prepared by melting agarose in a suitable volume of TBE buffer containing EtBr at a final concentration of 0.1 mg/ml. Routinely 0.8-1.5% (w/v) agarose gels were used and run at voltages up to 12 V/cm, until the bromophenol blue had migrated to the end of the gel. Often gels could be run for much shorter distances. After electrophoresis the gel was placed on a Chromato VUE UV source, model TM36. Gels were photographed using a Polaroid camera, with a Wratten 3A red filter.

2.2.3.7 Southern blotting.

Solutions:

1: 0.2M NaOH, 1.5M NaCl.
2: 3M NaCl, 0.5M Tris pH 7.5.
3: 20 x SSC - 3M NaCl, 0.3M Na_3Citrate.

Yeast DNA to be analysed by Southern blotting was isolated as described (2.2.3.3) and Southern blotting carried out as described by Southern (1975). 5µg of this DNA was restricted if required, and then subjected to gel electrophoresis, as described above. For plasmid DNA to be Southern blotted, 0.1-0.2 µg of DNA were used. After

electrophoresis, the gel was photographed with a ruler adjacent as a reference. The gel was then soaked in solution 1 for 30 mins to denature DNA. This was followed by soaking the gel in solution 2 for 1 hr to neutralise DNA. The DNA was transferred from the gel onto either a nylon or nitrocellulose membrane using a Pharmacia Vacugene XL vacuum blotter, by transferring with solution 3, for 5 hours at a pressure of 50 mbars. After transfer of the DNA, the DNA was chemically bound to the filter by, in the case of nylon through exposure to UV light (254nm) for 4 mins, and in the case of nitrocellulose by baking for 3 hrs at 80^{o}C. The filters were then ready to be probed.

2.2.3.8 Isolation of DNA fragments.

The isolation of DNA fragments was achieved either by using the Gene-clean procedure or by electroelution.

Gene clean procedure:

Solutions:

TAE: 89mM Tris-HCl pH 8.3, 40 mM NaOAc, 2.5 mM EDTA.
NaI: 6M NaI, 150mM Na_2SO_3, 70mM Na_2S).
NEW: 113mM NaCl, 10mM Tris, 1 mM EDTA pH 7.5 in 53% ethanol.

Digested DNA was electrophoresed on a TAE agarose gel until the fragment to be purified had been well resolved. The fragment was then sliced out using a scalpel and placed into a 1.5ml eppendorf tube where it was diced into small pieces in a volume of NaI equal to 3 times the weight of the gel slice. The gel mix was then placed in a waterbath at 55^{o}C for 10 mins in order to dissolve the agarose. 5µl of Gene clean glass milk were then added to the sample and the suspension vortexed. This was followed by placing the mix on ice for 5 mins to allow the glass milk to bind the DNA. The mix was centrifuged at 12000g at 4^{o}C for 5 sec to pellet the glass milk and DNA. The supernatant was discarded, while the glass milk/DNA was washed 3 times with 0.5ml of NEW solution. All NEW had to be removed after the last wash. The glass milk/DNA mix was resuspended in 50µl of TE and

placed in a 55°C waterbath to release DNA. The resulting suspension was centrifuged at 12000g for 30 secs at 4°C, with the supernatant which contains the DNA, transferred to a fresh tube. The yield of DNA was checked on a baby TBE gel.

Electroelution: While the gene clean procedure was very quick and does not involve an ethanol precipitation, the yield varied depending on the batch of glass milk used. Therefore, it was often necessary to isolate DNA fragments by electroelution.

Digested DNA was electrophoresed on a 0.5x TBE agarose gel, and the required DNA fragment sliced out of gel and placed in a dialysis tube containing a minimum amount of TBE buffer (approximately 0.5ml). The dialysis tubing was sealed using a pair of dialysis clips, placed in a gel tank containing 0.5 x TBE, and the DNA eluted from the agarose by applying a voltage of 10 v/cm for 2 hrs. The current was then reversed for two minutes, and the buffer containing DNA transferred to a fresh eppendorf tube. The DNA was ethanol precipitated, and resuspended in a suitable volume of TE.

2.2.3.9 Creation of Blunt ends on DNA fragments with recessed 3' ends.

In order to create blunt ends on digested DNA fragments, which contained 5' protruding ends, the DNA was treated as follows. 5µg of digested DNA was incubated with 10mM DTT, 0.3mM dATP, 0.3 mM dCTP, 0.3 mM dTTP, 0.3 mM dGTP, 50mM KPO_4(pH 7.5), 3mM $MgCl_2$, 1U of Klenow fragment and incubated at 37°C for 20 mins.

2.2.3.10 Phosphatase treatment of DNA.

CIP buffer - 10mM $ZnCl_2$, 10mM $MgCl_2$, 100mM Tris-HCl pH 7.5

To increase cloning frequencies it was often necessary to subject digested vector DNA to Calf Intestinal Phosphatase (CIP) which removes the 5' terminal phosphate groups and thus inhibits recircularisation during ligation reactions.

10-20 µg of vector DNA was digested, ethanol precipitated and resuspended in 100µl TE. To 90µl of this DNA, 10µl of 10 x CIP buffer

were added, along with 1µl of diluted CIP (10 U). The reaction mix was incubated at 37°C for 30 mins, and then 75°C for 10 mins to inactivate CIP. The inactivation of CIP was carried out in the presence of 5mM EDTA (pH 8.0). To remove all traces of CIP, the mix was extracted with phenol/chloroform/iso-amyl alchol, and then ethanol precipitated. The efficiency of CIP treatment was always assessed by comparing the abilities of treated and non-treated vector to self-ligate. Self-ligation was assessed by transformation of *E.coli* with equal quantities of the vectors in question.

2.2.3.11 Ligation of DNA.

10 x ligation buffer: 66mM Tris-HCl pH 7.6, 10mM DTT, 1mM ATP, 6.6mM $MgCl_2$.

Different ligation conditions were used for overhanging and blunt end ligations.

For "sticky-end" ligations, a 10µl reaction volume was used comprising of 200 ng of DNA, 1 x ligation buffer, and 0.5U of T4 ligase. The 200 ng of DNA was made up of insert and vector mixed at a 10 molar excess with respect to insert. The ligation mix was incubated at 14°C overnight and 2µl of the mix used to transform *E.coli* as described previously. In this study all of the blunt-end ligations involved only recirculisation of vector DNA. Thus, for these type of ligations, 1 µg of blunt ended vector was ligated by mixing DNA with 1U of T4 ligase, and 1 x ligation buffer in a final volume of 20µl. The ligation reaction was carried out at 37°C for 4 hrs. 2µl of this mix was then used to transform *E.coli*. Ligation of oligonucleotides.

2.2.3.12 Colony hybridisation.

For certain sub-clonings, it was necessary to screen many *E.coli* transformants in order to isolate the desired clone. Colony hybridisations were carried out when this was the case. Essentially the method described by Sambrook and co-workers (1989) was used.

Solutions:

A: 10% (w/v) SDS.
B: 0.2M NaOH, 1.5M NaCl.
C: 0.5M Tris pH 7.5.
D: 2 x SSC (0.3M NaCl, 0.03M Na_3Citrate).

Plates containing the *E.coli* transformants to be screened were incubated at 37°C overnight to give colonies approximately 5mm in diameter. The plates were then chilled for 30 mins as this was found to improve transfer. A dry nitrocellulose filter was placed on the surface of the agar in contact with the bacterial colonies until it was completely wet. The filter was then placed on separate pieces of Whatmann 3MM paper, soaked with the following series of solutions: A for 3 mins, B for 5 mins, C for 5 mins and D for 5 mins. The filter was left to dry for 30 mins at room temperature and then heat baked at 80°C for 2 hrs. The filter was now ready to be probed as described in 2.2.7. The plate from which the lift had been made, was placed in 37°C incubator for 5 hrs to allow colonies to reform.

2.2.3.13 Plasmid copy number analysis.

To determine the dosage of a particular gene, total genomic DNA was subjected to dot blot hybridisation with identical filters being probed separately for the gene of interest, and for a control gene.

This involved firstly resuspending 5 µg of restricted yeast DNA (as prepared in 2.2.3.3) in 100µl of TE. The DNA was chemically denatured by adding NaOH to a concentration of 0.1M, and incubating at 37°C for 15 mins. HCl was then added to a final concentration of 0.1M. A series of dilutions were then made and dotted out onto duplicate filters using the BRL Hybridot apparatus. The samples were washed with 100µl 15 x SSC, and then the filters heat baked at 80°C. One filter was probed (as described in 2.2.7) for a gene carried on the plasmid, while the other for a single copy gene. After hybridisation and autoradiography, individual dots were cut up and subjected to scintillation counting. The results were plotted on a graph displaying concentration of DNA per dot versus CPM per dot for each sample. The abundance of a particular single copy gene can then be calculated by

taking into consideration the CPM per µg of DNA and the specific activity of the probe. The abundance of the gene of interest can be calculated in the same manner. Finally, the dosage of the plasmid encoded gene can then be calculated relative to the abundance of the single copy gene.

2.2.4 RNA ISOLATION AND ANALYSIS.

When handling RNA, all materials used were RNase-free and gloves were worn at all times. All glassware used was heat baked at 200°C overnight, while plastic-ware was soaked in 0.1% (v/v) DEPC overnight and then rinsed in RNase-free H_2O. RNase-free solutions were prepared using chemicals solely for RNA work, treated with 0.1% DEPC overnight and then autoclaved. Solutions containing amine groups were not treated with DEPC, but were prepared in RNase-free H_2O in a heat baked bottle. Glass beads were soaked in concentrated HNO_3, rinsed in dH_2O, and then heat baked at 200°C overnight.

2.2.4.1 Isolation of RNA from *S.cerevisiae.*

RNA was isolated from yeast by the method of Lindquist (1981).

Extraction buffer: 0.1 M LiCl, 0.1 M Tris-HCl pH 7.5,
0.01 M DTT (added fresh).

Phenol: Phenol was saturated with 0.1M Tris-HCl (pH 7.5) and contained 0.1% (w/v) hydroxyquinoline.

A culture of the yeast strain from which RNA was to be extracted was grown to an absorbance at 600nm of 0.6 in 200 ml of rich or minimal media. At this stage cells were often stored under two volumes of ethanol at -20°C before isolation of RNA. Cells were not stored for longer than 3 days before carrying out extraction.

The cells were centrifuged at 960g for 5 mins at 20°C and the cell pellet resuspended in 5ml of extraction buffer, and transferred to a 50ml centrifuge tube containing the following mix: 14 g glass beads, 0.5ml 10% (w/v) SDS, 2.5ml phenol, 2.5ml chloroform. The mix was vortexed continuously for 5 mins and centrifuged at 960g, 20 mins, 4°C. The aqueous phase was removed and added to 2.5ml of phenol and 2.5ml of chloroform. This was mixed continuously for 1 min, and

centrifuged as before. This was repeated on the aqueous phase and followed by two ether extractions on the aqueous phase. The aqueous phase was then ethanol precipitated at -20°C.

2.2.4.2 Quantitation of RNA abundances.

Solution A: A 6:4 mix of 20 x SSC:37%(v/v) Formaldehyde.

In order to quantify the abundance of a specific mRNA, 1.5ml of ethanol precipitated RNA (prepared as described above) was centrifuged at 12000g for 15 mins at 4°C, and then after drying, resuspended in 200µl of RNase free H_2O. 200µl of solution A were added and the mix thoroughly vortexed. The RNA was then denatured by heating at 60°C for 15 mins, and from this the following deletion series made.

Dilution	Volume Denatured RNA	Volume 20 x SSC
1/2	200	200
1/4	100	300
1/8	50	350
1/16	25	375
1/32	12	388

All volumes are in µl.

Thus, the total volume prepared for each dilution prepared was 400µl. 95µl of each dilution was dotted in duplicate onto two separate nitrocellulose filters, using the BRL Hybridot apparatus. After all the samples had been loaded onto the filter, each sample was washed with 100µl of 15xSSC. Filters were heat baked at 80°C for 2 hrs and were ready to be probed (see 2.2.7). Hence, two duplicate filters were prepared for each dilution series of a specific RNA preparation. One of the duplicates was probed for the mRNA of interest while the other for a standard loading control RNA. When using 18S rRNA as a loading control, a further 1/1000 dilution was performed on each dilution prior to dotting out.

After hybridisation, washing, and autoradiography, dots were cut out individually from the filter, and the radioactivity bound quantified by liquid scintillation counting. The results for each probing were plotted graphically. Only when a straight line was generated for a dilution series, were the results considered quantifiably accurate. The abundance of the test mRNA (expressed as % of total mRNA) was calculated relative to a known standard.

When access to an AMBIS β-scanner became possible, mRNA abundances could be carried out accurately on Northern blots probed firstly for the mRNA of interest, followed by probing the same filter afterwards for a control mRNA. This was feasible only using the AMBIS β-scanner as it is possible to quantitate radioactivity on filters without the need for cutting and scintillation counting.

2.2.4.3 Northern blotting.

Northern blotting was carried out according to the modified procedures of Thomas (1980).

Solutions :

10 x MOPS : 0.2M MOPS, 0.05M NaOAc, 0.01M EDTA pH 7.0.
MMF : 1xMOPS, 50% Formamide, 12% Formaldehyde.
Loading buffer : 50% Glycerol, 1mM EDTA,
0.4% (w/v) xylene cyanol,
0.4% (w/v) bromophenol blue.

10-20 µg of RNA isolated as described in 2.2.4.1 was resuspended in 5µl of RNase free H_2O. 30µl of MMF was then added and the resulting mix heated at 60°C for 15 mins to denature the RNA. 3µl of loading dye were added and the RNA mix held on ice until loading of the gel. A 100ml, 1.5% formaldehyde agarose gel was prepared as follows: 1.5 g of agarose was melted in 73ml of RNase free H_2O and cooled to 60°C. Just prior to pouring of the gel, 10ml of 10x MOPS and 10ml of 37% formaldehyde were added. After the gel had set, the RNA samples were loaded and the gel run in circulated 1 x MOPS buffer

until the bromophenol blue had reached the bottom of the gel. The RNA was then blotted onto nylon or nitrocellulose membrane using the Pharmacia Vacugene apparatus for at least 4 hrs at 50 mbars pressure. After blotting, the RNA was chemically bound to the filter by either heat baking nitrocellulose filters at 80°C, or UV fixing nylon membranes. The filters were then ready to be probed as described in 2.2.7.

2.2.4.4 Primer extension analysis.

In order to determine the 5' end of a specific mRNA, the following primer extension method was used.

5-10 µg of RNA was mixed with [^{32}P] end-labelled primer oligonucleotide in such a way that the oligonucleotide was in molar excess. 3µl of reverse transcriptase buffer were then added and the volume made up to 25.5µl with H_2O. The primer oligonucleotide was then annealed to RNA by heating to 90°C, and cooling slowly to 37°C. After annealing, DNA was synthesised from the primer back to the 5' end of the mRNA by adding 1µl of 0.3M DTT, 1U of Moloney Murine Leukemia Virus reverse transcriptase, 1.5µl of each of dCTP, dATP, dGTP, and dTTP (each at 10mM). The reaction was carried out at 37°C for 45 mins and then 3.3µl of 1M NaOH added, followed by heating at 70°C for 10 mins to remove RNA. 3.3µl of 1M Tris pH 7.5 were then added, followed by the addition of 4µl of 3M NaOAc and 75 µl of ethanol. The DNA was precipitated at -20°C, and then centrifuged and resuspended in 10µl of TE. 2 µl of this was then electrophoresed on a 8% SDS polyacrylamide gel containing 7.5M Urea, along side a sequence ladder. The gel was electrophoresed at 45 constant watts for 2hrs, dried and exposed to X-ray film for 24 hrs. The film was developed and the length of the extended DNA product determined. From this it was possible to determine the 5' end of the mRNA.

2.2.5 YEAST POLYSOME GRADIENTS.

2.2.5.1 Preparation of polysome gradients.

Solutions:

A: 1M Sorbitol, 25mM EDTA pH 8.0, 50mM DTT (added fresh).
B: 1.2M Sorbitol, 10 mM EDTA, 100mM NaCitrate pH 5.8
3% ß-glucouronidase (added fresh).
C: 1.2M Sorbitol in YPG.
D: 0.1M NaCl, 30 mM $MgCl_2$, 10mM Tris-HCl pH 7.4.

To analyse how efficiently specific yeast mRNAs were translated *in vivo*, yeast polysomes were separated by sucrose density centrifugation using a modification of the Hutchinson and Hartwell (1967) method (Moore *et al.*, 1991a).

A stationary culture of the yeast strain to be studied was prepared in selective media with 100-1000 µl used to inoculate 400 ml of prewarmed YPG which grown at 30°C until an absorbance at 600 nm of 0.6 was reached. In cases where yeast transformants were very slow growing, a higher inoculum was used.

At this point a sample was plated out onto a YPG plate (100µl of a 10^{-4} dilution) and allowed to grow for 2-3 days at 30°C. 100 colonies were patched out onto minimal agar plates which select for plasmid-containing yeast to give an indication of the proportion of cells in the culture which contained plasmid.

The remaining cells were centrifuged at 960g for 5 mins at 20°C, resuspended in 50ml of solution A, and incubated at 30°C for 20 mins. After this incubation, the cells were again centrifuged as above, resuspended in 50ml of solution B, and incubated at 30°C for 45 mins. Spheroplasts were centrifuged as before, resuspended in 50ml of solution C, and placed in a shaking waterbath at 30°C for 2 hrs. After this 2 hr recovery phase, the cells were centrifuged as above, resuspended in 500µl of solution D, and transferred to an RNAse-free microfuege tube. At this stage it was critical that all materials were RNase-free and pre-cooled, and that all stages were carried out as quickly as possible. 50µl of 10% (w/v) sodium deoxycholate were added and the suspension vortexed for 5 secs. 50µl of 10% (w/v) Brij 58 were

added, the suspension vortexed for 5 secs and centrifuged at 12000g for 1 min at 4°C. 600µl of the lysate were then layered onto a 38ml 10-50% (w/w) sucrose gradient and the gradients centrifuged at 25500 rpm using an SW28 Beckman rotor for 2 hrs 50 mins at 4°C. After centrifugation, the gradient was fractionated through a spectrophotometer monitoring absorbance continuously at 260nm, using a 2.5mm diameter flow cell. The gradient was fractionated by either of two methods described below.

2.2.5.2 Dot blot analysis.

Solutions:

A: A 6:4 solution of 20xSSC:37% Formaldehyde

For dot blot analysis, the gradient was dripped into 40-50 microfuege tubes containing 400µl of solution A. Approximately 800µl of the gradient were dripped into each tube and heated at 60°C for 15 mins. Fractions were stored at -70°C until they were to be analysed. When being analysed, 20-100µl of each fraction was dotted onto nitrocellulose using a BRL Hybridot apparatus. The samples were washed with 100µl of 15 x SSC, and the filter heat baked at 80°C for 2 hrs. The filter was then probed for the mRNA of interest. After hybridisation, each dot on the filter was cut out and subjected to scintillation counting in order to quantify the CPM per dot. Hence, it was possible to determine the distribution of an mRNA across a polysome gradient.

2.2.5.3 Northern blot analysis.

Solutions:

Phenol : Phenol was saturated with 0.1M Tris-HCl pH 7.5
and contained 1mg/ml hydroxyquinoline.

When fractions were to be analysed by Northern blotting, the gradient was dripped into twelve 15ml RNase-free centrifuge tubes containing 3ml RNase-free H_2O, 1.5ml phenol, 1.5ml chloroform, 150µl 10% (w/v) SDS, and 12µl 0.5M EDTA. The mix was well vortexed, placed at -70°C overnight, and then centrifuged at 1000g for 30 mins, at 20°C.

The aqueous phase was taken and one further phenol chloroform extraction performed followed by a chloroform extraction. The aqueous phase was then ethanol precipitated and stored for Northern analysis. Quantification of Northern bands was performed using the AMBIS β-scanner.

2.2.6 PROTEIN ISOLATION AND ANALYSIS.

2.2.6.1 Preparation of protein extracts from *S.cerevisiae.*

A 200ml mid-logarithmic culture of the yeast strain to be analysed was prepared. In most experiments involving the preparation of protein extracts, RNA was also extracted in order to compare specific protein levels with specific mRNA levels. Thus at this stage, 100ml of the yeast cells were stored under ethanol at -20^{o}C until RNA was being extracted and 50ml used to prepare protein extracts. The latter was centrifuged at 960g for 5 mins at 20^{o}C, and resuspended in 1ml of 25 mM $NaPO_4$, pH 7.0. The yeast cell suspension was then transferred to a sterile 1.5ml microfuege tube, and washed twice with 25 mM $NaPO_4$ buffer. Following the last wash, the cells were microfueged at 12000g, 4^{o}C for 3 mins, and the cell pellet placed at -70^{o}C for 10-15 mins. The pellet was then resuspended in 50µl of 25mM $NaPO_4$, and glass beads added to the level of the meniscus. The extract was then vortexed for 1.5 mins, placed at -70^{o}C for a further 15 mins, and then thawed on ice. Cells were centrifuged at 12000g for 5 mins, at 4^{o}C, and the supernatant stored at -70^{o}C. When enzyme assays were being performed, PMSF was included in the extraction buffer at a concentration of 50mM.

2.2.6.2 Bradford Assay.

To determine the protein concentration in a given protein extract, a sample was subjected to the Bradford assay, (Bradford, 1976).

Solutions:

Bradford's reagent: 100 mg of Coomassie Brilliant Blue was dissolved in 50ml of 95% ethanol. 100ml of 85% Phosphoric acid was then added and the final volume made up to 1 litre with dH_2O. The resulting solution was then filtered through Whatmann filter paper and stored in a dark bottle.

Standard curve: A 1 mg/ml solution of Bovine Serum Albumin was prepared in 150 mM NaCl. In the assay 0, 10, 20, 30, 40, and 50µl of the standard solution were used to generate a standard curve of protein concentration against absorbance at 595nm. Each standard was tested in duplicate, and the final volume in each case was made up to 50µl with 150 mM NaCl.

Assay: 50µl of the sample to be assayed was mixed with 2.5 ml of Bradford's reagent and incubated at room temperature. The absorbance at 595nm of each sample was measured after 2 mins and before 20 mins. Yeast extract samples were tested in triplicate. By using the standard curve it was possible to convert the absorbance at 595 nm to concentration of protein.

2.2.6.3 SDS.Polyacrylamide Gel Electrophoresis.

Protein samples were analysed qualitatively by SDS-Polyacrylamide gel electrophoresis.

Solutions:

Acrylamide stock: 30% w/v acrylamide
(acrylamide : bisacrylamide = 19 : 1).

Stacking buffer : 0.5M Tris-HCl pH 6.8.

Resolving buffer: 3M Tris-HCl pH 8.8.

Running buffer : 0.02M Tris-HCl pH 8.3, 0.192M Glycine,
0.1% (w/v) SDS.

Loading buffer : 63mM Tris-HCl pH 6.8, 10%(v/v) glycerol,
2%(w/v) SDS, 5% β-mercaptoethanol,
0.0015%(w/v) Bromophenol blue.

Stain : 0.1%(w/v) Coomassie Brilliant blue-R
40%(v/v) methanol, 10%(v/v) acetic acid.

Destain : 40%(v/v) methanol, 10%(v/v) acetic acid.

Sample preparation: 5 mg of yeast protein extracts were routinely run per lane. The volume of sample was made up to 20µl with H_2O, mixed with 10µl of loading buffer, and boiled for 3 mins to denature proteins. The samples were then held on ice until the gel was run.

Preparation of gel: SDS-PAGE gels were run with both a stacking and a resolving gel. The following gel mixes were used for all SDS-PAGE gels carried out.

	Resolving gel (ml)	Stacking gel (ml)
Acrylamide stock	8	2.5
10%(w/v) SDS	0.3	0.2
1.5% $NH_4(SO_4)2$	1.5	1.0
TEMED	0.015	0.015
Stacking buffer	-	5
Resolving buffer	7.5	-
H_2O	12.7	11.3

The constituents of the resolving gel were scaled up when larger gels were being run. Both resolving and stacking gels were prepared and degassed prior to the inclusion of $NH_4(SO_4)_2$ and TEMED. The resolving gel was poured to 3/4 of the height of the glass plate sandwich, and allowed to polymerise. H_2O saturated butanol was layered on top of the gel to prevent it from drying out. After the resolving gel had set, the stacking gel was layered on top and allowed to polymerise with comb in place.

Running of gel: Gels were electrophoresed vertically in running buffer at 125 V until the bromophenol blue had reached the bottom of the gel.

Staining: Gels were stained for protein by submerging the gel in stain for 3-4 hrs and then destained in destain for 2-3 hrs.

2.2.6.4 Pyruvate kinase assays.

An assay for pyruvate kinase activity in yeast cell extracts was developed based on the method of Kaslow and Garrison (1983). The development of this method is described in chapter 5. The method involves the use of a radioactively labelled substrate ([^{14}C]-labelled

phosphoenolpyruvate; PEP), which is incubated with the sample to be tested, along with a series of co-factors. Pyruvate kinase cleaves the phosphate group from the [^{14}C]-PEP to give [^{14}C]- pyruvate. Unreacted substrate was then separated from [^{14}C]-pyruvate by hydroxyapatite chromatography.

The following reaction mix was used: 50mM Immidazole-HCl pH 7.5, 8 mM ADP, 100mM KCl, 100 µg/ml BSA, 0.025 mM [^{14}C]-PEP, 4mM PEP, 10mM $MgCl_2$, 100mM fructose-1,6-diphosphate. 320µl of the reaction mix was incubated with 80µl of the sample to be tested. Yeast extracts were prepared as described in 2.2.6.1 except 10ml of cells were used, and the yeast extract taken up in a final volume of 1 ml of TE. 80µl of this extract was then used to test for pyruvate kinase activity. A second sample comprising of 8µl of extract with 72µl of TE was also tested. The reaction was carried out at 37^{o}C in the case of rabbit muscle PYK (used as standard) and at 30^{o}C in the case of yeast cell extracts. After 10 and 30 mins, duplicate 100µl samples were removed and added to 50µl of 0.5M EDTA to stop the reaction. The reaction could be terminated in the case of rabbit muscle with 30 µl of 0.1M EDTA, but when using yeast extracts higher concentrations of EDTA appeared to be needed. After the reactions had been stopped, samples were purified through hydroxyapatite. Due to the poor flow properties of hydroxyapatite, it was used in batch form rather than in a column. Prior to use, the hydroxyapatite was resuspended in 0.1M Immidazole-HCl pH 6.0 buffer, and 500 µl aliquots dispensed into microfuege tubes. 125µl of the terminated reaction mix was added to 500µl of hydroxyapatite, vortexed, and incubated at room temperature for 5 mins, to allow any PEP to bind to the hydroxyapatite. The mix was then centrifuged at 12000g for 5 mins at 4^{o}C, 200µl of the supernatant removed and added to a second aliquot of hydroxyapatite, and the above repeated. 200µl of the supernatant was then added to 1ml of Escoscint and subjected to scintillation counting. When reactions were being carried out on yeast extracts, a positive control with rabbit muscle PYK, and a negative control (a duplicate of the test reaction, but with EDTA added at beginning of reaction) were performed.

2.2.6.5 β-Galactosidase assays.

Solutions :

Z Buffer : 60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol, 0.025% (w/v) SDS, pH 7.0

ONPG : 4 mg/ml ONPG in 0.1M Potassium phoshpate pH 7.0

To determine the level of β-galactosidase expressed by a particular strain of yeast, 0.1-1 ml of mid-logarithmic cells grown as described in 2.1.3.2 were subjected to the assay described by Legrain and Rosbach (1989). The cells were sedimented by centrifugation at 4100g for 2 mins, suspended in 200µl of 50mM potassium phosphate, pH7 and incubated at 28°C for 5 mins. 200µl of Z buffer were then added, along with 2.5µl of chloroform, and the sample vortexed for 10 secs. The sample was then re-incubated at 28°C for 5 mins. 200µl of ONPG were then added and the sample mixed and re-incubated at 28°C, until a yellow colour formed. The reaction was stopped by the addition of 250µl of 1M Na_2CO_3, the cell debris removed by centrifugation at 4100g for 2 mins, and the β-galactosidase activity quantified by measuring the absorbance at 420nm of the supernatant. The units of β-galactosidase were calculated using the formula given by Miller (1972).

Units of β-galactosidase activity

$= OD\ 420nm / (t * OD\ 600nm * v) * 1000$

OD 420nm reflects the hydrolysis of ONPG by β-galactosidase

OD 600nm reflects the cell density of the culture just prior to assay

t is the time of the reaction in minutes

v is the volume of culture used in the assay, in ml.

This formula is not a measure of the specific activity of β-galactosidase, which is normally defined as β-galactosidase units/mg of protein. However, these units are proportional to the increase in O-nitrophenol per minute per yeast cell and, as it is assumed that the protein content of an exponentially growing culture is proportional to its cell density (ie. its cell number), it is adequate for the purposes for which it was used.

2.2.7 RADIOLABELLING OF DNA AND FILTER HYBRIDISATIONS.

2.2.7.1 Radiolabelling of plasmid DNA.

Klenow buffer: 10mM $MgCl_2$, 0.066% Gelatin, 10mM Tris pH 7.4

Random-primer mix: 250 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 5 mM B-mercaptoethanol, 2mM each dATP, dGTP, dTTP, 1M HEPES (adjusted to pH 6.6 with 4N NaOH), 1 mg/ml random hexanucleotides.

Plasmids used as probes for specific genes or mRNAs are listed in Table 2.1. Plasmid DNA was labelled with [^{32}P] by either nick-translation, or by random prime labelling.

Nick-translation: This method described by Rigby and co-workers (1977) was used when 1 µg of DNA or more was to be labelled. This was particularly pertinent to dot blot hybridisations, where an excess of probe was imperative to achieve quantitatively linear hybridisations. The following reaction mix was used.

Plasmid DNA (0.5-1µg)	5 µl.
Klenow buffer (x1)	45 µl.
DNase (0.5µg/ml)	0.7 µl.
[^{32}P]-dCTP (10µCi/µl)	5 µl.
dATP (10mM)	0.2 µl.
dTTP (10mM)	0.2 µl.
dGTP (10mM)	0.2 µl.
ß-mercaptoethanol (2.5%v/v)	1 µl.
DNA polymerase (1U/µl)	1 µl.

DNase was added to the reaction tube last to initiate the reaction. The reaction was performed at 14^{o}C for 1hr. 4µl of 0.25M EDTA was added to stop the reaction.

Random priming: When DNA with a very high specific activity was required, plasmid DNA was labelled by random priming (Feinberg and Vogelstein, 1983). A prerequisite to this procedure was the preparation of the DNA to be labelled as a linear fragment (this was achieved by digestion with a restriction enzyme). Prior to labelling the

TABLE 2.1 **Plasmids used to probe for specific genes.**

Plasmid	Gene	Description
pSPK2	*PYK1*	Contains a 550bp *Xba*1/*Bgl*II base pair fragment of the *PYK1* gene sub-cloned into the *Bam*H1/*Xba*1 sites in the polylinker of pSP64
Source: A.Brown Reference: Santiago *et al.*, 1987		
pSP65R	18SRNA	Contains a 1kb *SSt*1/*Xba*1 fragment of the yeast 18S rRNA gene sub-cloned into the *SSt*1/*Xba*1 sites in the polylinker of pSP65.
Source: A.Brown Reference: Santiago *et al.*, 1986		
pSPGK2	*PGK*	Contains the entire coding region of the *PGK* gene on a 2.95 kb *Hind*III fragment inserted into the *Hind*III site of pSP64.
Source: A.Brown Reference: Unpublished		
CDNA 10	*ENO2*	Contains enolase cDNA sub-cloned into the *Pst*1 site of pBR322.
Source: A.Brown Reference: Santiago *et al.*, 1986		
pPFK1	*PFK1*	Contains a 3.4kb EcoR1 fragment carrying the entire *PFK1* gene sub cloned into the *Eco*R1 site of pBR322.
Source: J.Heinsch Reference: Heinsch, 1986		
pPFK2	*PFK2*	Contains a 3.2kb EcoR1 fragment carrying the entire *PFK2* gene sub cloned into the *Eco*R1 site in pBR322.
Source: J.Heinsch Reference: Henisch, 1986		

TABLE 2.1. continued

Plasmid	Gene	Description
pSPUR1	*URA3*	Contains a 1.1kb HindIII fragment carrying the entire *URA3* gene sub-cloned into the *Hin*dIII site of pSP65.
Source : A.Brown Reference : unpublished		
pSPACT9	*ACT1*	Contains a 1.5kb *Bam*H1/*Hin*dIII fragment of the actin gene sub-cloned into the *Bam*H1/*Hin*d III sites in the polylinker of pSP64.
Source : A.Brown Reference : Moore *et al.*, 1990a		
pSP56RT7	*RAP1*	Contains *RAP1* coding region and downstream sequences cloned into pSP56.
Source : A.Chambers Reference : Chambers *et al.*, 1989		
pPDC1 E	*PDC1*	Contains a 1.2kb *Eco*R1 fragment of the *PDC1* coding region cloned into pUC18.
Source : S.Hohmann Reference : Hohmann and Cederberg, 1990		
pHGSOO1	*FBA1*	Contains a 600 bp fragment of *FBA1* cloned into pGEM1.
Source : S.D.Kohlwein Reference : Schwelberger *et al.*, 1989		
pGP.R1	*LPD*	Contains a 3.6kb *Xho*1-*Xho*1 fragment of the *LPD* gene.
Source : Z.Zaman Reference : Roy and Dawes (1987)		
pMA300	*LEU2*	Contains the entire *LEU2* gene on a 1.2 kb *Pst*1 fragment in pBR325.
Source : A.Kingsman Reference: Kingsman *et al.*, 1981.		

linear DNA was boiled for 3 mins to denature the template. The following reaction mix was then used:

H_2O	32 µl
Random primer mix	10 µl
BSA (DNase free)	2 µl
[^{32}P]-dCTP (10µCi/µl)	3 µl
DNA (20-30 ng)	5 µl
Klenow (1 U/µl)	1 µl.

The reaction was carried out at 37^{o}C for 30 mins, and then terminated by the addition of 2µl of 0.25M EDTA.

2.2.7.2 Radiolabelling of oligonucleotides.

10 x kinase buffer: 100mM KCl, 70mM Tris-HCl pH 7.5,
10mM $MgCl_2$, 5mM DTT.

The sequence of the oligonucleotides used to probe for specific genes or mRNAs are given in Table 2.2., along with the percentage of formamide used in each hybridisation. Hybridisations involving oligonucleotide probes were carried out at 37^{o}C, unless otherwise stated. The following reaction was prepared and the reaction carried out at 37^{o}C for 60 mins, and terminated by the addition of 2µl of 0.25M EDTA.

[τ-^{32}P] ATP (10µCi/µl)	5 µl
10x kinase buffer	4 µl
T4 kinase (1 U/µl)	1 µl
Oligonucleotide (1 µg/µl)	1 µl
H_2O	29 µl.

TABLE 2.2. **Oligonucleotides used to probe for specific genes.**

Gene	Sequence
TDH	5' GGACGAGTGATGACAACCTTCTTGGCACCAGCG 3'

This oligonucleotide hybridises to all 3 of the *TDH* genes, and was found to hybridise optimally at 20% formamide.

RP1	5' CCTCTTGGGGTTTCGACGTAACCGACAACACC 3'

This oligonucleotide hybridised optimally at a formamide concentration of 20%, specifically to the *RP1* mRNA.

HXK	5' GATTGAGTGGTGTCAAAGGTACGG 3'

This oligonucleotide hybridised to both *HXK1* and *HXK2*, and was found to hybridise optimally at formamide concentration of 6%.

TPI	5' GTTACCACCGACAAAGAAAGTTCTAGC 3'

This oligonucleotide hybridised specifically to the *TPI* gene and was found to hybridise optimally at 12% formamide.

PFK1	5' CCACCTTGTTGAACATGGCCTGGGATAGC 3'

This oligonucleotide hybridised specifically to the *PFK1* gene,and was found to hybridise optimally at 12% formamide.

PFK2	5' CGGTATCAGAACCCAAAGAGTATTCAGTACC 3'

This oligonucleotide hybridised specifically to the *PFK2* gene, and was found to hybridise optimally at 6% formamide.

Table 2.2. continued

Gene	Sequence
PGM	5' GACCGTGTCTAACTAAAACTAACTTTGG 3'

This oligonucleotide was used to probe specifically for *PGM* and was found to hybridise optimally at 42^{o}C with a formamide concentration of 20%.

PYK	5' CTCGACATTGTGATGATGTTTTATTTGTTTTGATTGGTGTCT TGATG 3'

This oligonucleotide was used to hybridise to the 5' untranslated leader of the *PYK1* gene. It was found to hybridise optimally at a concentration of 20%

PYK	5' GGTCAATCTTTCTAATCTAGA 3'

This oligonucleotide hybridised to the *PYK1* mRNA between bases +3 and +24, and was used to map the 5' end of the *PYK1* mRNA.

PGI	5' CGGTGATCTTCTTACCGGTATAACCCTTCC 3'

This oligonucleotide hybridises to the *PGI* mRNA at an optimal formamide concentration of 20%.

ADH1	5' CCAGAATCCAGAAAGTTG 3'

This oligonucleotide hybridises specifically to the *ADH1* mRNA. It was found to hybridise optimally at a formamide concentration of 20%.

2.2.7.3 Purification of probes.

Both plasmid and oligonucleotide probes were purified by chromotography through a 10ml G-50 Sephadex vertical column, eluting with TE buffer. Separation was monitored by the addition of 10µl of Dextran blue (10mg/ml) and 2µl of phenol red (10mg/ml) to the reaction mix prior to loading sample onto column. The incorporated nucleotides ran at the same rate through the Sephadex column as the dextran blue, and thus the probe was collected in the blue fraction eluted from the column. The unincorporated nucleotides which co-eluted with the phenol red were discarded.

2.2.7.4 Determination of Probe Specific activity

After purification through Sephadex G-50, 1µl of the probe was added to 1ml of H_2O and subjected to liquid scintillation counting to determine the CPM/µl. From this, and the amount of DNA labelled, the specific activity of the probe was determined.

2.2.7.5 Filter hybridisations.

Solutions:

100 x Denhardt's: 1% (w/v) Ficoll, 1% Polyvinylpyrrolidone,
1% (w/v) BSA (Pentax Fraction V).

20 x SSPE : 3M NaCl, 0.3M $NaH_2PO_4.H_2O$,
20 mM EDTA. pH 7.4.

Filters prepared for probing were firstly pre-hybridised for 2-3 hrs before being exposed to the probe of interest. This involved placing the filter in a heat sealed plastic bag, and then adding pre-hybridisation fluid. For most filters (12 cm x 10 cm in size) 10ml of pre-hybridisation solution was used. The following prehybridisation fluid was used for all hybridisations involving plasmid probes.

20 x SSPE	6.25 ml
100 x Denhardt's solution	1.25 ml
10% (w/v) SDS	1.25 ml
Formamide	12.5 ml
H_2O	3.75 ml.

The sealed filter was placed in a waterbath at 42°C for 3 hrs, denatured probe (by boiling for 5 mins) was added to give approximately 10^6 cpm per ml of hybridisation fluid and hybridisations carried out at 42°C for at least 12 hrs using a shaking waterbath.

For oligonucleotide probes, the same pre-hybridisation mix was used, except that the concentration of formamide was varied to optimise the hybridisation for each specific oligonucleotide. Also, reactions were carried out at 37°C, rather than 42°C.

2.2.7.6 Post-hybridisation conditions.

Washing of filters:

20 x SSCP : 3.3M NaCl, 0.3M NaCitrate,
0.3M $Na_2HPO_4.NaH_2PO_4.H_2O$. pH 7.4

Filters which had been probed with a plasmid probe were washed as follows. The first wash was carried out at 20°C, with 2 x SSCP, 0.1% (w/v) SDS, for 10-15 mins. This wash was repeated, and the filters then washed with 0.5x SSCP, 0.1%(w/v) SDS at 42°C for 15 mins. This wash was repeated, and a final wash with 0.1 x SSCP, 0.1% SDS at 50°C carried out. If necessary, washings were repeated at this stringency until the background cpm were minimal.

For filters which had been probed with a labelled oligonucleotide, more gentle washes were performed. Routinely two washes were performed with 6 x SSCP at 37°C and further washes were only performed if there appeared to be substantial background. If this was the case, filters were washed progressively more stringently until most background cpm were removed.

Autoradiography: After adequate washing, the filter was exposed to X-Ray film at -70°C, in the presence of an intensifying screen. The X-ray film was then developed automatically using a Kodak XO-mat M6-tabletop processor.

Stripping of filters: In some cases it was necessary to reprobe Northern filters with a second probe. This was only carried out when a nylon membrane was used. The initial probe was removed by pouring a boiling solution of 0.1% (w/v) SDS over the membrane, and then

allowing the solution to cool to room temperature. Filters were then subjected to autoradiography to ensure the probe had been removed.

CHAPTER 3

RESULTS (I)

CARBON SOURCE REGULATION OF GLYCOLYTIC GENE EXPRESSION

3.1 INTRODUCTION.

It is well documented that the expression of many yeast genes is repressed in the presence of glucose (Wills, 1990). Many of these glucose-repressed genes encode enzymes which metabolise carbon sources other than glucose. As pointed out by Gancedo (1987), the physiological meaning for this phenomenon is clear. Since glucose is a carbon source readily metabolised by most organisms, it is advantageous for yeast to metabolise the available glucose, before metabolising alternative carbon sources for which the competition is less intense.

This phenomenon has been shown to operate largely at the level of transcription (Gancedo, 1987), and through analyses of mutants which constitutively express catabolite-repressed genes at a high or low level, the network of *trans*-acting factors are beginning to unfold (Celenza and Carlson, 1989). One of the proteins which mediates catabolite repression is hexokinase II. Certain mutations in the *HXK2* gene result in loss of catabolite regulation. Therefore, hexokinase II has two roles in the cell; it is needed for glycolysis, and also to mediate carbon source regulation (Ma *et al.*, 1989).

Thus, when yeast grows on glucose, synthesis of the TCA cycle enzymes are repressed and fermentative metabolism predominates. This involves the metabolism of glucose by the glycolytic pathway to ethanol. When yeast cells are grown on non-fermentative carbon sources such as ethanol, respirative metabolism predominates and involves activation of the TCA cycle enzymes. However, while glycolysis is apparently redundant during growth on non-fermentable carbon sources, many of the glycolytic enzymes are still required for gluconeogenesis (Fraenkel, 1982), a process which is essentially the reverse of glycolysis. Gluconeogenesis is necessary as intermediates of the glycolytic pathway are required for biosynthesis of macromolecules required by the growing yeast (Fraenkel, 1982).

The two glycolytic enzyme reactions which are specific to glycolysis in yeast are the steps catalysed by phosphofructokinase and pyruvate kinase (Fraenkel, 1982). During gluconeogenesis, oxaloacetate (an intermediate in the TCA cycle) is converted to pyruvate by phosphoenolpyruvate carboxykinase. This step circumvents

the step catalysed by pyruvate kinase during glycolysis. The phosphoenolpyruvate is then metabolised by the glycolytic enzymes operating in "reverse", until the phosphofructokinase step which is catalysed instead by fructose-1,6-diphosphatase during gluconeogenesis (Gancedo and Serrano, 1989).

Apparently contradictory evidence exists as to whether the synthesis of glycolytic enzymes is affected by the presence of glucose in the growth medium. In direct contrast to the glucose repressed genes, it has been demonstrated that the level of many glycolytic enzymes is increased when yeast cells are transferred from gluconeogenic media to glycolytic media, (Maitra and Lobo, 1971; Entian *et al.*, 1984). However, Clifton and Fraenkel (1981) and Baker (1986), demonstrated that the levels of the majority of glycolytic enzymes remained constant when yeast is grown on either a non-fermentative or fermentative carbon source. These differences may be due to variation in the strain of yeast used, its genetic background, and the precise conditions of growth.

While there is debate as to whether the exact levels of the glycolytic enzymes change in response to carbon source, there is evidence that the genes themselves are expressed by alternative means depending on the carbon source available. Clifton and Fraenkel (1981) have isolated a yeast mutant strain (*gcr1*) which shows a decrease in the level of the glycolytic enzymes compared to the parent strain. Interestingly, this decrease in glycolytic enzyme levels is greater when *gcr1* yeast are grown on gluconeogenic carbon sources (to 5% of wild type) than when they are grown fermentatively (to 20-50% of wild type; Clifton and Fraenkel, 1981). In addition the *gcr1* mutant grows slower than the wild type strain when grown on glucose, whereas there is no difference in the growth rates of the mutant and wild-type on gluconeogenic media. This suggests that relatively high levels of the glycolytic enzymes are required for efficient growth on glucose, (Holland *et al.*, 1990). Thus it appears that in a *gcr1* strain most of the glycolytic enzymes are differentially regulated in response to carbon source, with up to 10 times higher level of expression observed during fermentative growth compared with gluconeogenic conditions. This result suggests that in wild-type strains the glycolytic genes are

indeed expressed by alternative (however slight) mechanisms, with this difference being highlighted in a *gcr1* strain.

This study has attempted to clarify the confusion concerning the question of glucose-induced regulation of glycolytic gene expression. This was achieved by isolating RNA from both fermentatively and non-fermentatively grown yeast, and then measuring the abundance of every glycolytic mRNA on both carbon sources. This allowed a direct comparison of the relative induction levels of each glycolytic mRNA to be made. The regulation of *PYK1* in response to carbon source was then studied in greater detail.

Previous workers in this laboratory have demonstrated that there is an element within the *PYK1* coding region which is required for optimum levels of *PYK1* transcription (Purvis *et al.*, 1987a). The *PYK1* DAS element resides in the first 850 bp of the *PYK1* coding region and preliminary evidence suggested that it may be involved in mediating carbon source regulation (Lithgow *et al.*, 1990). Experiments in this study were carried out to analyse this further.

3.2 RESULTS.

3.2.1 GROWTH OF *S.CEREVISIAE* ON A GLYCOLYTIC AND GLUCONEOGENIC CARBON SOURCE.

The haploid yeast strain DBY746 was used to carry out the analysis of carbon-source regulation of glycolytic mRNAs. Lactate was used as the gluconeogenic carbon source and glucose as a fermentable carbon-source (Fig. 1.3). DBY746 was grown as described in 2.2.1.2, separately in 200ml of YPL and YPG (in a 500ml conical flask) to the mid-logarithmic phase of growth (to an absorbance at 600nm = 0.6). At this stage, 100 ml of the yeast culture were used for RNA extraction, and 50 ml for protein extraction.

Growth curves of yeast on each carbon source are shown in Fig. 3.1. In the case of the glucose grown culture, the doubling time during exponential phase was approximately 90 mins, while on lactate the doubling time was about 275 mins. Thus, under these conditions DBY746 grew 3 times faster on glucose compared with lactate.

Figure 3.1 Growth curve of DBY746 on glucose and lactate.

DBY746 was grown separately in 200ml of YPL and YPG. Doubling time was monitored by measuring absorbance at 600 nm of duplicate samples taken at hourly intervals. Arrows denote point at which cultures were harvested for RNA and protein extractions.

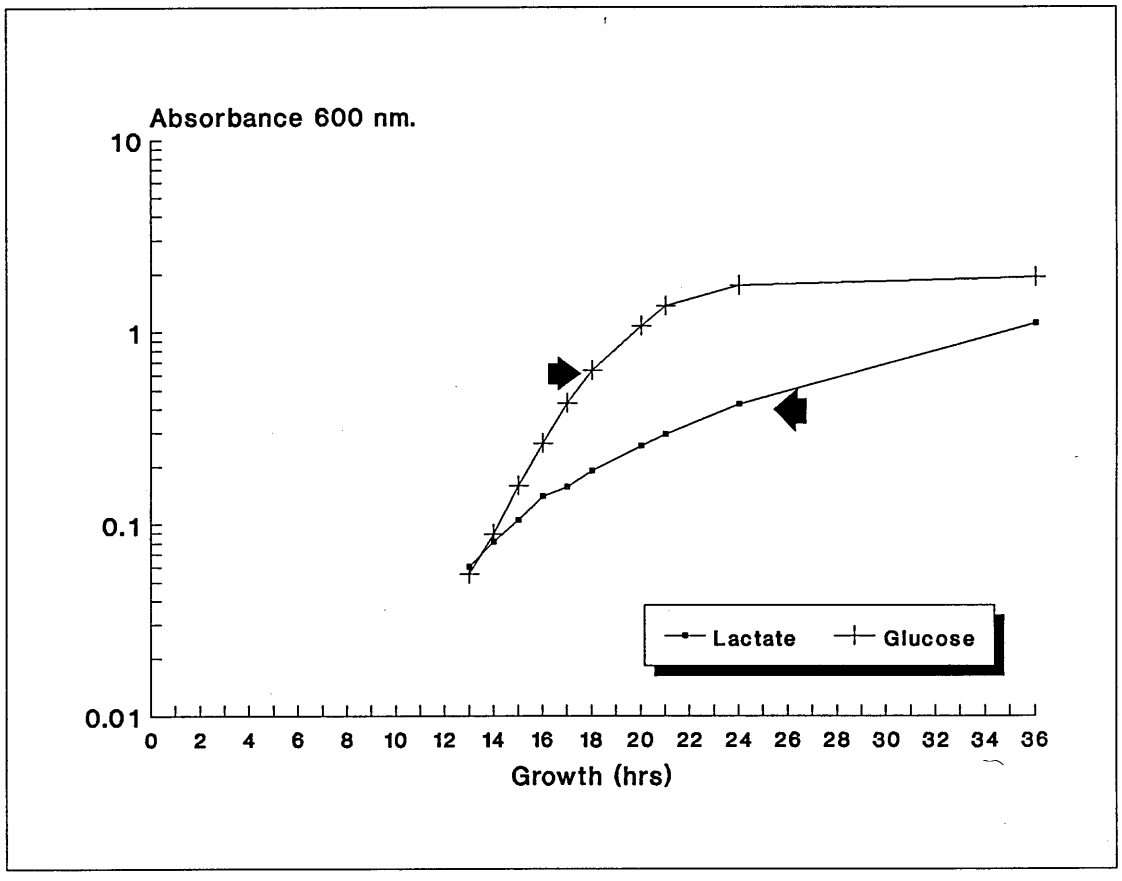
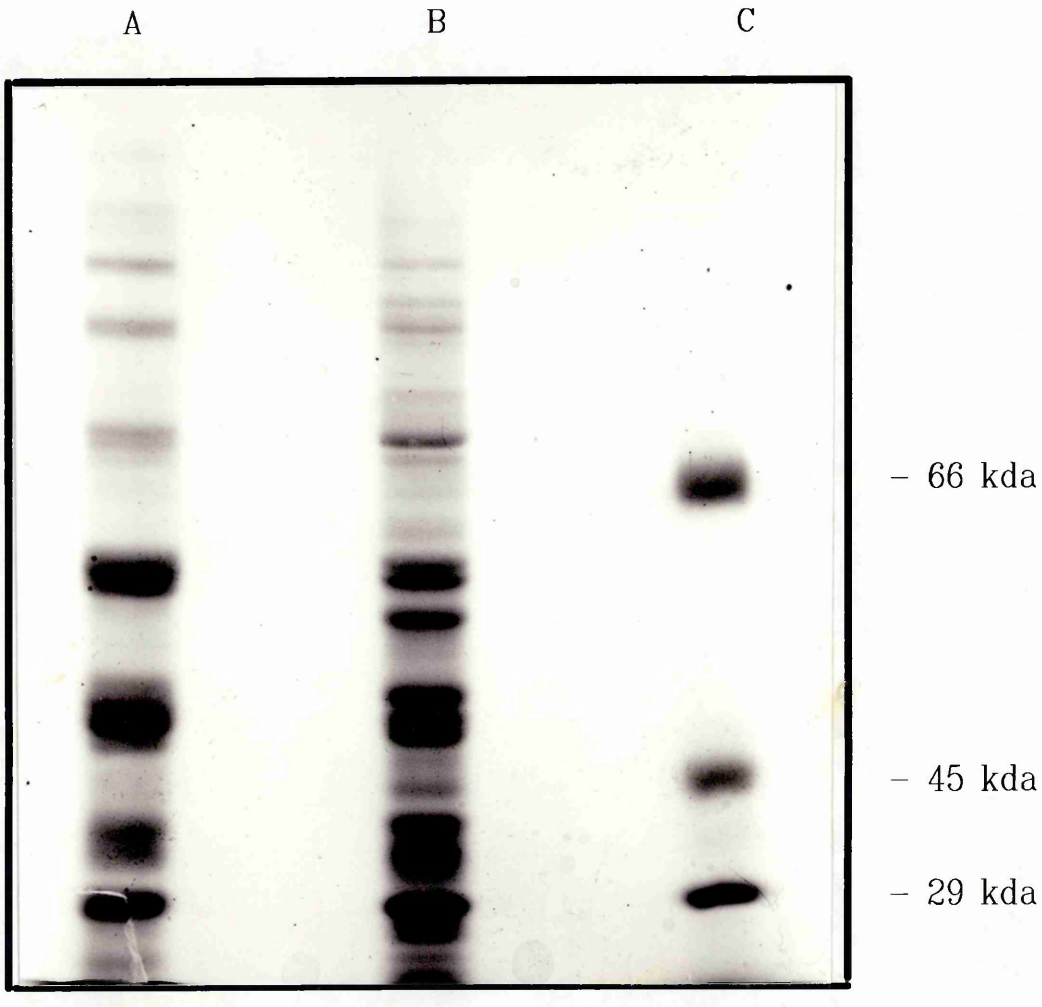

Absorbance 600 nm.
10
1
0.1
0.01
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Growth (hrs)
Lactate
Glucose

Figure 3.2 SDS PAGE analysis of protein extracts.

Protein extracts were prepared from DBY746 grown on (A) glucose and (B) lactate and subjected to SDS-PAGE together with protein size markers (C). The gel was stained using Coomassie blue.


A
B
C
– 66 kda
– 45 kda
– 29 kda

3.2.2 QUALITATIVE ANALYSIS OF PROTEIN SYNTHESIS ON DIFFERENT CARBON SOURCES.

Soluble cellular protein was extracted from both cultures as described in 2.2.6.1 and analysed by SDS-PAGE as described in 2.2.6.3. 50 mg of each protein preparation was electrophoresed on an 8% polyacrylamide gel. The gel was stained using Coomassie blue and is shown in Fig. 3.2 calibrated with size markers. Clearly there are major differences in the levels of specific intracellular proteins expressed on the different carbon sources demonstrating that protein synthesis in *S.cerevisiae* is regulated in response to carbon source.

Visualisation of the gel reveals that several proteins are highly expressed on lactate but are absent or expressed at a relatively low level on glucose These proteins are presumably under catabolite repression. Conversely, there are no clear examples of proteins which are expressed exclusively on glucose. However, the resolution of the gel is not optimal, and it is very likely that some proteins co-migrate. Although the repression of many proteins is evident, it is not possible to conclude from this analysis whether the intracellular level of glycolytic enzymes are elevated on glucose.

3.2.3 REGULATION OF SPECIFIC mRNA LEVELS IN RESPONSE TO CARBON SOURCE.

Total yeast RNA was isolated from the two cultures by the method described in 2.2.4.1. The RNA was examined on a formaldehyde gel by EtBr staining before further analysis to give both an indication of integrity and yield. The abundance of each individual glycolytic mRNA on both carbon sources was then determined by Northern analysis of RNA isolated from the fermentative and non-fermentative yeast cultures. Quantitation was achieved using the AMBIS β-scanner. Filters were then stripped of probe and reprobed for the actin transcript to adjust for differences in RNA loading.

3.2.3.1 Quantitation analysis using the AMBIS β-scanner.

Prior to mRNA abundance studies, several preliminary tests were conducted to ensure that the AMBIS β-scanner was suitable for mRNA quantitation from Northern blots. It was imperative that the AMBIS β-scanner displayed a linear response across the range of cpm to be detected on the Northern blots. This was checked by analysing a serial dilution of radioactive material: this dilution was created by dotting out a dilution series of a radioactive probe onto a nylon membrane and leaving to air-dry. This filter was sealed in a plastic bag, scanned by the AMBIS β-scanner, and the radioactivity per dot quantified by the AMBIS software. Using the AMBIS software it is possible to determine the level of activity in any particular part of the filter; thus the level of radioactivity in any dot or slot can be accurately determined. The results of the scan are shown in Fig. 3.3a. This graph shows that the AMBIS β-scanner displays a linear response to increases in cpm from 0 cpm to 1400 cpm. Thus, it is clear that the AMBIS β-scanner can be confidently used as a quantitative tool in this study

3.2.3.2 Quantitation of glycolytic mRNA levels.

A range of identical Northern filters were prepared using RNA from the same two RNA preparations (RNA isolated from lactate-grown yeast and RNA from glucose-grown yeast). Thus, a direct comparison of the relative levels of induction of each glycolytic mRNA was possible. Before blotting, these gels were stained with EtBr to identify the 18S and 25S rRNA bands to determine the approximate lengths of the transcripts of interest. The Northern filters were then probed individually for a specific glycolytic mRNA using the plasmids or oligonucleotides described in Tables 2.1 and 2.2. The following mRNAs were probed using random-primed linearised plasmid: *PYK1, PGK, ENO2, PFK1, PFK2, PDC1, FBA1* (see 2.2.7 for random-prime labelling). The remaining glycolytic mRNAs were probed using [^{32}P]-end-labelled oligonucleotides: *TDH, HXK, TPI, PGM, PGI, ADH1* (see section 2.2.7. for oligonucleotide labelling). The *PFK1* and *PFK2* mRNAs were analysed using oligonucleotides as well as random-primed probes because the

Figure 3.3 Linear quantitation by the AMBIS β-scanner.

A serial dilution series of radioactive material was analysed by the AMBIS β-scanner. Each point on the graph is the average of two measurements.

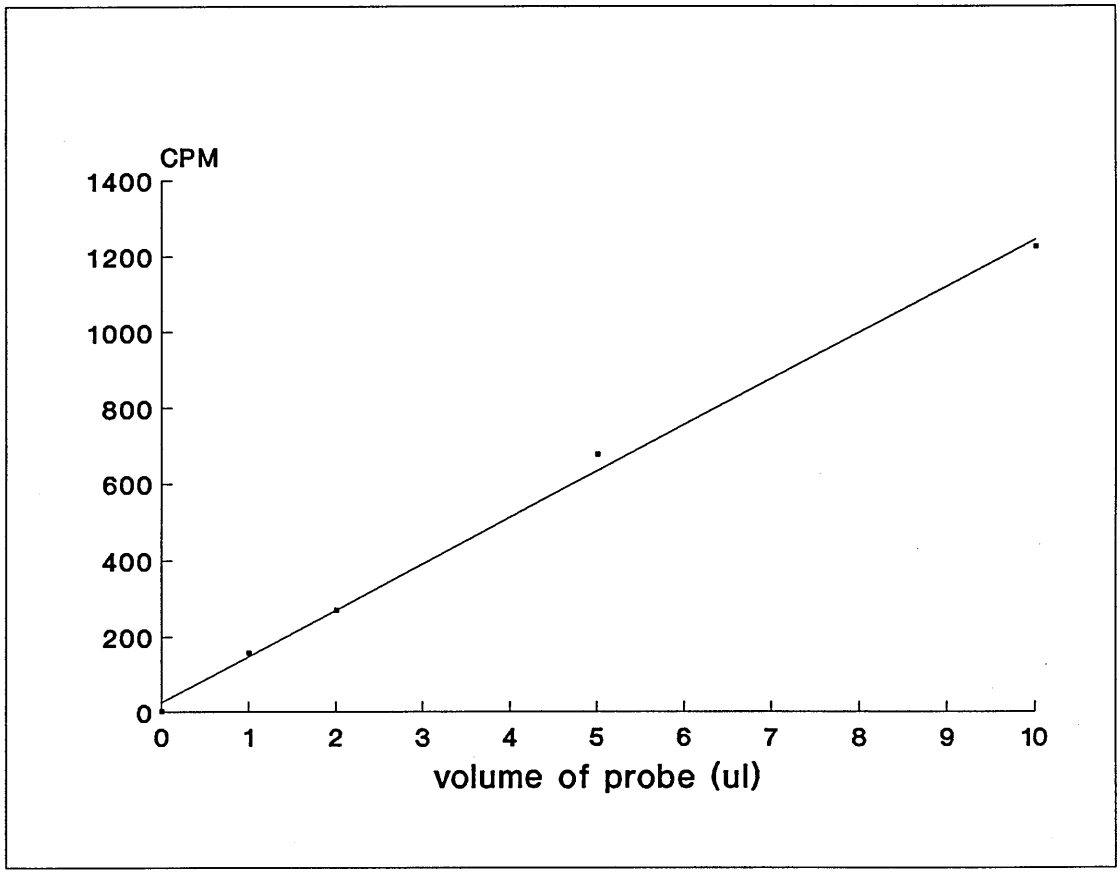

CPM
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
9
10
volume of probe (ul)

Figure 3.4a Induction of Yeast Glycolytic mRNAs by Glucose.

The abundance of each glycolytic mRNA was determined with respect to the abundance of the actin mRNA in yeast grown on glucose and lactate. See list of abbreviations for complete gene names. The activation of glucose (relative level on glucose / relative level on lactate) for each of these mRNAs and *LPD1* is presented graphically and the actual value of induction shown above each lane. The values for *PFK1* and *PDC1* were determined from two separate Northern analyses, while *PFK2* was determined from three separate analyses. The variation between these was +/- 8%.

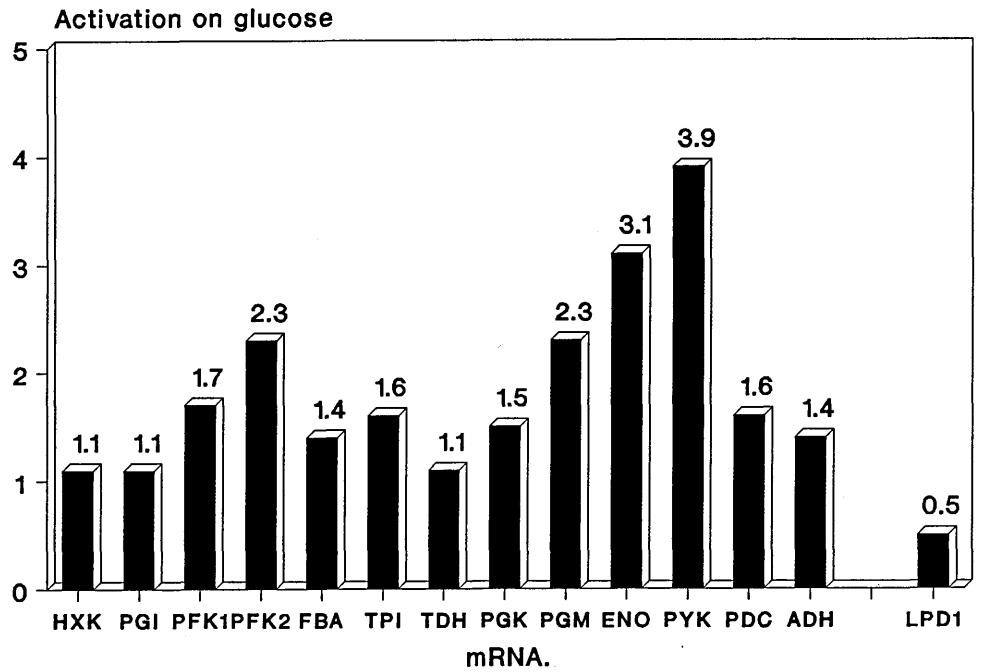

Activation on glucose
5
4
3
2
1
0
1.1
1.1
1.7
2.3
1.4
1.6
1.1
1.5
2.3
3.1
3.9
1.6
1.4
0.5
HXK
PGI
PFK1
PFK2
FBA
TPI
TDH
PGK
PGM
ENO
PYK
PDC
ADH
LPD1
mRNA.

Fig 3.4b Determination of *PYK1* and *LPD1* abundance.

RNA isolated from yeast cultured on lactate (A) and glucose (B) was electrophoresed and Northern blotted. The filter was analysed sequentially for *PYK1*, *LPD*, and *ACT1* mRNAs. The amount of bound radioactivity was determined by the AMBIS β-scanner.

Fig 3.4c Determination of *PDC1* mRNA abundance.

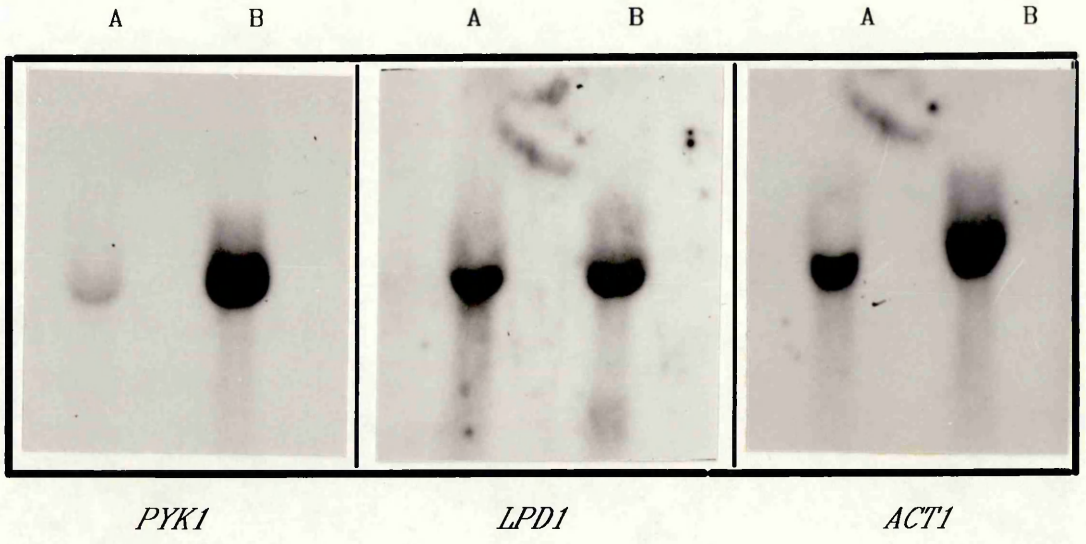
RNA isolated from yeast cultured on glucose (A, C) and lactate (B) was electrophoresed and Northern blotted. The filter was analysed sequentially for the *PDC1* and *ACT1* mRNAs. The amount of bound radioactivity was determined by the AMBIS β-scanner.

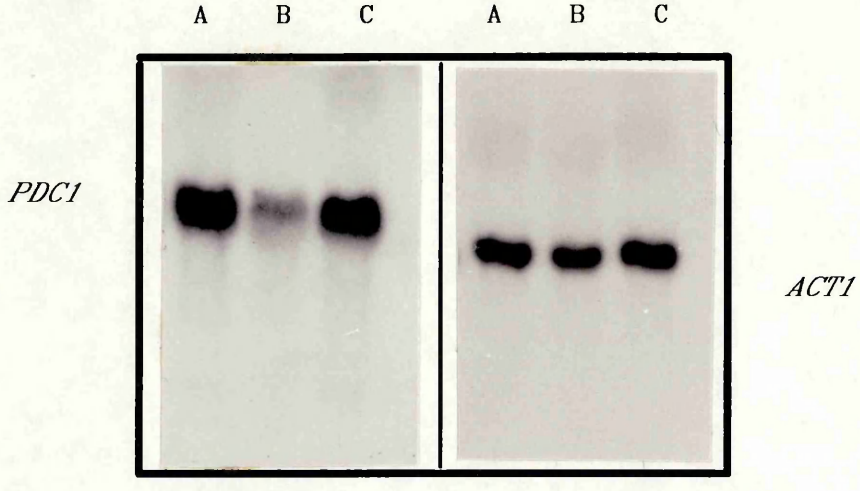
Fig 3.4d Determination of *TDH 1-3* mRNA abundance.

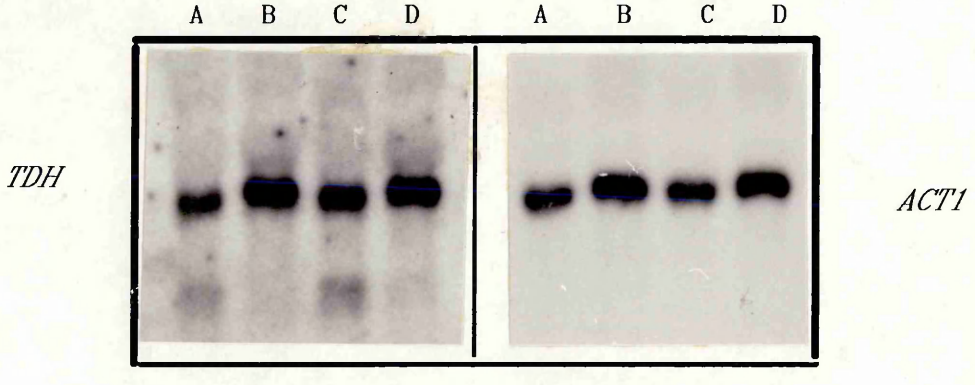
RNA isolated from yeast cultured on lactate (A, C) and glucose (B, D) was electrophoresed and Northern blotted. The filter was analysed sequentially for *TDH1-3* and *ACT1* mRNAs. The amount of bound radioactivity was determined by the AMBIS β-scanner.

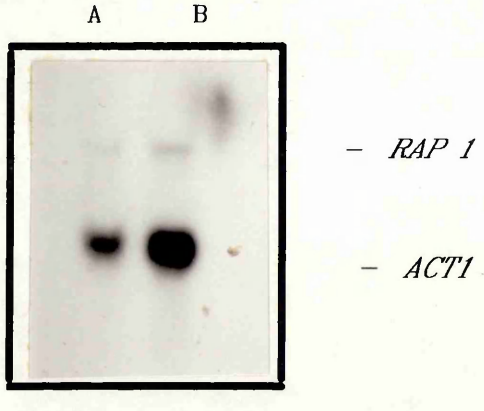
Fig 3.4e Determination of *RAP1* mRNA abundance.

RNA isolated from yeast cultured on lactate (A) and glucose (B) was electrophoresed and Northern blotted. The filter was analysed for *RAP1* and *ACT1* mRNAs. The filter was probed first for *RAP1*, and without stripping, then analysed for the *ACT1* mRNA. The amount of bound radioactivity was determined by the AMBIS β-scanner.


A
B
A
B
A
B
PYK1
LPD1
ACT1


A
B
C
A
B
C
PDC1
ACT1


A
B
C
D
A
B
C
D
TDH
ACT1


A
B
– RAP 1
– ACT1

PFK1 and *PFK2* genes are homologous and it was possible that the plasmid probes may have cross-hybridised (Heinisch, 1986). However, similar results were obtained with the gene-specific oligonucleotides and the plasmid probes suggesting that any cross-hybridisation which may have occurred did not affect the result obtained. The levels of *LPD1* mRNA (a carbon catabolite-repressed gene) and *RAP1* mRNA (a protein involved in glycolytic gene expression) on both lactate and glucose were also determined. *LPD1* mRNA was analysed using a 3.6kb Xho1-Xho1 fragment purified from plasmid pGP.R1, which was random-prime labelled. *RAP1* mRNA was probed using random-prime labelled plasmid (see Table 2.1). After probing each filter for a particular mRNA, the filter was scanned using the AMBIS ß-scanner to determine the CPM bound to the mRNA of interest in both the lactate lane and glucose lane. The hybridised probe was then stripped from these filters and the filters re-probed with random-prime labelled actin probe. In some cases, filters were probed for a second mRNA before analysing for the actin mRNA. The actin mRNA probing was used as a loading control, as it is claimed that the level of the actin transcript does not change in response to carbon source (Mayer and Dieckmann, 1989). After determination of the level of actin, the glucose activation was quantified using the following formula.

$$\frac{\text{[cpm for mRNA of interest on glucose]}}{\text{[cpm for mRNA of interest on lactate]}} * \frac{\text{[cpm for actin mRNA on glucose]}}{\text{[cpm for actin mRNA on lactate]}}$$

The results of this analyses are presented in Fig. 3.4a. Multiple analyses of several mRNAs demonstrated the reproducibility of the measurements (Fig. 3.4a). It is apparent from the results of this experiment that while some mRNA levels increase 4-fold on addition of glucose (eg *PYK1*; see Fig. 3.4b), others are moderately induced (eg. *PDC1*; see Fig. 3.4c), and some are constitutively expressed (eg *TDH*; see Fig. 3.4d). Therefore glycolytic mRNAs are differentially induced in response to glucose. As expected, the *LPD1* mRNA was found to be

repressed approximately 2-fold on glucose (see Fig. 3.4b). On the other hand, the level of the *RAP1* mRNA remained constant under the conditions used in this study (see Fig. 3.4e).

3.2.4 CARBON SOURCE REGULATION OF *PYK1* EXPRESSION.

From the Northern analysis described above, it is apparent that *PYK1* mRNA levels are elevated 4-fold during growth on glucose. Indeed, *PYK1* appears to be the most induced of all the glycolytic genes. While it is possible that this is caused by differential mRNA stability on the two carbon sources, it is likely that this regulation is mediated largely through transcription. Thus, regulation of the *PYK1* gene on the two carbon sources was characterised further. Possible differences in the transcriptional start point of the *PYK1* gene on both of the carbon sources was tested. Also chromosomal *PYK1:lacZ* fusions were created in order to determine if a 3'-element is involved in the carbon source regulation of *PYK1* (Lithgow *et al.*, 1990). Using the *PYK1:lacZ* fusions, the influence of carbon-source on *PYK1* translation was also determined.

3.2.4.1 5' end mapping of the *PYK1* mRNA.

The primer extension method used to map the 5' end of the *PYK1* mRNA is described in 2.2.4.4 and the oligonucleotide used is described in Table 2.2. This oligonucleotide is designed to hybridise between bases +3 and +24 of the *PYK1* mRNA on the basis of the known sequence of *PYK1* (Burke *et al.*, 1983).

10µg of the same lactate or glucose RNA preparations described in 3.2.2.2 were used for this analysis. The reaction products of the primer extension reaction were electrophoresed on a sequencing gel calibrated with a sequencing ladder. This gel (Fig. 3.5a) shows that the same major transcript and minor transcript start sites are present in both lanes. Thus, it appears there is no differential use of *PYK1* transcript start sites in response to carbon source.

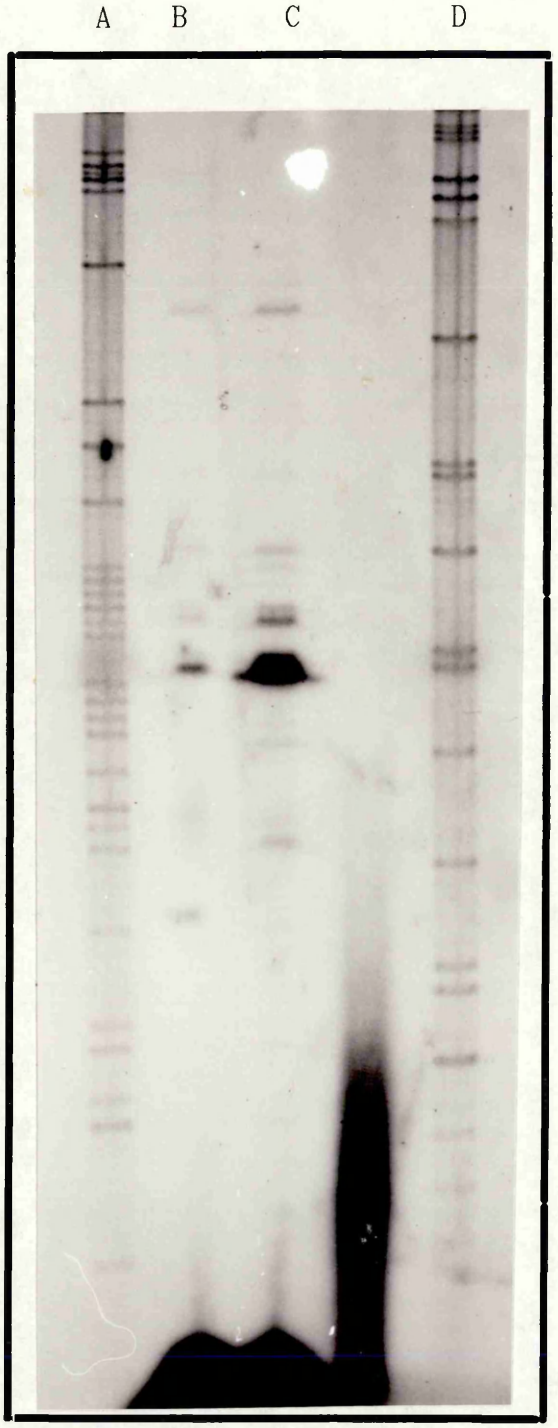
Interestingly, the intensity of the bands are considerably stronger for the glucose RNA than for the lactate RNA even though

Figure 3.5a Primer Extension analyses of *PYK1* mRNA.

RNA isolated from yeast grown on lactate (B) and glucose (C) was subjected to primer extension analysis to determine the 5'-transcriptional start site of the *PYK1* mRNA. Adenine (A) and Thymidine (T) tract sequencing ladders of known length were used as size markers. The position of the major and minor transcripts and the corresponding length of the extended products are denoted.

Figure 3.5b *PYK1* **Transcriptional start sites.**

The DNA sequence of the 60 nt immediately 5' to the *PYK1* coding region are presented. The positions of the start sites are denoted below the sequence with the major transcript depicted by (≥) and the minor transcript by (>). The lengths of the 5' untranslated region for each transcript are also given. The sequence underlined corresponds to the region previously mapped by Burke and co-workers to where *PYK1* transcription initiates.



- minor transcript 82 nt
- minor transcript 61 nt
- minor transcript 55 nt
- major transcript 52 nt
- unextended primer 21 nt

ATTATTCTCTCTTGTTTCTATTTACAAGACACCAATCAAAACAAATAAAACATCATCACA ATG

> > > ≥

58 37 31 28

similar levels of RNA were used in each reaction. This confirms the induction of *PYK1* mRNA levels in response to glucose.

The major extension product from the *PYK1* transcripts in each culture is 52 nt in length, while the minor extension products are 55, 61, and 82 nt in length. The sequence of the 5' untranslated leader for each of the transcripts and the nucleotide at which the transcript initiates is shown in Fig. 3.5b.

3.2.4.2 Construction of *PYK1:lacZ* gene fusion plasmids.

PYK1:lacZ gene fusions were made to determine if the sequences required for carbon source regulation of *PYK1* reside in the 5' promoter region or whether sequences 3' to the start codon are involved. To avoid the complication of plasmid copy number, constructs were integrated into the chromosome where they are stably inherited (Struhl, 1983). An outline of the route taken to create the desired vectors is given in Fig. 3.6a. Fortunately, *PYK1:lacZ* gene fusions were already available in the lab, having been made by Dr I.J. Purvis. However, these were carried on an unsuitable vector, and the first stage of the plasmid constructions involved the creation of an integrating construct. The essential plasmids involved in this half of the plasmid construction are shown in Fig. 3.6b.

Creation of pLD10: The original plasmid from which all the constructs were made was YCpKev1333, which contains a 6.3 kb genomic insert containing the PYK1 gene inserted into YCp50. The plasmid, shown in Fig. 3.6b, contains a slightly modified *PYK1* gene, having a polylinker sequence immediately 3' to the coding region. This sequence does not affect *PYK1* expression (Purvis *et al.*, 1987c) but makes the manipulation of the *PYK1:lacZ* fusions more straight-forward.

Previous attempts in this laboratory to integrate *PYK1* sequences into the genome have been foiled due to the existence of an origin of replication at the 5' end of the genomic fragment (A. Brown personal communication.) This element has not been finely mapped, but analysis of available DNA sequence (Burke *et al.*, 1983) showed that no strong homology to the 11bp consensus sequence (Newlon, 1988) for the yeast

Figure 3.6a Creation of plasmids pLD305 and pLD405.

The steps involved in the creation of the integrating vectors, pLD305 and pLD405, and the plasmids involved (boxed) are presented. Maps of each plasmid are presented in Figs. 3.6b and 3.6c.

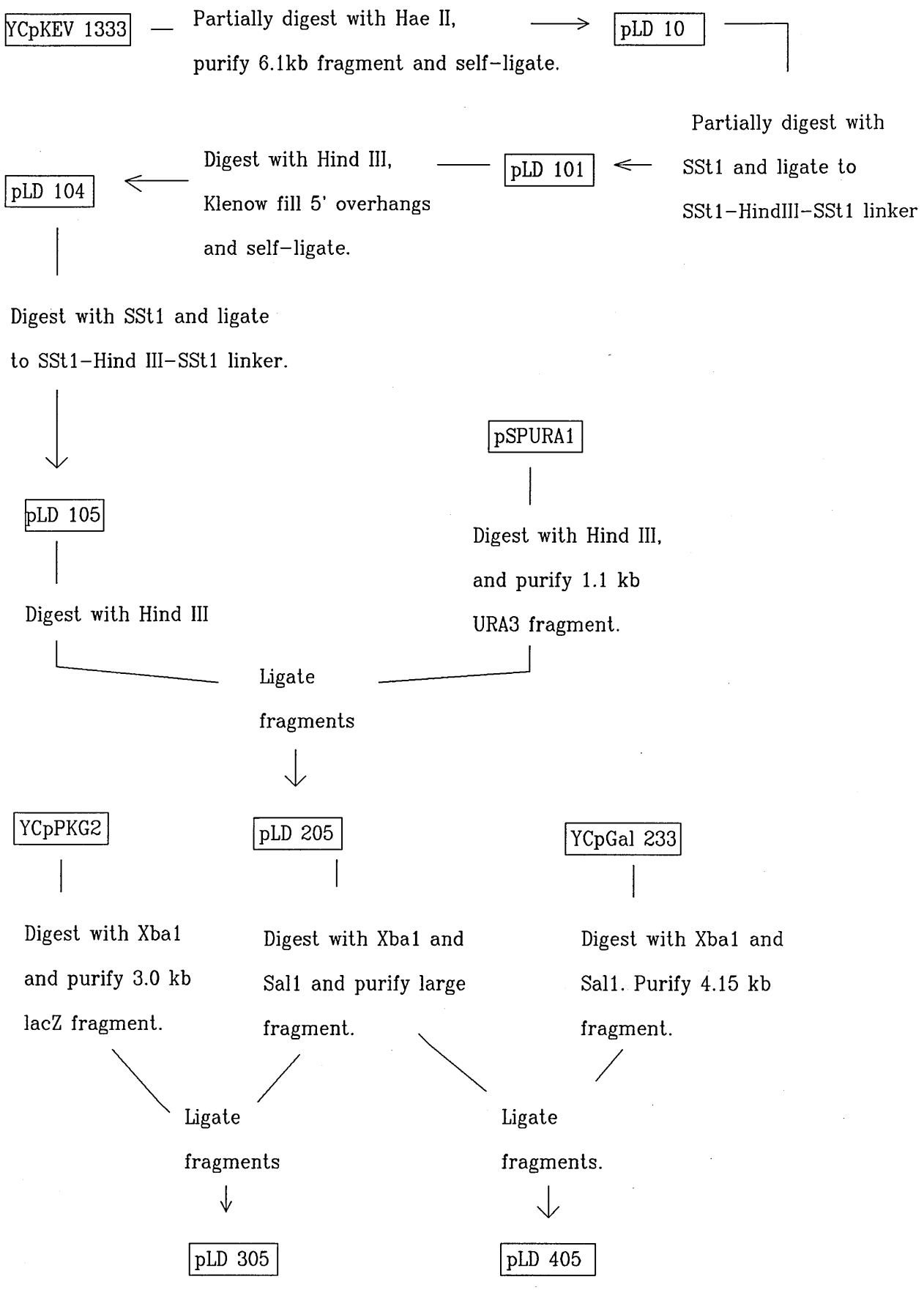
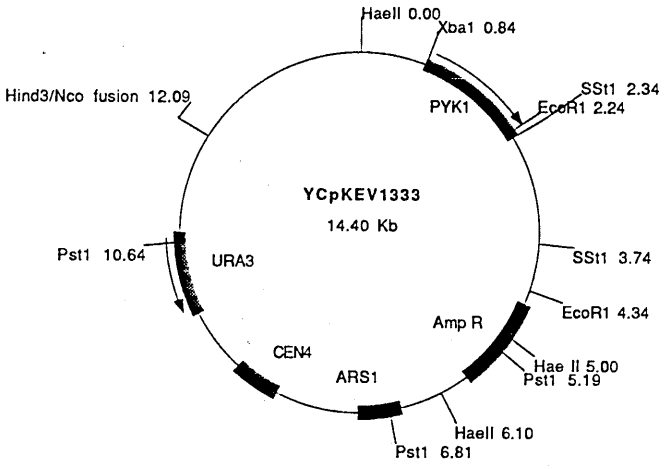
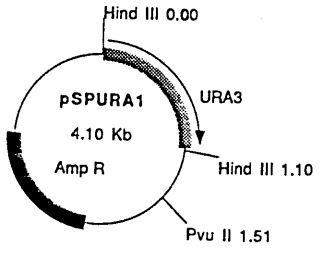
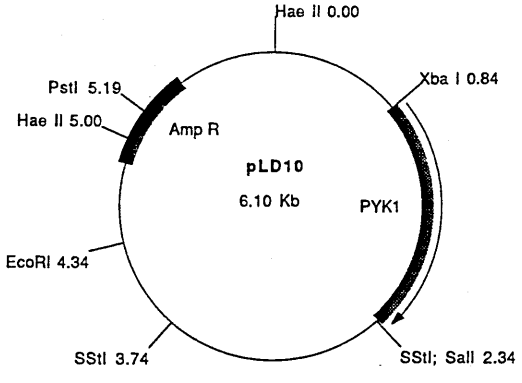

YCpKEV 1333
Partially digest with Hae II,
purify 6.1kb fragment and self-ligate.
pLD 10
Partially digest with
SSt1 and ligate to
SSt1-HindIII-SSt1 linker
pLD 101
Digest with Hind III,
Klenow fill 5' overhangs
and self-ligate.
pLD 104
Digest with SSt1 and ligate
to SSt1-Hind III-SSt1 linker.
pLD 105
Digest with Hind III
pSPURA1
Digest with Hind III,
and purify 1.1 kb
URA3 fragment.
Ligate
fragments
YCpPKG2
Digest with Xba1
and purify 3.0 kb
lacZ fragment.
pLD 205
Digest with Xba1 and
Sal1 and purify large
fragment.
YCpGal 233
Digest with Xba1 and
Sal1. Purify 4.15 kb
fragment.
Ligate
fragments
Ligate
fragments.
pLD 305
pLD 405

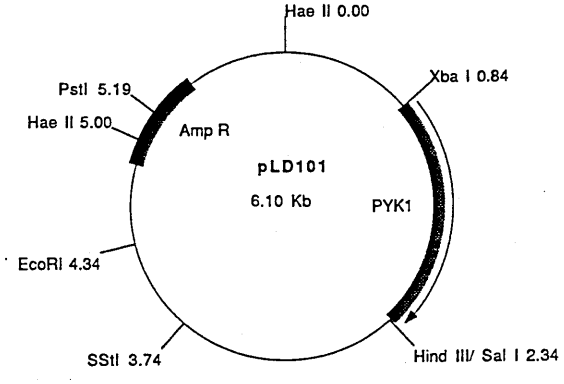
Figure 3.6b Plasmids involved in the creation of pLD205.

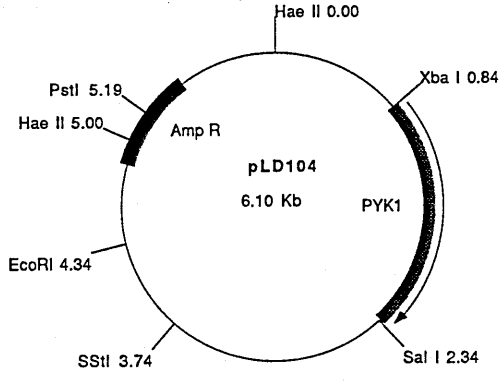
Plasmid maps of the vectors involved in the creation of the *PYK1* YIp vector (pLD 205) are shown. Restriction sites marked are not unique, and their distance from an arbitrary origin (0.00) restriction site are given in kb.

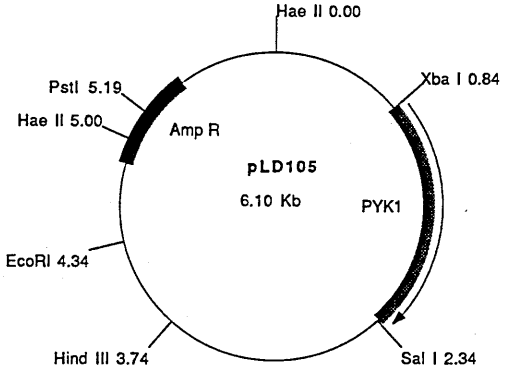

HaeII 0.00
Xba1 0.84
SSt1 2.34
EcoR1 2.24
PYK1
Hind3/Nco fusion 12.09
YCpKEV1333
14.40 Kb
Pst1 10.64
URA3
SSt1 3.74
EcoR1 4.34
Amp R
CEN4
ARS1
Hae II 5.00
Pst1 5.19
HaeII 6.10
Pst1 6.81


Hind III 0.00
pSPURA1
4.10 Kb
URA3
Amp R
Hind III 1.10
Pvu II 1.51


Hae II 0.00
PstI 5.19
Hae II 5.00
Amp R
Xba I 0.84
pLD10
6.10 Kb
PYK1
EcoRI 4.34
SStI 3.74
SStI; SalI 2.34


Hae II 0.00
PstI 5.19
Hae II 5.00
Amp R
Xba I 0.84
pLD101
6.10 Kb
PYK1
EcoRI 4.34
SStI 3.74
Hind III/ Sal I 2.34


Hae II 0.00
PstI 5.19
Hae II 5.00
Amp R
Xba I 0.84
pLD104
6.10 Kb
PYK1
EcoRI 4.34
SStI 3.74
Sal I 2.34


Hae II 0.00
PstI 5.19
Hae II 5.00
Amp R
Xba I 0.84
pLD105
6.10 Kb
PYK1
EcoRI 4.34
Hind III 3.74
Sal I 2.34

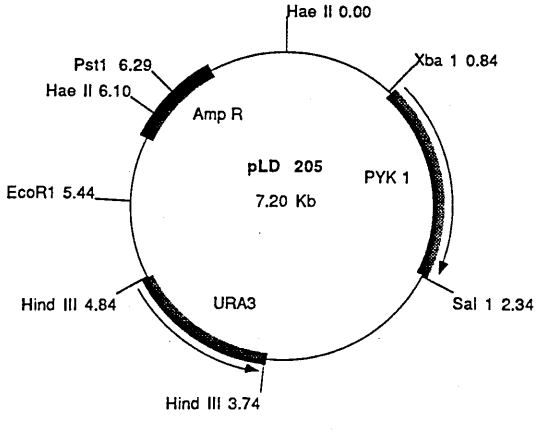
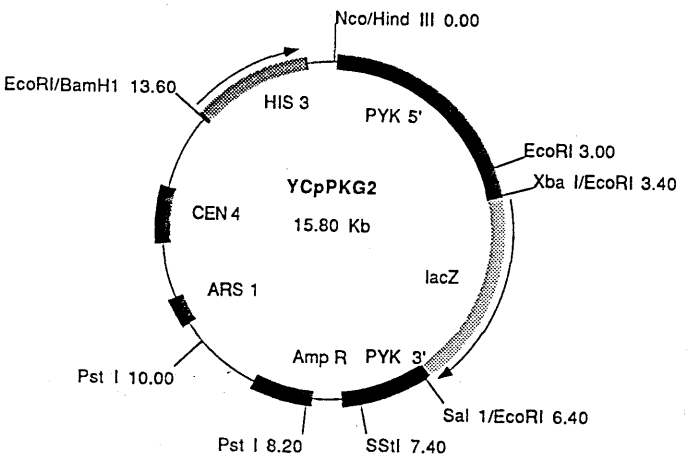
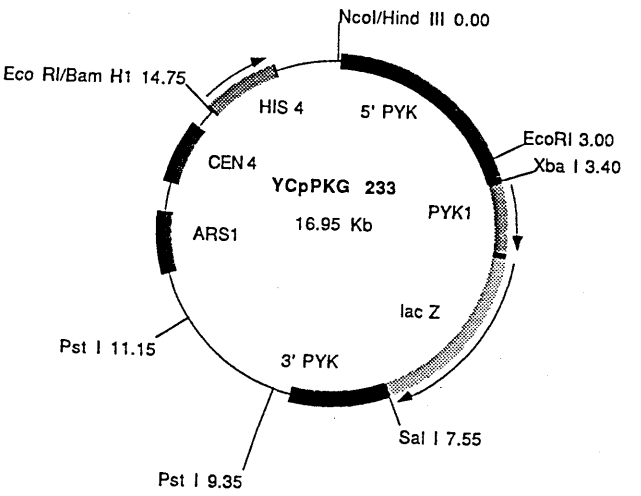
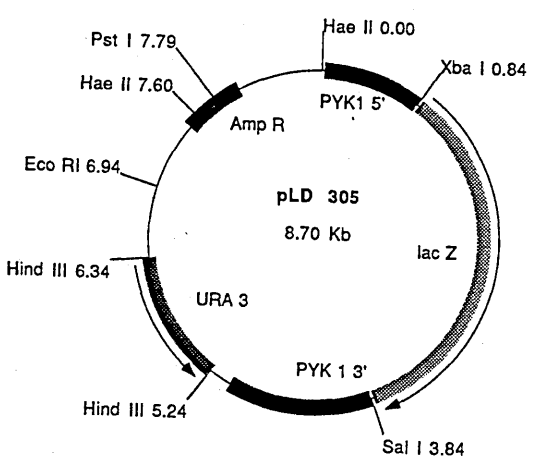

Hae II 0.00
Pst1 6.29
Hae II 6.10
Amp R
Xba 1 0.84
pLD 205
7.20 Kb
PYK 1
EcoR1 5.44
Hind III 4.84
URA3
Sal 1 2.34
Hind III 3.74

Figure 3.6c *PYK1:lacZ* containing plasmids.

The vectors YCpPKG2 and YCpPKG233 were used as the source of *lacZ* and *PYK1:lacZ* fusion genes in the creation of pLD305 and pLD405 respectively.


Nco/Hind III 0.00
EcoRI/BamH1 13.60
HIS 3
PYK 5'
EcoRI 3.00
Xba I/EcoRI 3.40
YCpPKG2
CEN 4
15.80 Kb
ARS 1
lacZ
Amp R
PYK 3'
Pst I 10.00
Sal 1/EcoRI 6.40
Pst I 8.20
SStI 7.40


NcoI/Hind III 0.00
Eco RI/Bam H1 14.75
HIS 4
5' PYK
EcoRI 3.00
Xba I 3.40
CEN 4
YCpPKG 233
16.95 Kb
PYK1
ARS1
lac Z
Pst I 11.15
3' PYK
Sal I 7.55
Pst I 9.35


Pst I 7.79
Hae II 0.00
Hae II 7.60
Xba I 0.84
PYK1 5'
Amp R
Eco RI 6.94
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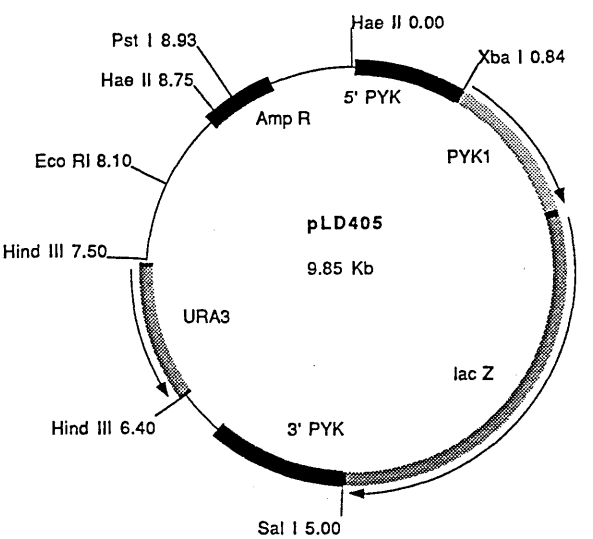
8.70 Kb
lac Z
Hind III 6.34
URA 3
PYK 1 3'
Hind III 5.24
Sal I 3.84


Pst I 8.93
Hae II 0.00
Hae II 8.75
Xba I 0.84
5' PYK
Amp R
PYK1
Eco RI 8.10
pLD405
9.85 Kb
Hind III 7.50
URA3
lac Z
Hind III 6.40
3' PYK
Sal I 5.00

origin of replication exists in the 1000 bp immediately 5' to the *PYK1* coding sequence. This suggested that the origin of replication resides further upstream from the *PYK1* coding region. Therefore, upstream sequences were removed using the *Hae* II site 824bp 5' to the *PYK1* coding region, leaving all the 5' sequences required for wild-type *PYK1* expression (Nishizawa *et al.*, 1988). YCpKev1333 was partially digested with *Hae* II and the desired 6.1kb *Hae* II fragment containing 824 bp of *PYK1* 5' UTR, 1.5 kb of *PYK1* coding region, 1.4 kb of *PYK1* 3' UTR, the Amp^r gene, and the bacterial origin of replication was isolated (see Fig. 3.6b). The gel purified fragment was self-ligated to create pLD10 (see Fig. 3.6b) which replicates in *E.coli* but not in yeast.

Creation of pLD 101: The next step in the cloning strategy was the insertion of a selectable yeast marker. The *URA3* gene was isolated as a 1.1kb *Hind* III fragment from pSPURA1 (see Fig. 3.6b). Unfortunately no *Hind* III sites exist in pLD10. Therefore an *Sst* I site which resides 1.4 kb downstream of the *PYK1* coding region was converted to a *Hind* III site by subcloning the self-complementary oligonucleotide 5'-GCAAGCTTGCAGCT-3' into the *Sst* I site. A complication with this sub-cloning was the presence of a second *Sst* I site in the polylinker region, immediately 3' of the coding region. Thus, pLD10 was partially digested with *Sst* I and ligated with a 40 molar excess of the annealed oligonucleotide. The oligonucleotide was self-annealed by heating 150ng of the oligonucleotide in 100µl TE at 65°C for 5 mins and cooling slowly to 37°C. Unfortunately, all of the plasmids which had oligonucleotide insertions, had the oligonucleotide inserted at the *Sst* I site immediately 3' to the *PYK1* coding region giving rise to plasmid pLD101. Therefore, it appeared that the pLD10 was always cut first at this *Sst* I site and not at the desired site. Bearing this in mind, it was decided to purify pLD101 and modify this plasmid to allow sub-cloning of the *URA3* gene.

Creation of pLD104: This plasmid is identical to pLD101 with the exception that the *Hind* III site 3' to the *PYK1* coding region is destroyed. This was achieved by digesting the new unique *Hind* III site in pLD101, followed by Klenow filling of the cohesive ends and

religation. As a result, pLD104 has a unique *Sst* I site in a suitable position for the insertion of the *URA3* gene (Fig. 3.6b).

Creation of pLD105: This plasmid was created by digesting pLD104 to completion with *Sst* I and ligation with the annealed oligonucleotide described above. This generated a unique *Hind* III site 1.4kb 3' to the *PYK1* gene. A diagram of this plasmid is given in Fig. 3.6b.

Creation of pLD205: The 1.1kb *Hind* III *URA3* fragment isolated from pSPURA1 was inserted into the unique site in pLD105 to give pLD205 (Fig. 3.6b). The desired clone was isolated by colony hybridisation using the *URA3* fragment as a probe. The orientation of *URA3* gene was checked by diagnostic restriction analysis (not shown). This vector now contains all the necessary requirements for a *PYK1* integrating plasmid vector.

The next stage in the creation of the *PYK1:lacZ* reporter system involved the removal of *PYK1* sequences and the insertion of the *lacZ* coding region. The plasmids involved in this half of the construction are shown in Fig. 3.6c.

Creation of pLD305: The *PYK1* coding region was removed from pLD205 by digestion with *Xba* I and *Sal* I, and the large vector fragment purified as described in section 2.2.3.8. This fragment was then ligated with the *lacZ* coding region which had been gel purified as an *Xba* I-*Sal* I fragment from the plasmid YCpPKG2 (Fig. 3.6c). The resulting plasmid pLD305 contains the *lacZ* gene under the control of the *PYK1* 5' promoter region and is shown in Fig. 3.6c. Therefore, in this construct, the *PYK1* coding region was replaced with that from the *lacZ* gene, with the translational fusion occurring after the second codon of the *PYK1* gene (Purvis *et al.*, 1987c).

Creation of pLD405: pLD205 was digested with *Xba* I and *Sal* I, and the 5.7kb vector fragment gel purified. This fragment was ligated with a fragment of DNA containing the 5' 850bp of *PYK1* coding region fused in frame to the *lacZ* coding region. This second fragment had been isolated and purified as an *Xba* I-*Sal* I fragment from the plasmid YCpKG233 (Fig. 3.6c). The resulting plasmid (pLD405) contains the *lacZ*

gene under the transcriptional control of the *PYK* 5' coding region and also the first 850 bp of coding region (previously shown to contain a DAS element, Purvis *et al.*, 1987a). A diagram of this plasmid is given in Fig. 3.6c.

3.2.4.3 Transformation of yeast and confirmation of plasmid integration.

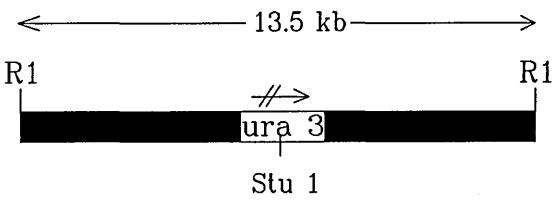
Before transformation of DBY746, both pLD305 and pLD405 were linearised at the *Stu* I site in the middle of the *URA3* gene. This site is unique to the plasmid. The purpose of this is to direct integration to the *ura3* locus of DBY746. An outline of the postulated integration event for pLD305 and pLD405 are shown in Fig. 3.7a and Fig. 3.7b respectively. The data on the positions of the *Eco* RI sites relative to the *ura3* locus is taken from Rose and co-workers (1984).

10µg of each linearised plasmid were used to transform DBY746, with six pLD305 and three pLD405 transformants obtained. This low frequency of transformation is characteristic of integrative transformations. The transformants were patched out onto selective minimal agar to check their auxotrophic markers and also onto X-gal indicator plates. This verified that they were authentic transformants which expressed ß-galactosidase. To verify that integration of the plasmid sequences had occurred at the *ura3* locus, total yeast DNA was extracted from both a pLD305 and a pLD405 transformant, digested with *Eco* RI, and Southern blotted. The filter was probed with random-primed 1.1 kb *Hind* III *URA3* fragment isolated from pSPURA1, and the results of the blot are shown in Fig. 3.8a along with a photograph of the DNA gel calibrated with lambda/*Hind* III standards.

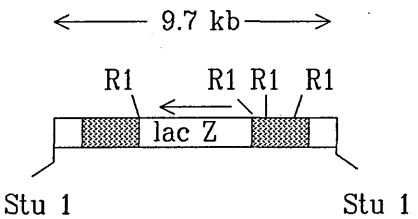
Rose and co-workers (1984) demonstrated that the *URA3* gene is present as a 13.0 kb *Eco* RI fragment on chromosome 5. A similar sized band is seen with the *Eco* RI digest of untransformed DBY746 (see lane 2 of Fig. 3.8a). If integration had occurred at the *ura3* locus then it was anticipated that this 13.0kb fragment should be disrupted upon integration to give two bands of 6.35kb and 10.25kb (see Fig. 3.7a; 3.7b). Analyses of lanes C and D of the Southern blot shown in Fig. 3.8a shows that two bands approximately of this size were observed in the two transformants. Furthermore, the 13.0kb band seen in lane 1

Figure 3.7a **Chromosomal Integration of pLD 305 at the *ura3* locus of *S.cerevisiae*.**

The anticipated results of plasmid integration (single and double) of pLD 305 at the chromosomal *ura3* locus of DBY746 are presented. Integration to the *ura3* locus was directed by linearising pLD405 with *Stu*1 which resides in *URA3* alleles. The lengths of the *Eco*R1 (R1) fragments incorporating *URA3* sequences in both the host and integrant strains are given in kb. In the case of the double integration, the second plasmid copy can integrate at either of the two *URA3* alleles present. Only one of these possibilities is shown.

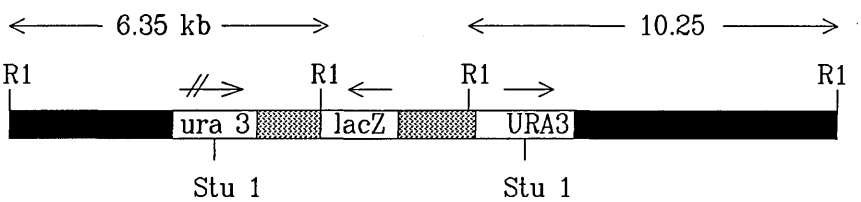


Chromosomal ura3 locus. :

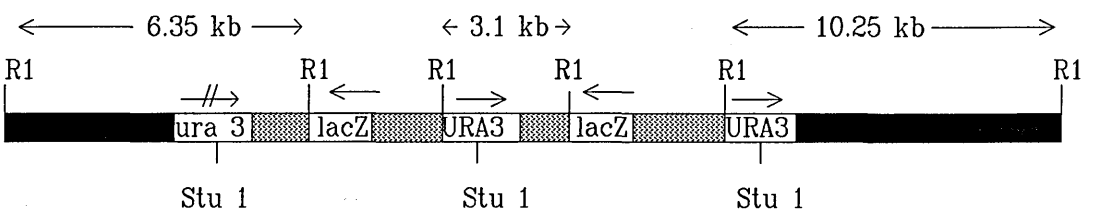


pLD 305 linearised by Stu1 which resides in URA 3. :

Single Integration of plasmid into the Stu1 site present in chromosomal ura3.



Double integration event at the chromosomal locus.

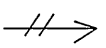


– Chromosomal sequences.

– Plasmid sequences.

– Genes

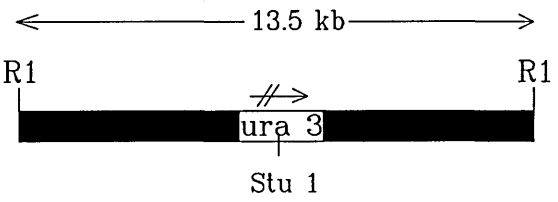
Direction of transcription



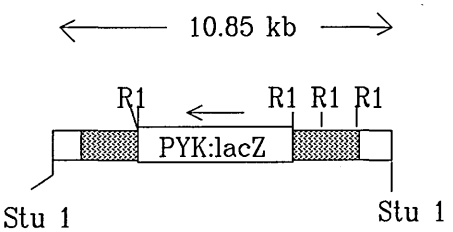
Non-functional gene

Figure 3.7b Chromosomal Integration of pLD 405 at the *ura3* locus of *S.cerevisiae.*

The anticipated result of a single pLD405 integration at the *ura3* locus of DBY746 is presented. Integration at the *ura3* locus was directed by linearising pLD405 with *Stu*1 as before (Fig 3.7a).

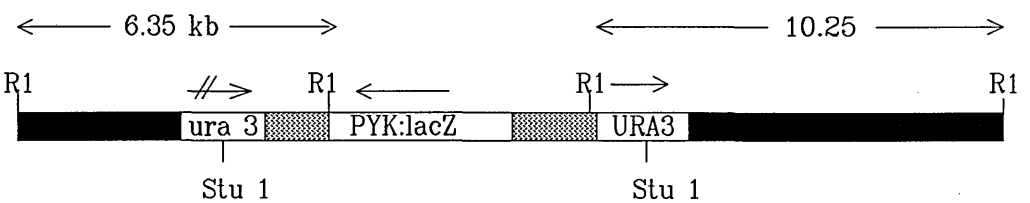


Chromosomal ura3 locus. :



pLD 405 linearised by Stu1 which resides in URA 3. :

Single Integration of plasmid into the Stu1 site present in chromosomal ura3.



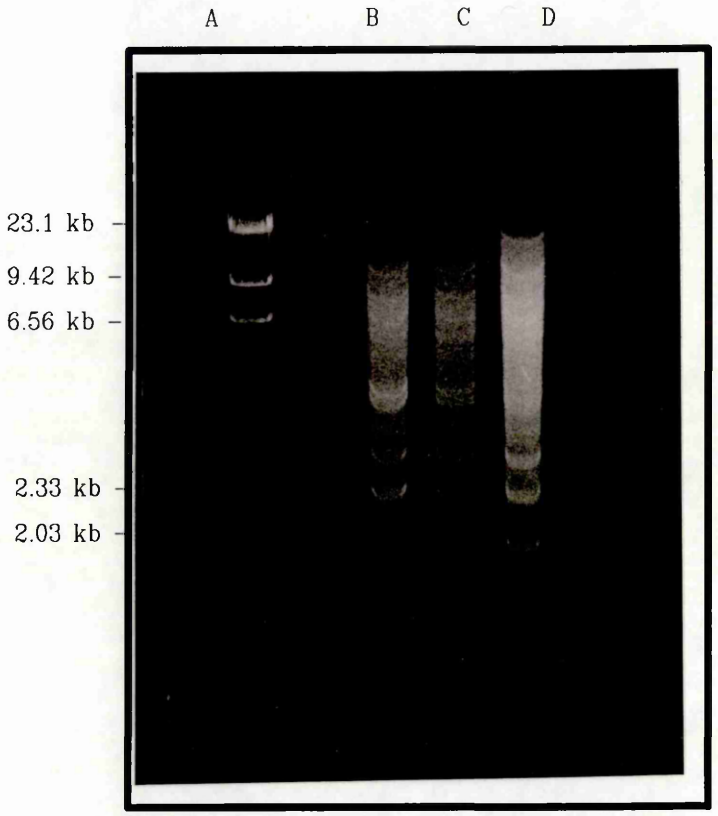
- Chromosomal sequences.
- Plasmid sequences.
- Genes
- Direction of transcription
- Non-functional gene

Figure 3.8a Confirmation of plasmid integration (i)

DNA was isolated from DBY746 and from both a pLD305 and pLD405 yeast transformant to confirm integration had taken place at the *ura3* locus. The DNA was digested with *Eco*R1 (I), transferred to a Hybond-N membrane and analysed with a *URA3* probe (II). The filter was exposed to X-ray film for 6hr at -70°C. The sizes of bands obtained correspond with the sizes of bands anticipated in Fig 3.7 if multiple integration events had occurred.

A: *Hind* III digested lamda size markers
B: *Eco* RI digested DNA extracted from DBY746
C: *Eco* RI digested DNA extracted from DBY746:pLD305(I)
D: *Eco* RI digested DNA extracted from DBY746:pLD405(I).

I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B
C
D
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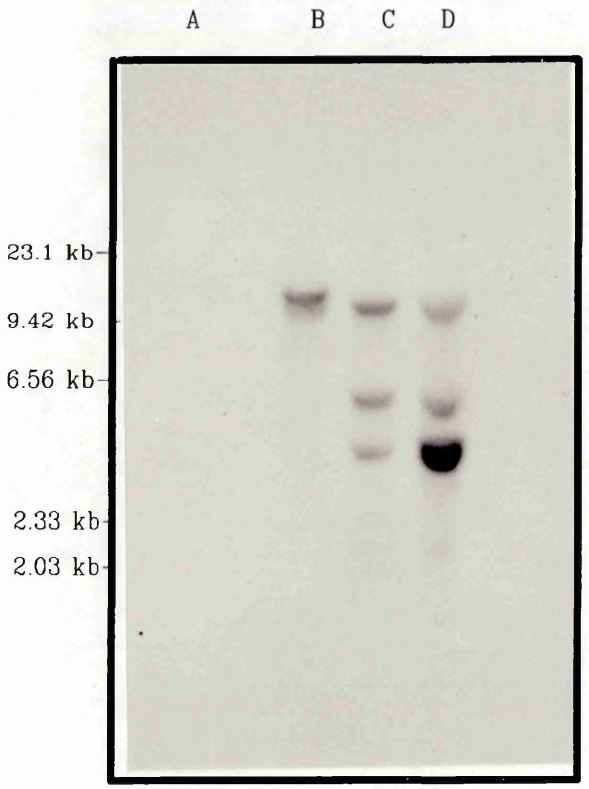
6.56 kb
2.33 kb
2.03 kb

II


A
B
C
D
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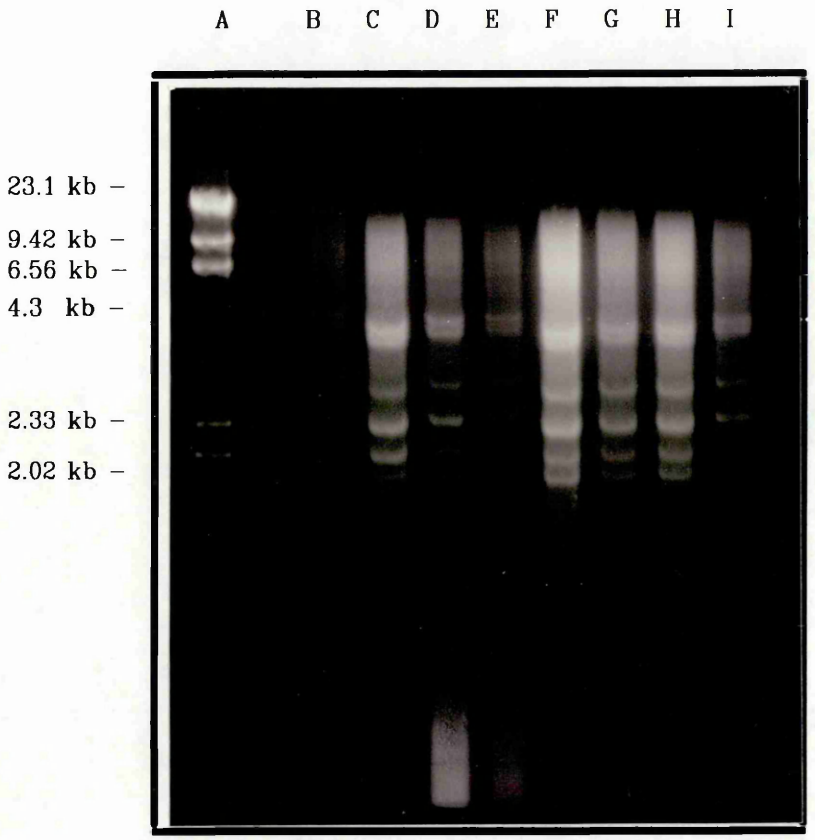
9.42 kb
6.56 kb
2.33 kb
2.03 kb

Figure 3.8(b) Confirmation of plasmid integration (ii)

DNA isolated from DBY746 and from 4 pLD305 and 2 pLD405 yeast transformant was digested with *Eco*R1 (I), transferred to a Hybond-N membrane and analysed with a URA3 probe (II). The filter was exposed to X-ray film for 6hr at -70°C. The sizes of bands obtained correspond with the sizes of bands anticipated in Fig 3.7 if single or multiple integrations had occurred.

A: *Hind* III digested lambda size markers
B: *Eco* RI digested DNA extracted from DBY746
C: *Eco* RI digested DNA extracted from DBY746
D: *Eco* RI digested DNA extracted from DBY746:pLD305(II)
E: *Eco* RI digested DNA extracted from DBY746:pLD305(III)
F: *Eco* RI digested DNA extracted from DBY746:pLD305(IV)
G: *Eco* RI digested DNA extracted from DBY746:pLD305(V)
H: *Eco* RI digested DNA extracted from DBY746:pLD405(II)
I: *Eco* RI digested DNA extracted from DBY746:pLD405(III)

I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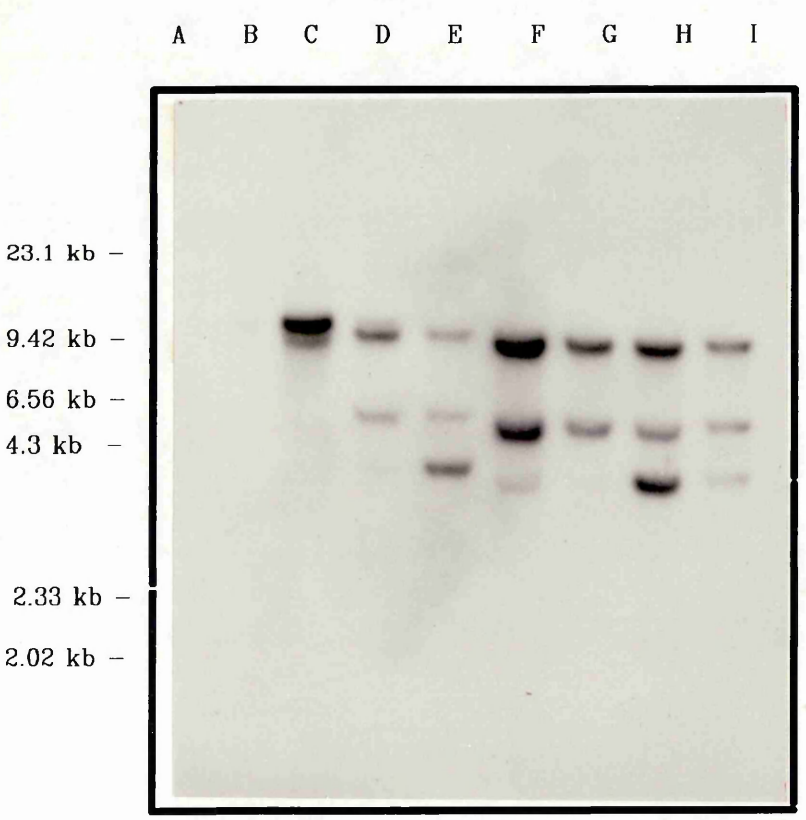
9.42 kb
6.56 kb
4.3 kb
2.33 kb
2.02 kb

I I


A B C D E F G H I
23.1 kb
9.42 kb
6.56 kb
4.3 kb
2.33 kb
2.02 kb

was no longer present, suggesting that integration had indeed occurred at the *ura3* locus. However, there was an extra band of 3.1 kb present in both lanes 2 and 3. This gel was scanned by the AMBIS β-scanner to determine the intensity of the 3.1kb band relative to the other bands in these lanes. In the case of the pLD305 transformant, the 3.1kb band was of similar intensity to the other bands in the lane, while in the case of the pLD405 transformant the 3.1kb band was three times more intense than the other bands.

It is likely that this band was generated by multiple integration events occurring at the *ura3* locus as shown in Fig. 3.7a for pLD 305 (the same pattern of bands would be anticipated if multiple integrations of pLD405 had occurred). Additional integration events would give rise to additional 3.1kb bands leaving unique 6.35 and 10.25 kb fragments upon hybridisation with *URA3* sequences.

Alternatively, the 3.1kb band could have originated from an autonomously replicating pLD305 or pLD405 containing the *URA3* gene. However, Southern analysis of uncut DNA isolated from the two transformants using a *URA3* probe did not reveal the presence of autonomous plasmid (blot not shown). Thus the latter explanation is invalid.

It appears therefore, that in the case of the pLD305 transformant analysed, there have been two integration events. In the pLD405 transformant analysed there have been four integration events.

The remaining transformants were then analysed in a similar way in an attempt to identify a strain which had only a single plasmid integrated. DNA was isolated from the four remaining pLD305 transformants and the two remaining pLD405 transformants, southern blotted, *Eco* RI digested and probed for *URA3*. The resulting autoradiogram and a photograph of the gel are shown in Fig. 3.8b. This gel was then scanned by the AMBIS β-scanner to determine the number of plasmid integration events for each transformant. The result of this is shown in Table 3.1 together with data gleaned from the gel shown in Fig. 3.8a.

In the case of pLD305, transformant (5) was used for analysis of *lacZ* expression as it had only one copy of the plasmid integrated. pLD405 transformant (3) was used for analysis of *lacZ* expression under

TABLE 3.1 Number of pLD305 and pLD405 integration events obtained in individual DBY746 transformants

Transformant	No of integrated plasmid copies
DBY746:pLD305 (1)	2
DBY746:pLD305 (2)	1
DBY746:pLD305 (3)	2
DBY746:pLD305 (4)	1
DBY746:pLD305 (5)	1
DBY746:pLD405 (1)	4
DBY746:pLD405 (2)	2
DBY746:pLD405 (3)	2

the control of the *PYK1* DAS element. The presence of two copies of the reporter gene was noted and taken into consideration when analysing data. (Insufficient time remained for the isolation and analysis of a single copy integrant for pLD405). From this point I refer to the pLD305 (5) transformant as the *lacZ* (DAS-) strain and the pLD405 (3) transformant as the *lacZ* (DAS+) strain.

In order to determine how stable the integration events had been, both the *lacZ*(DAS-) and the *lacZ*(DAS+) were grown for approximately 20 generations in non-selective liquid YPG, a dilution of the broth spread onto a YPG plate and colonies allowed to form. 200 colonies from both the *lacZ*(DAS-) and *lacZ*(DAS+) populations were then checked for uracil prototrophy to determine the percentage which retained plasmid sequences. As all colonies grew from both populations, the percentage of plasmid loss is less than 0.025% per generation.

3.2.4.4 Localisation of *PYK1* sequences required for carbon source regulation.

Both the *lacZ* (DAS-) and *lacZ* (DAS+) strains created above were grown separately to mid-logarithmic phase of growth in both 200ml of YPG and 200ml of YPL (in 500ml conical flask) as described in 2.1.3.2. As with the untransformed host strain, the transformants grew approximately three times faster on glucose than on lactate.

At this stage 100ml of culture was used for RNA extraction. This RNA was Northern blotted and probed for the *lacZ* mRNA. A purified 3.0 kb *Eco* RI fragment isolated from YCpPKG2 which contained the *lacZ* coding region (see Fig. 3.6c.) was labelled by random priming and used as the probe. The resulting Northern blot is presented in Fig. 3.9 and demonstrates the presence of *lacZ* mRNA in the two transformants but not in the untransformed host. Also, the difference in the lengths of the *lacZ* mRNA transcripts synthesised in each of the transformants (due to different amounts of *PYK1* sequences present) is apparent (Fig. 3.9).

The levels of *lacZ* mRNA were quantified by scanning with the AMBIS β-scanner. A second filter containing the same amounts of RNA samples was analysed for the *ACT1* mRNA to control for loading (Fig. 3.9). The results of these analyses are shown in Table 3.2.

Figure 3.9 Determination of *lacZ* mRNA abundance in *PYK:lacZ* integrants.

RNA was isolated from the following cultures:

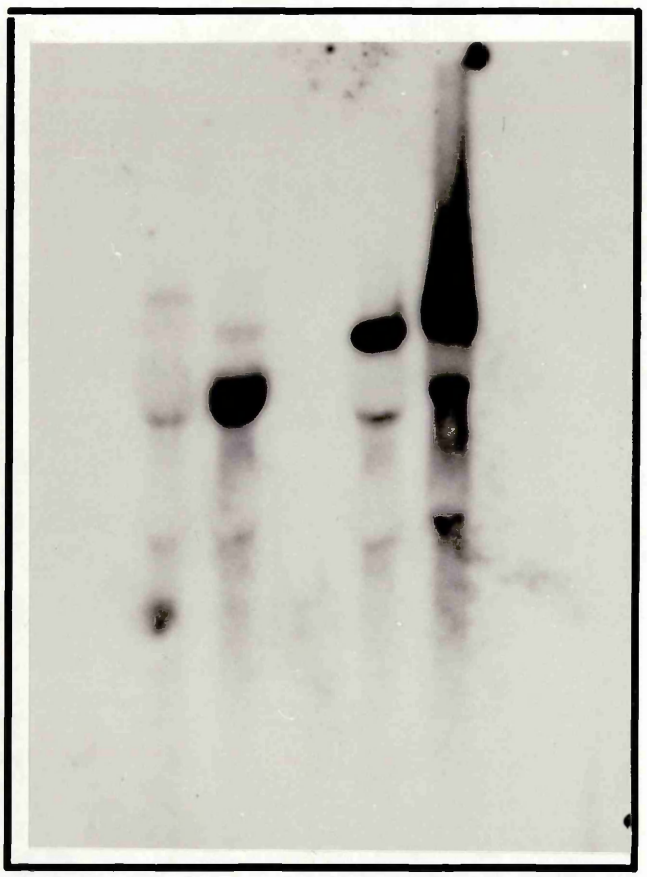
A: DBY746 grown on glucose,
B: *lacZ*(DAS-) grown on lactate,
C: *lacZ*(DAS-) grown on glucose,
D: *lacZ*(DAS+) grown on lactate,
E: *lacZ*(DAS+) grown on glucose.

Equal volumes of samples were loaded onto two denaturing gels and Northern blotted. One filter was analysed for the *lacZ* mRNA using a 3kb *Eco* RI fragment isolated from the vector YCpKG2, which contains the complete *lacZ* coding region (I). The positions of the *lacZ*(DAS-) transcript and *lacZ*(DAS+) transcripts are shown. The positions of the 18S rRNA and 25S rRNAs are shown (these were pinpointed by EtBr staining of the gel prior to blotting). In lanes B-E other RNAs hybridised with the *lacZ* probe. Subsequent probings demonstrated that thes minor bands contain pBR322 sequences, and therefore are created by readthrough from alternative promoters. The major *lacZ* containing transcripts do not contain pBR322 sequences.

The other filter was blotted and probed for the *ACT1* mRNA, to control for loading (II)

Hybridisation to each filter was determined using the AMBIS β-scanner.

I


A
B
C
D
E
25S rRNA –
18S rRNA –
– lac Z (DAS+) mRNA
– lac Z (DAS–) mRNA

II

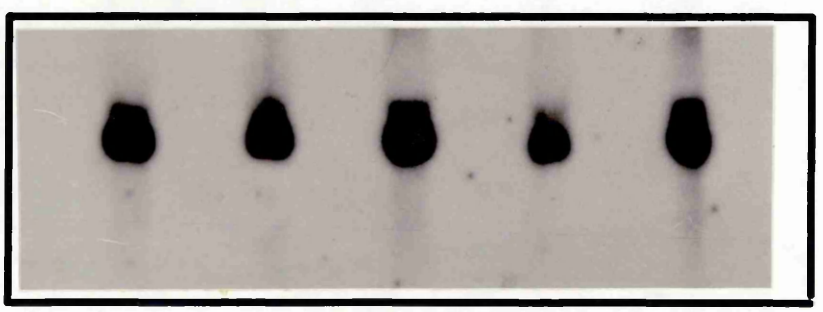

A
B
C
D
E
ACT 1 mRNA

TABLE 3.2 Expression levels of *lacZ* mRNA in *PYK1:lacZ* transformants on different carbon sources.

Strain	Carbon source	*PYK1:lacZ* mRNA level[a]	*PYK1:lacZ* mRNA per gene copy[b]
DBY746	Glucose	NA	-
lacZ(DAS-)	Lactate	<0.05	-
lacZ(DAS-)	Glucose	0.14	0.14
lacZ(DAS+)	Lactate	0.36	0.18
lacZ(DAS+)	Glucose	1.01	0.51

a: The abundance of the *PYK1:lacZ* mRNA is expressed relative to the abundance of the *ACT1* mRNA and was determined by Northern analysis combined with quantitaion using the AMBIS β-scanner.

b: The level of the *PYK1:lacZ* mRNA was corrected to the level synthesised per integrated gene copy of *PYK1:lacZ* fusion.

TABLE 3.3 Expression levels of β-galactosidase in *PYK1:lacZ* transformants on different carbon sources.

Strain	Carbon source	β-gal activity[a]	β-gal activity per gene copy[b]
DBY 746	Glucose	0	0
LacZ(DAS-)	Lactate	81	81
LacZ(DAS-)	Glucose	805	805
lacZ(DAS+)	Lactate	2713	1356
lacZ(DAS+)	Glucose	22157	11078

a: The units of β-galactosidase activity are proportional to the increase in O-nitrophenol per minute per yeast cell (as described in 2.2.6.5).
b: The level of β-galactosidase activity was corrected to the level synthesised per integrated gene copy of *PYK1:lacZ* fusion.

Unfortunately, insufficient radioactivity was present on the filter to determine the abundance of the *lacZ* mRNA in the *lacZ*(DAS-) strain. At the same time that cells were harvested for RNA preparations, samples were also taken for β-galactosidase assays (see 2.2.6.1). These assays were carried out in duplicate and the results are shown in Table 3.3.

Analysis of Table 3.3 demonstrates several important points. Firstly, the activation of β-galactosidase activity by the *PYK1* DAS element is observed when yeast is grown on either glucose or lactate. When yeast is grown on lactate there is approximately a 17-fold increase in the level of β-galactosidase activity observed when *lacZ* expression is under the control of the *PYK1* DAS element (see Table 3.3). A similar increase in β-galactosidase activity (about 14-fold) was also observed when yeast was grown on glucose. Thus, the DAS element is active on both a fermentative and a non-fermentative carbon source.

This DAS effect has been shown to act at least partly at the level of mRNA abundance. Table 3.2 shows that on glucose, the level of *lacZ* mRNA synthesised was 0.51 (ratio of *lacZ/ACT1* mRNA) when the *PYK1* DAS element was present while when it is absent the level synthesised was decreased to 0.14. Thus, a 3-fold increase in the level of *PYK1:lacz* mRNA accompanies the introduction of the *PYK1* DAS element.

Secondly, Table 3.3 shows that carbon source regulation has been imparted on *lacZ* expression in both the *lacZ*(DAS-) and *lacZ*(DAS+) strain. In the case of the *lacZ*(DAS-) strain, there is a 10-fold increase in β-galactosidase activity when the transformant is grown on glucose (805 U) than when it is grown on lactate (81 U). A similar level of carbon source regulation (8-fold) was also observed in the *lacZ*(DAS+) strain: when grown on glucose 11078 U of β-galactosidase activity were observed, while on lactate only 1356 U of β-galactosidase were recorded. This induction in *lacZ* activity is mediated partly by a 2.8-fold increase in *lacZ* mRNA levels as shown in Table 3.2.

In conclusion, it appears that all of the *PYK1* sequences required to impart carbon source regulation onto a heterologous gene reside in the 5' promoter region. The DAS element present in the first 850 bp of

the *PYK1* coding region is necessary for maximal levels of expression, but is not required for carbon source regulation.

Interestingly, while carbon source regulation has been imparted on *lacZ*, the level of induction observed for the *PYK1:lacZ* mRNA in the *lacZ*(DAS+) strain was 2.8-fold, which is lower than the 3.9-fold increase previously observed for the chromosomal copy of *PYK1* in DBY746 (see Fig. 3.4a). To clarify this matter, the abundance of the *PYK1* mRNA was determined in each of the four RNA preparations used to determine the level of *PYK1:lacZ* mRNAs in order to calculate glucose activation. This allowed a direct comparison of regulation of the wild-type *PYK1* with the *PYK1:lacZ* transcripts to be made in the same transformants. The four RNA samples were Northern blotted and probed for the *PYK1* mRNA, using random-primed pSPK2 (see Table 2.1.) as a probe (. After hybridisation and washing, the filter, the filter was scanned by the AMBIS β-scanner to quantify the level of hybridisation to the *PYK1* mRNA. This filter was then stripped of *PYK1* probe and rehybridised with an *ACT1* probe to adjust for differences in RNA loading and the filter rescanned by the AMBIS β-scanner. The level of *PYK1* mRNA originating from the *PYK1* locus in each transformant is presented in Table 3.4.

Analyses of these results, demonstrates that in the *lacZ*(DAS-) and *lacZ* (DAS+) strains, the level of induction of the *PYK1* mRNA (2.7 and 2.35 respectively) is similar level to that for the *PYK1:lacZ* mRNA observed in the *LacZ*(DAS+) strain (2.8-fold; Table 3.3) and lower than the level of induction previously observed for *PYK1* in an untransformed DBY746 (3.9 -fold; Fig. 3.4a).

3.2.4.5. The translation of a *PYK1:lacZ* gene fusion is more efficient when yeast is grown fermentatively.

Further analysis of the data presented in Tables 3.2 and 3.3 allows the amount of β-galactosidase synthesised per *lacZ* mRNA in both the glucose and lactate-grown cultures to be calculated. This value serves a measure of the relative translatability of the *PYK:lacZ* fusion mRNA, and is presented in Table 3.5.

Information for this table was only available for the *lacZ* (DAS+) construct since the abundance of the *PYK1:lacZ* mRNA in the *lacZ*(DAS-)

TABLE 3.4 Levels of *PYK1* mRNA in *lacZ*(DAS-) and *lacZ*(DAS+) strains on different carbon source.

Strain	Carbon source	*PYK1* mRNA levels[a]	Activation by glucose[b]
lacZ(DAS-)	Lactate	1	
lacZ(DAS-)	Glucose	2.7	2.7
lacZ(DAS+)	Lactate	1.2	
lacZ(DAS+)	Glucose	2.8	2.3

a: The levels of the *PYK1* mRNA are relative to the level of the actin mRNA and are standardised to the level present in the *lacZ*(DAS-) strain. They were determined by Northern analysis followed by quantitation using the AMBIS β-scanner.
b: Glucose activation is the ratio of *PYK1* mRNA on glucose to the level expressed on lactate.

TABLE 3.5. Effect of carbon source on the translation of *PYK1:lacZ* mRNA.

Carbon source	*lacZ* mRNA[a]	β-gal activity[b]	β-gal activity/ *lacZ* mRNA	Ratio[c]
Glucose	1.01	22 200	21 900	2.91
Lactate	0.36	2 700	7 540	

a: The abundance of the *PYK1:lacZ* mRNA is expressed as a ratio relative to the abundance of the *ACT1* mRNA. The levels were determined by Northern analyses followed by quantitation using the AMBIS β-scanner.

b: The units of β-galactosidase activity are proportional to the increase in O-nitrophenol per minute per yeast cell (as described in 2.2.6.5).

c: Ratio of β-galactosidase/*lacZ* mRNA on glucose compared to β-galactosidase/*lacZ* mRNA on lactate.

strain was to low to measure accurately. The *lacZ*(DAS+) construct directs the transcription of an mRNA with the *PYK1* untranslated leader followed by 850 bp of *PYK1* coding region, the *lacZ* coding region and *PYK1* 3' untranslated region. Thus, the efficiency of translational initiation on this construct presumably depends on *PYK1* sequences while translational elongation efficiency depends on both *PYK1* and *lacZ* sequences.

From Table 3.5, it appears that the *PYK1:lacZ* mRNA is translated nearly three times more efficiently on glucose than on lactate as there is three times more ß-galactosidase synthesised per *lacZ mRNA* when the *lacZ*(DAS+) construct was grown on glucose. This suggests that either translational initiation or translational elongation of the *PYK1:lacZ* mRNA is more efficient on glucose than on lactate. Alternatively, the ß-galactosidase protein is more stable in yeast during growth on glucose.

3.3 DISCUSSION.

It has been demonstrated in this study that *S.cerevisiae* regulates genes differentially in response to fermentative or gluconeogenic growth. This is obvious from Fig. 3.2 which demonstrates that there is a clear disparity in the proteins expressed when yeast is grown on glucose (a fermentative carbon source), compared to when grown on lactate (a gluconeogenic carbon source). This difference in gene regulation is accompanied by a change in growth rate: yeast grows about three times faster when growing on glucose than when utilising lactate as a carbon source under the conditions used in this study (Fig. 3.1).

The main objective of this study however, was to determine if the glycolytic genes were amongst those genes whose expression responded to a change from gluconeogenic to fermentative growth. As stated in the introduction there is apparently conflicting evidence in the published literature as to whether glycolytic mRNAs are induced by the presence of glucose and to what level.

The Northern analysis conducted in this study is the first analysis which has measured each individual glycolytic mRNA within the same RNA preparations. It has revealed that the expression of some

of the glycolytic genes does indeed respond to carbon source, while others appear to be unaffected (Fig. 3.4). The level of induction observed in this study for the glycolytic mRNAs on glucose ranges from 1.1- to 3.9-fold. The quantitation of the relative transcript levels was carried out using an AMBIS ß-scanner which was tested as a quantitative tool before this analysis (Fig. 3.3). The RNA loading control used throughout, was the actin mRNA, which is thought to be constitutively expressed (Mayer and Dieckmann, 1989).

The elevation of mRNA levels on glucose could occur due to either increased levels of transcription or decreased levels of mRNA degradation. Considering the evidence concerning glucose-repressed genes and studies which have dealt with the regulation of the glycolytic genes themselves, it is likely that this increase is mediated by changes in the level of transcription. Furthermore, recent experiments conducted in this laboratory have demonstrated that there are no significant changes in the stability of glycolytic mRNAs when yeast is cultured on glucose or lactate (Moore *et al.*, 1991b)

In certain previous studies a greater level of induction of individual glycolytic mRNAs has been demonstrated. However, this appears to be observed during a transient response to the addition of glucose to a gluconeogenically growing yeast culture (*ADH1*, Santangelo and Tornow, 1990; *PDC1*, Schmitt *et al.*, 1983). This study has addressed the levels of glycolytic mRNAs in "steady-state" batch cultures in exponential growth phase. Prior to the analysis, the yeast strains had been grown on the respective carbon source for at least 20 generations. The essence of this study was to determine the relative induction of each of the glycolytic genes in the presence of glucose.

Importantly in this study, the 2-fold induction observed for *LPD1* on lactate is in the range of induction observed for the glycolytic mRNAs on glucose (Fig. 3.4a.; Fig. 3.4e.). *LPD1* encodes Lipoamide dehydrogenase, an enzyme involved in the TCA cycle. Carbon catabolite genes such as *LPD1* are transcriptionally repressed in the presence of glucose. Thus, it appears that the level of induction observed for the induced glycolytic mRNAs in response to carbon source is similar to the level of repression observed for a catabolite repressed gene in the presence of glucose. Furthermore the repression

of *LPD1* on glucose seen in this study confirms the relevance of conditions to study regulation of glycolytic genes.

Fig. 3.4a which summarises the Northern analysis data, presents a very interesting pattern: it appears that there are two peaks in the induction of glycolytic mRNAs. These coincide with genes which are used exclusively for glycolysis and are not necessary for gluconeogenesis: *PFK2* and *PYK1*. Considering that both of these genes catalyse steps considered important in controlling the metabolic flux through glycolysis (Muirhead, 1983), this differential induction of mRNA levels may have physiological relevance in the regulation of glucose metabolism through glycolysis.

As presented in Fig. 3.4, *PYK1* appears to be the most highly induced of all of the glycolytic genes with a 3.9-fold increase on glucose. Previous studies have shown similar levels of activation (Nishizawa *et al.*, 1989; Albig and Entian, 1988; Entian *et al.*, 1984). Taking this together with the fact that the regulation of *PYK1* is studied in the remainder of this thesis, it was decided to study the carbon source regulation of *PYK1* in greater detail.

Firstly, the 5' end of the *PYK1* mRNA was precisely mapped on both carbon sources to determine if the decrease in the *PYK1* mRNA level observed on lactate is accompanied by a change in the transcriptional start point. Previously, the transcriptional start point was mapped within 5 nt of -33 (Burke *et al.*, 1983). To map the start point a smaller primer oligonucleotide than the one used by Burke and co-workers was employed. In addition, as the oligonucleotide hybridised close to the translational start site the length of the extended DNA product should be short enough to resolve easily on a sequencing gel.

No difference in the *PYK1* transcriptional start point was observed on either of the two carbon sources (Fig. 3.5). In both cases the major transcriptional start point was finely mapped to 28 nt upstream of the translational start codon. There were also three minor start sites which were common to both cultures (see Fig 3.5). A motif shown for other genes to be important in directing transcriptional initiation is CAAG (Rathgen and Mellor, 1990). Such a sequence resides

in the *PYK1* 5' untranslated region between nt -36 and -32, and perhaps is important in directing *PYK1* transcriptional initiation.

As stated in the introduction *PYK1* contains an element 3' to its transcriptional start point which is required for full transcriptional activation. Preliminary evidence suggested that this element is regulated in response to carbon source (Lithgow *et al.*, 1990). Thus, a pair of *PYK1:lacZ* constructs were made to test whether this DAS element was required for carbon source regulation. One of the test plasmids carried *lacZ* as a reporter gene fused to either only 5' *PYK1* sequences and is designated *lacZ*(DAS-). The second plasmid contained the *lacZ* gene fused to 850 bp of *PYK1* coding sequences as well as the 5' *PYK1* sequences and is designated *lacZ*(DAS+). These plasmids were linearised at the unique *Stu* I site in *URA3* and transformed into yeast. After transformation into yeast and confirmation of integration by Southern blotting, the *lacZ*(DAS-) strain and *lacZ*(DAS+) strain were examined for their levels of *PYK1:lacZ* expression on both glucose and lactate. The levels of *PYK1:lacZ* mRNA and ß-galactosidase activity are summarised in Tables 3.2 and 3.3. The results of this analysis verify that the DAS element is necessary for optimum transcription since without the DAS sequences, the level of ß-galactosidase activity decreases approximately 15-fold. This study also demonstrates that the DAS element functions when integrated into the chromosome. Previously it had only been shown to operate on plasmid vectors (Purvis *et al.*, 1987a).

The DAS element is not required for carbon source regulation as *PYK1:lacZ* expression is subject to carbon source regulation in the *lacZ*(DAS-) strain (Table 3.3). Thus, all the *cis*-acting elements necessary for carbon source regulation of *PYK1* at the level of mRNA abundance appear to reside in the 5' untranslated region (and/or in the 3' untranslated region).

However, the level of induction by glucose is lower in the *lacZ*(DAS+) and *lacZ*(DAS-) strains than for *PYK1* in the untransformed host strain. In the host strain the level of *PYK1* induction observed was 3.9-fold (Fig. 3.4a), while in the *lacZ*(DAS+) strain the induction of *PYK1:lacZ* was 2.8-fold (Table 3.2). The level of glucose activation of the *PYK1* chromosomal copy was thus determined for both the *lacZ*(DAS-)

and *lacZ*(DAS+) strains and the results of this are presented in Table 3.4. In both strains the level of glucose activation of the *PYK1* gene is lower than was previously observed in DBY746. Furthermore, it is interesting that in the *lacZ*(DAS+) strain, a slightly lower level of *PYK1* induction is observed (2.3-fold; Table 3.4) than in the *lacZ*(DAS-) strain (2.7-fold; Table 3.4). This is perhaps due to the fact that in the *lacZ*(DAS-) strain there are two copies of the *PYK1* promoter, while there are three copies of the promoter in the case of the *lacZ*(DAS+) transformant. This characteristic of *PYK1* gene expression is addressed in detail in Chapter 5.

Further analysis of the ß-galactosidase activity and the corresponding *lacZ* mRNA abundance exhibited by the *lacZ*(DAS+) strain on both of the carbon sources tested (Table 3.5), shows that the *PYK1:lacZ* mRNA is translated almost three times more efficiently on glucose than on lactate. Thus, in addition to being transcriptionally activated on glucose, *PYK1* also appears to be translationally activated. However, this translational increase may not be specific to *PYK1* (and possibly other glycolytic genes) but may be a general increase in protein synthesis on glucose. Maicas and co-workers (1990) noted that when yeast is grown on glycerol (a gluconeogenic carbon source) the average polyribosome size is 3-4 ribosomes per mRNAs and the doubling time is 6 hrs. On glucose however, the polyribosome size was 8-10 ribosomes with a doubling time of 2 hrs. Thus, it appears the rate of protein synthesis might be related to the doubling time. If so, the increase in *PYK1* mediated translation on glucose may be due to a general increase in protein synthesis. Certainly in this study, yeast grew 3 times faster on glucose than on lactate.

Recently, an extensive deletion analysis of the *PYK1* 5' promoter was undertaken in an attempt to uncover the *cis*-acting elements which direct transcription. Nishizawa and co-workers (1989) identified two UAS elements and a URS element. Interestingly, the URS element which was mapped between -344 and -468 represses transcription of the *PYK1* gene to a greater extent when yeast is grown on gluconeogenic media than when on fermentative media. When the URS was deleted, there was a 2.7-fold increase in *PYK1* transcription when yeast was grown on glycerol and ethanol. On glucose when the URS

was deleted, only a 1.6-fold increase was observed (Nishizawa *et al.*, 1989). This suggested that the increase in *PYK1* mRNA levels may be achieved via negative regulation. However, considering there is approximately a 4-fold difference in the level of *PYK1* transcription between fermentative and gluconeogenic grown yeast (Fig. 3.4a; Nishizawa *et al.*, 1989), it would appear that there must be additional *cis*-acting sequences required for carbon source regulation.

Similar deletion analyses have also been conducted on other glycolytic genes including the *ENO1* (Cohen *et al.*, 1987) and *ENO2* genes (Cohen *et al.*, 1986). In the studies carried out in this thesis, *ENO* gene expression was induced on glucose 3.3-fold at the mRNA level (see Fig. 3.4a). Determination of the level of *ENO* mRNA expression was achieved using a cDNA clone as a probe. Although the sequence of this clone corresponds to the published sequence for *ENO2* it is also 95% homologous to *ENO1*. Thus, it seemed likely that this probe hybridised to both the *ENO1* and *ENO2* mRNAs and that the level of induction observed represented an average of both genes.

Previous studies have demonstrated that while *ENO1* is constitutively expressed, *ENO2* expression is induced on glucose (Cohen *et al.*, 1987). Interestingly if the *ENO1* promoter is fused to *ENO2* coding region then ENO2 protein becomes constitutively expressed. Likewise, when the *ENO2* 5' promoter is fused to the *ENO1* coding region then the ENO1 protein becomes glucose inducible (Cohen *et al.*, 1987). Deletion analysis of the promoters of these genes has thrown light on how this regulation operates. Both *ENO1* and *ENO2* have two UAS elements required for full transcription, but *ENO1* has an additional URS element. This 38 bp URS element represses transcription of the *ENO1* gene in the presence of glucose. If the URS element is deleted from *ENO1* then the gene is no longer constitutively expressed but is induced on glucose. However, this URS sequence is not sufficient to repress transcription on glucose on its own, because if it is placed in the 5' untranslated region of *ENO2*, then *ENO2* is still highly expressed on glucose. Therefore, it appears that the ability of the *ENO1* URS to repress transcription depends on the nature of the surrounding sequences (Cohen *et al.*, 1987).

In the case of *PGK* expression it has been postulated that the RAP1 protein plays an important role in mediating carbon source regulation (Chambers *et al.*, 1989). This protein has been shown to bind to the 5' untranslated regions of several glycolytic genes in addition to *PGK* (Chambers *et al.*, 1989). Binding has also been demonstrated with *ENO1, PYK1* (Buchman *et al.*, 1988), *ADH1* (Santangelo and Tornow, 1990), and *TPI* (Scott *et al.*, 1990). In the case of *PYK1* and *ENO1* it has been shown that "CT blocks" located close to the RAP1 binding site are integral for transcriptional activation by the RAP1 protein even though they are not required for RAP1 binding (Buchman *et al.*, 1988). With regard to *PGK* expression, it has been shown that a 62 bp fragment of the *PGK* UAS containing the RAP1 binding site (the RPG box) and 3 "CT blocks" can impart carbon source regulation onto a reporter gene (Chambers *et al.*, 1989). Furthermore, it appears that RAP1 binding to the *PGK* UAS is carbon source regulated: while nuclear extracts prepared from glucose-grown yeast cultures possessed RAP1 binding activity, nuclear extracts prepared from pyruvate cultures did not show any RAP1 binding activity (Chambers *et al.*, 1989). As substantiated in this study (see Fig. 3.4e; also Chambers *et al.*, 1989) the levels of *RAP1* mRNA are the same on both fermentative and gluconeogenic carbon sources. Furthermore Tsang and co-workers (1990) claim there is no change in the level of the RAP1 protein on different carbon sources. They also demonstrate that the ability of the RAP1 protein to bind DNA *in vitro* is dependent on its phosphorylation state of the RAP1 protein. Perhaps phosphorylation of RAP1 plays a role in the regulation of glycolytic gene expression in response to different carbon sources. However, this remains to be confirmed and recently Scott and co-workers (1990) demonstrated RAP1 binding activity in nuclear extracts isolated from gluconeogenic grown yeast to the RPG box in the *TPI* 5' untranslated region.

Certainly, the RPG box on its own is insufficient to mediate carbon source regulation, as deletion of the sequence from the promoters of several glycolytic genes does not disrupt carbon source regulation (*PYK1,* Nishizawa et al., 1989; *ADH1,* Tornow and Santangelo, 1990; *PDC1,* Butler *et al.,* 1990). However, its deletion does result in a

dramatic decrease in the abundance of the mRNA on both carbon sources.

Thus, despite intensive analysis of the 5' regions of glycolytic genes, no common sequence has been uncovered which mediates carbon source regulation. Indeed, any sequences which have been identified (such as the URS of *ENO1* and *PYK1*) which are integral for carbon source regulation appear to require the presence of other sequences in the 5' region. However, as pointed out in the introduction (3.1), the isolation of the *gcr1* mutation suggests that the glycolytic genes are indeed co-ordinately expressed (Clifton and Fraenkel, 1981). This mutation results in a decrease in the levels of glycolytic enzymes both on fermentative and gluconeogenic carbon sources. However, a greater decrease is observed when yeast is grown on a gluconeogenic carbon source than on a fermentative carbon source (Clifton and Fraenkel, 1981).

While the GCR1 protein has not been shown to bind DNA or interact with DNA binding proteins, it has been shown to affect the transcription of *ENO1*, *ENO2*, and *TDH1*-3 (Holland *et al.*, 1987). Thus, it is thought to be a transcriptional activator. Recent studies with *ENO2* have identified at least one of the *cis*-acting sequences through which GCR1 operates (Holland *et al.*, 1990). An earlier analysis of various 5' promoter deletions mutations of *ENO2* identified an element which acts as a positive *cis*-acting sequence in a *GCR1* strain (Cohen *et al.*, 1987). Interestingly, this same sequence (-479 to -461) appears to act as a negative element in a *gcr1* background since deletion of this element results in restoration of *ENO2* mRNA levels to the level observed in the *GCR1* strain (Holland *et al.*, 1990). Thus, it appears that in the case of *ENO2* expression at least, the *GCR1* gene product functions to overcome repression of gene expression mediated by sequences located between -479 and -461 (Holland *et al.*, 1990). This sequence has since been shown to bind both the ABF1 and RAP1 proteins (Brindle *et al.*, 1990). These proteins are believed to act as positive activators of *ENO2* transcription in *GCR* strains. Interestingly the GCR1 protein has also been shown to mediate its effect on the *ADH1* gene through the RPG box, a site which binds the RAP1 protein (Santangelo and Tornow, 1990). This has led to the possibility that GCR1 mediates its affect via DNA binding proteins

such as RAP1 or ABF1. However, it has been shown that GCR1 disruption neither reduces the level of RAP1 or altered its DNA binding ability *in vitro* (Santangelo and Tornow, 1990; Scott *et al.*, 1990).

Thus, the way in which the *GCR1* gene product functions remains unclear. Until this becomes known, the mechanism by which glycolytic genes are co-ordinately expressed and how they are regulated by carbon source will also remain a mystery. This study has established that glycolytic genes are differentially regulated in response to the presence of glucose, with *PYK1* being the most highly induced. However, while there is a substantial change in the steady state level of *PYK1* mRNA, there is no change in the *PYK1* transcriptional start site when yeast is grown on either lactate or glucose. Sequences which mediate carbon source regulation of *PYK1* reside in the 5' untranslated region. The *PYK1* DAS sequence which is required for full activation of *PYK1* transcription is not necessary for glucose induction. Finally, in addition to the induction of *PYK1* at the mRNA level in response to carbon source, the translation of *PYK1:lacZ* fusion mRNAs is also higher on glucose than on lactate. Whether this induction at the translational level on glucose is specific to *PYK1* and other glycolytic mRNAs or is a general increase in protein synthesis of all genes remains to be determined.

CHAPTER 4

RESULTS (II)

MULTIPLE COPIES OF THE PYRUVATE KINASE GENE AFFECT YEAST CELL GROWTH

4.1. INTRODUCTION

The glycolytic genes are amongst the mostly efficiently expressed genes in *S.cerevisiae*. Taken together, the glycolytic enzymes comprise between 30 and 65% of total soluble protein (Fraenkel, 1982). As a result much attention has been paid towards understanding how the glycolytic genes are so well expressed. Owing to the high level of expression achieved by glycolytic genes, the promoters from several of them have been used to drive the expression of a large number of heterologous proteins in yeast (Kingsman *et al.*, 1985; Goodey *et al*, 1987; King *et al.*, 1989).

Many of the glycolytic genes themselves have been over-expressed in yeast on multi-copy vectors (Kawasaki and Fraenkel, 1982; Schaaf *et al.*, 1989). Phosphoglycerate kinase has been shown under some conditions to comprise up to 50% of soluble protein, without any detrimental effect on cell growth (Mellor *et al.*, 1985). Similarly, Schaaf and co-workers (1989) showed that the expression of many glycolytic genes can be markedly increased without affecting yeast growth or viability.

However, evidence from this laboratory (Bettany, 1988; Moore *et al*, 1990b) and from others (Andrew Goodey, personal communication; Teresa M^{C}Nally and Linda Gilmore, personal communication) has demonstrated that when yeast is transformed with *PYK1* on a multi-copy vector, transformants display a range in colony size and growth rate. Furthermore, slow growing transformants revert to faster growth during growth or storage (Bettany, 1988; Moore *et al.*, 1990). This chapter describes experiments which have been conducted to study the growth effects characteristic of transformants containing multiple copies of *PYK1* borne on a 2-micron based plasmid, and to determine whether these effects are mediated by the *PYK1* gene itself.

4.2 RESULTS.

4.2.1 TRANSFORMATION OF *S.CEREVISIAE* WITH MULTIPLE COPIES OF *PYK1*.

4.2.1.1 Structure of pLD1(35).

Multiple copies of the *PYK1* gene were introduced into yeast using the vector pLD1(35) (Fig. 4.1). This plasmid contains a 6.3 kb yeast genomic fragment cloned between the *Hind* III and *Nde* I sites of the multi-copy vector pJDB207 (Parent *et al.*, 1985; Bettany *et al.*, 1989). The fragment carries the *PYK1* coding region (1.5kb), approximately 1.6 kb of DNA immediately 3' of the *PYK1* coding region, and 3.2 kb of DNA located immediately 5' of the *PYK1* coding region. The wild-type *PYK1* gene on this plasmid has been shown to complement the conditionally-defective *pyk1* alleles *pyk1-5* and *cdc19* at their restrictive temperatures (Bettany *et al.*, 1989). As the vector is 2-micron based, it replicates autonomously in yeast. It also contains the *LEU2d* allele, which allows selection for the plasmid in a *leu2* yeast auxotroph.

4.2.1.2 pLD1(35) transformants differ in their growth rates.

Transformation of pLD1(35) into yeast was carried out by the spheroplast method described by Beggs (1978). The frequency of transformation obtained with this plasmid was always lower than that anticipated for a 2-micron based plasmid. For two separate strains of yeast (DBY746 and X4003-5B) the transformation frequency was routinely between 5 and 50 transformants per µg of DNA. When these strains of yeast were transformed with other vectors, including pJDB207 itself, routinely 10-100 times higher frequencies of transformation were obtained. Also, pLD1(35) transformants took longer to form colonies than other plasmid transformations. Normally when either DBY746 or X4003-5B were transformed, transformants appeared after 3-7 days. However, many of the pLD1(35) transformants took 3-4 weeks to form colonies.

Figure 4.1 Structure of pLD1(35).

Plasmid pLD1(35) carries a 6.3kb yeast genomic fragment, containing the *PYK1* gene, between the *Hind* III and *Nde* I sites of the multi-copy vector pJDB207. Not all the restriction sites marked are necessarily unique, and their distance from an arbitrary origin (0.00) are given in kb. All five *Eco* RI sites are shown.

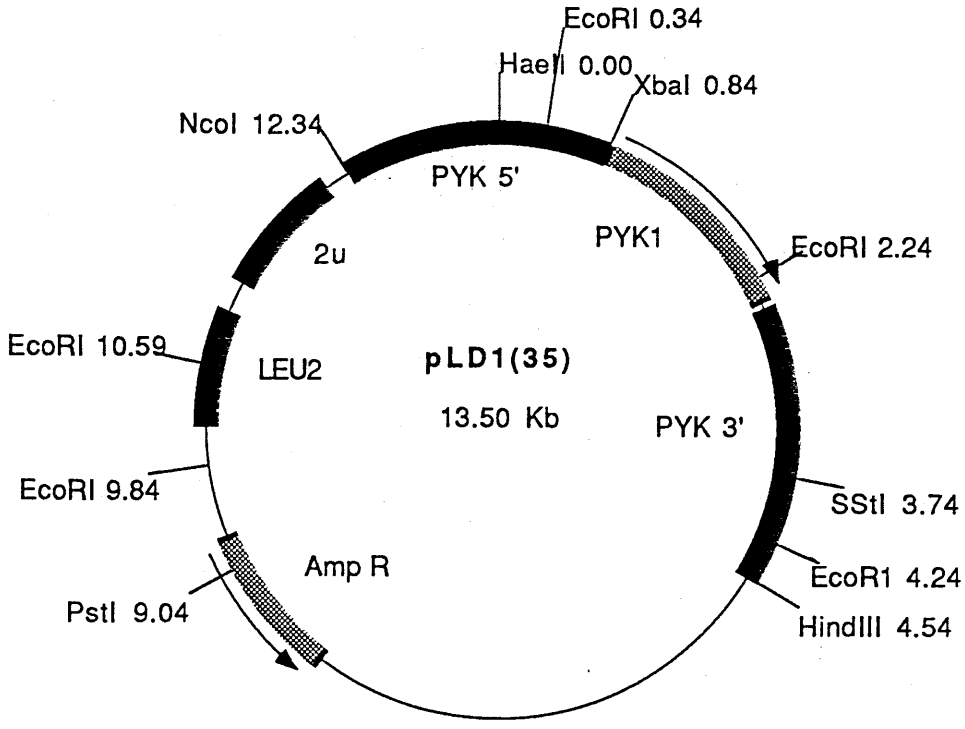
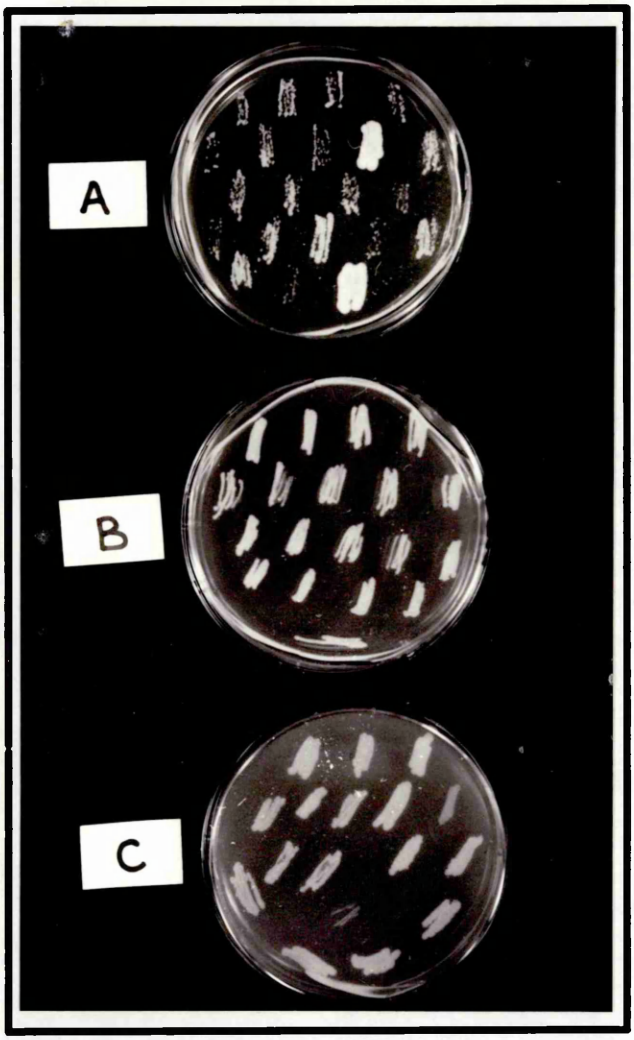

EcoRI 0.34
HaeII 0.00
XbaI 0.84
NcoI 12.34
PYK 5'
PYK1
EcoRI 2.24
2u
EcoRI 10.59
LEU2
pLD1(35)
13.50 Kb
PYK 3'
EcoRI 9.84
SStI 3.74
Amp R
EcoR1 4.24
PstI 9.04
HindIII 4.54

Figure 4.2 **Growth of transformants carrying multi-copy *PYK1* or *pyk1* plasmids.**

A, B: DBY746 was transformed with pLD1(35). Fresh transformants were patched onto minimal agar lacking leucine and incubated at 30°C for 5 days (A). This plate was then stored for 1 week at 4°C, after which each transformant was then repatched onto fresh minimal agar minus leucine, and grown at 30°C for a further 5 days (B).
C: Fresh DBY746:pLD1(35) transformants treated identically to (a).


A
B
C

When pLD1(35) transformants were patched out onto selective plates, a wide range of growth rates were displayed. Fig. 4.2a shows pLD1(35) transformants of DBY746 patched onto minimal agar lacking leucine after 5 days of growth at 30°C. Clearly, while some transformants showed strong growth on minimal plates, the majority grew weakly. This heterogeneity in growth rate was also observed when pLD1(35) transformants were grown in liquid GYNB medium lacking leucine. Doubling times were monitored for at least 20 different transformants and were found to range from 2 hr to over 5 hr. In contrast, no such effects were observed when either DBY746 or X4003-5B were transformed with the vector alone (pJDB207).

4.2.1.3. Slow growing pLD1(35) transformants revert to faster growth.

Routinely it was observed that slow growing pLD1(35) transformants reverted to more rapid growth with high frequency, even under conditions that selected for the plasmid-borne *LEU2d* marker. This has been shown for transformants grown in either liquid medium or on plates. The plate shown in Fig. 4.2a was stored at 4°C for 1 week and then an inoculum from each colony was patched onto a fresh GYNB plate lacking leucine. The fresh plate which was incubated at 30°C for 5 days (Fig. 4.2b) shows that <u>all</u> of the transformants sub-cultured grew normally. Thus, it appears there is a strong selection for rapid growth in relatively slow growing pLD1(35) transformants.

This phenomenon has previously been observed by other workers in this laboratory (Bettany, 1988) and in other laboratories (Andrew Goodey, personal communication; Teresa McNally and Linda Gilmour, personal communications, Murcott *et al.*, 1991).

4.2.1.4 Slow growth correlates with high *PYK1* expression levels.

To determine if there was a correlation between slow growth and the level of *PYK1* gene expression in the pLD1(35) transformants, both these parameters were assayed in individual pLD1(35) transformants.

Fresh pLD1(35) transformants were picked directly from the original transformation plate onto fresh selective plates and their

auxotrophic markers checked. Authentic transformants were grown in 5ml of minimal medium lacking leucine at 30°C for 3 days to select for plasmid-containing cells. The cells were then sub-cultured into 200 ml of minimal medium lacking leucine and grown to an absorbance at 600nm of between 0.4-0.8. At this stage 100 ml of cells were harvested and RNA isolated. The remaining cells were allowed to continue to grow until stationary phase. Throughout, the absorbance at 600nm of the yeast cultures was monitored to permit the calculation of the yeast strains' doubling time.

RNA was isolated from the transformants as previously described (2.2.4), and the integrity of the RNA preparations checked by agarose gel electrophoresis (2.2.4). mRNA abundance measurements were then carried out by dot-blot hybridisation (Moore *et al.*, 1990a).

The use of dot-blotting as a quantitative tool is featured extensively throughout this thesis. It was used both to calculate mRNA abundances and plasmid copy numbers before there was access to an AMBIS β-scanner. The method is described in detail in section 2.2.4.2.

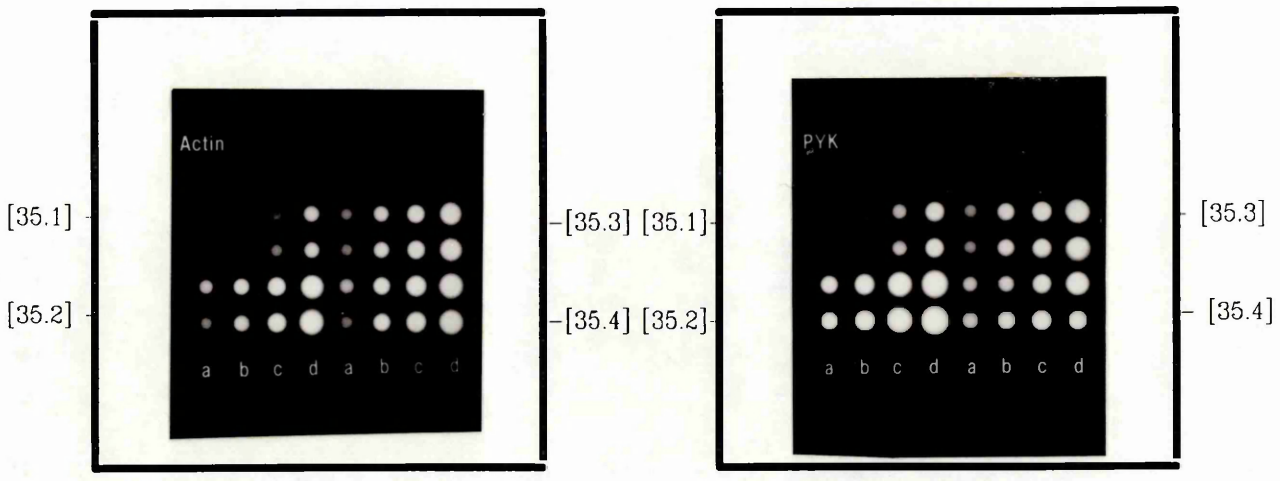
A serial dilution of each RNA preparation to be analysed was prepared and dot-blotted in duplicate onto two separate nitrocellulose filters. One of the filters was probed for the mRNA of interest and the other for an mRNA such as *ACT1* which serves as a loading control. Fig. 4.3a shows the results of such an analysis on four pLD1(35) transformants (designated [35.1-4]), for which the *PYK1* mRNA abundance was required. The dots were then cut out and subjected to liquid scintillation counting to determine the amount of radioactivity in each dot. These data were then plotted against the amount of RNA dotted, and were used to calculate mRNA abundances only when a linear relationship was obtained, ie. when the hybridisation had occurred under conditions of probe excess.

The abundance of the *PYK1* mRNA has previously been determined to be 0.6% of total mRNA in the untransformed host (Purvis *et al.*, 1987a). Thus, a DBY746 RNA control dilution series was routinely dotted out with the samples to be tested. By comparing the ratio of *PYK1* mRNA to *ACT1* mRNA calculated for each transformant with that obtained for DBY746, it was then possible to express the abundance of *PYK1* mRNA in transformants as % of total mRNA.

Figure 4.3 Measurement of *PYK1* mRNA abundance by dot blotting.

a: RNA was prepared from the DBY746:pLD1(35) transformants [35.1], [35.2], [35.3] and [35.4] . Serial dilutions of each RNA preparation (a, b, c, d; 8x, 4x, 2x, 1x, respectively) were dotted in duplicate onto two nitrocellulose filters, which were analysed separately for the *ACT1* and *PYK1* mRNAs. The amount of radioactivity per dot was determined by cutting out dots and subjecting them to liquid scintillation counting.

b: For the purposes of presentation, the data for the untransformed DBY746 and DBY746:pLD1(35) transformants [35.1] (1) and [35.5] (5) have been corrected for differences in loading (using the actin mRNA data). RNA concentrations are therefore in arbitrary units. Also, the *PYK1* mRNA levels (full triangles) which are routinely calculated relative to the actin mRNA data (hollow triangles) have been presented relative to that of the untransformed DBY746 (0.6% of total mRNA).


Actin
[35.1]
[35.2]
[35.3] [35.1]
[35.4] [35.2]
a b c d a b c d
PYK
[35.3]
[35.4]
a b c d a b c d

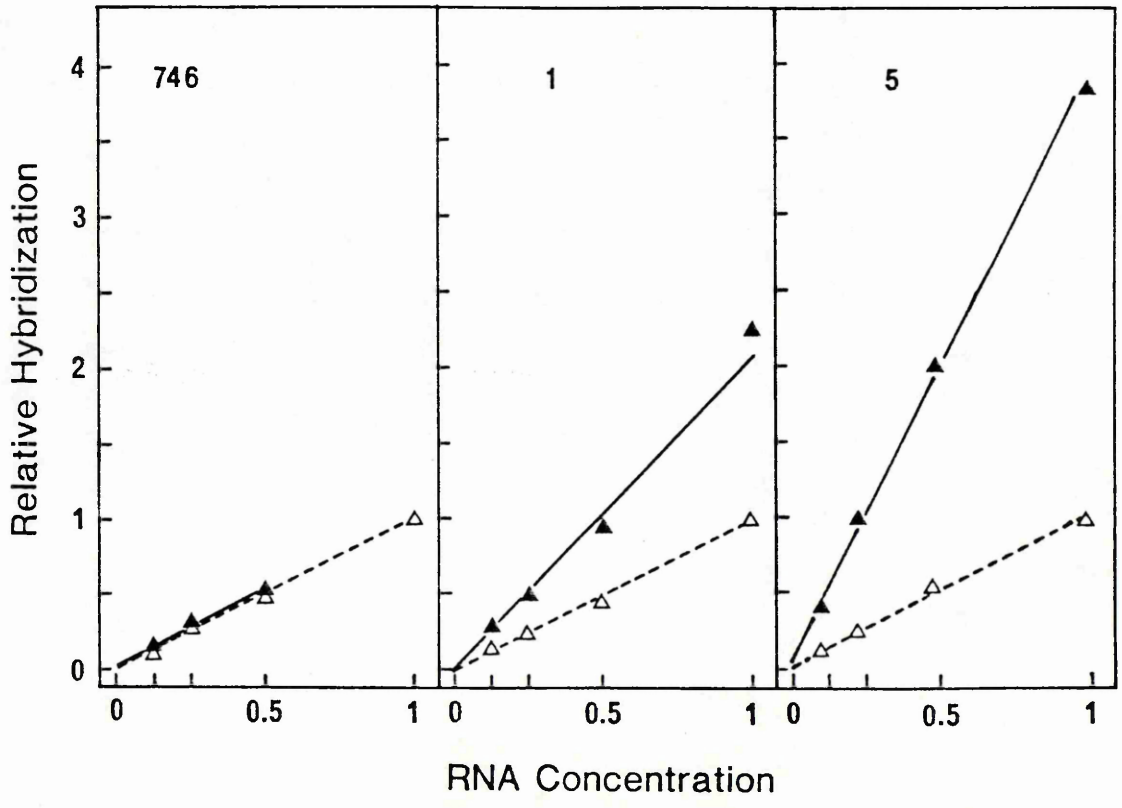
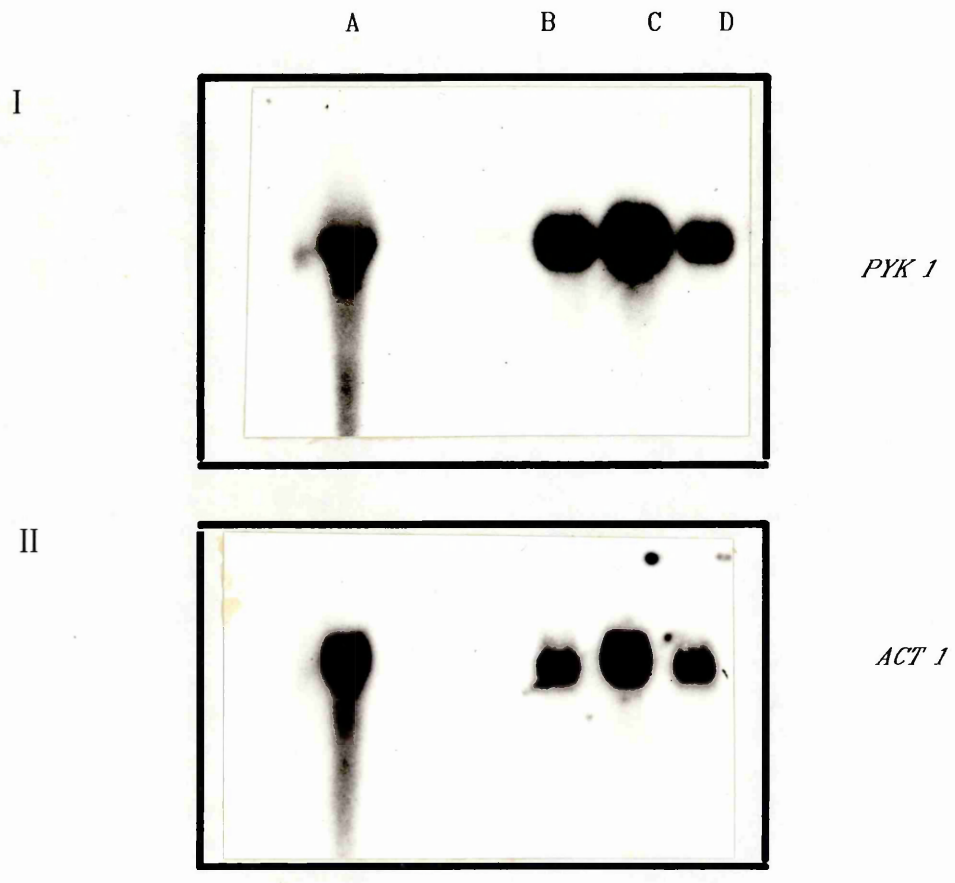

746
1
5
Relative Hybridization
RNA Concentration
0
1
2
3
4
0
0.5
1

Figure 4.4 Northern analysis of the *PYK1* mRNA in pLD(1)35 transformants.

RNA isolated from DBY746 (A) and three slow growing pLD1(35) transformants ([35.6-8]; (B-D)) was subjected to Northern analysis. The filter was probed for *PYK1* mRNA **(I)**, the probe stripped, and the filter then re-probed for actin mRNA **(II)**. At each stage the amount of bound radioactivity in each band was determined using an AMBIS 2D-Radioimaging System. Note that the amount of total RNA loaded in each lane differs for each sample.


A
B
C
D
I
PYK 1
II
ACT 1

The data from a typical analysis is shown in Fig. 4.3b. For the purposes of clarity, the data for the untransformed DBY746 and the two pLD1(35) transformants [35.1] and [35.5] have been corrected for differences in loadings (using the actin mRNA). RNA concentrations are therefore in arbitrary units. In addition, the *PYK1* mRNA levels have been presented relative to that of the untransformed host of DBY746, and are expressed as a percentage of total mRNA. This standardisation of the data with respect to DBY746, facilitates the comparison of these strains which have approximately 2-fold ([35.1]) and 4-fold ([35.5]) higher *PYK1* mRNA levels compared with DBY746.

RNA was isolated from approximately thirty pLD1(35) transformants to compare their *PYK1* mRNA abundances. For ten of these transformants, the doubling time during exponential phase was also determined. These transformants showed a considerable variation in their steady state levels of *PYK1* mRNA, ranging from less than 1.0% to over 6% of total mRNA. Typically, the faster growing strains had less than 1.5% *PYK1* mRNA, while slower growing strains had more than 1.5% *PYK1* mRNA.

These quantitative measurements of *PYK1* mRNA abundance obtained by dot-blotting were qualitatively confirmed for three transformants by Northern analysis (Fig. 4.4). RNA from DBY746 and three pLD1(35) transformants displaying different growth rates was electrophoresed on a denaturing gel. This gel was transferred to a Hybond N filter and probed for *PYK1* (Fig. 4.4a) and then stripped of *PYK1* probe and re-probed for *ACT1* (Fig. 4.4b). The abundance of the *PYK1* mRNA was then re-calculated by scanning the filter with the AMBIS β-scanner after hybridisation with *PYK1* probe, and then again after probing for the *ACT1* probe. The values obtained for *PYK1* mRNA abundance by this method of quantitation was found to be similar to the values determined by dot-blot hybridisation. The summary of these results are given in Table 4.1, along with the doubling times of the strains.

Table 4.1 demonstrates that strains with elevated *PYK1* mRNA levels have greater doubling times. However, there does not appear to be a strong correlation between the doubling time and the *PYK1* mRNA level for each transformant. For example, strain [35.6] has a doubling

TABLE 4.1 : Doubling time and *PYK1* mRNA levels of pLD1(35) transformants

Strain	Doubling time (hr) [a]	*PYK1* mRNA level[b]
DBY746	2.0	0.6
35.6	3.3	2.7
35.7	4.1	1.8
35.8	2.7	1.6

a: The doubling time of each strain was determined by monitoring the A_{260} of the culture during the exponential phase of growth.
b: The *PYK1* mRNA level was determined by analyses with the AMBIS β-scanner, using the level of the *ACT1* mRNA as a loading control.

time of 3.3 hr and a *PYK1* mRNA level of 2.7%, while [35.7] has greater doubling time (4.1 hr) but a lower *PYK1* mRNA level (1.8%). Thus, it is possible that the *PYK1* mRNA level is not the only parameter which affects cell growth rate. Alternatively, this apparent discrepancy could be due to the fact that, by necessity, the growth rate was measured over a long period, whereas the mRNA abundance was determined at a single point of growth. Nevertheless, it was consistently observed that transformants containing significantly elevated *PYK1* mRNA levels, grew more slowly than the untransformed host.

4.2.2 ABNORMALLY HIGH *PYK1* TRANSCRIPT LEVELS DECREASE DURING PROLONGED GROWTH OF A pLD1(35) TRANSFORMANT.

The results described so far suggested that elevated *PYK1* expression levels resulted in slow growth of the transformed yeast and showed that slow growing transformants reverted to faster growth. Therefore, it was decided to determine if this reversion to more rapid growth correlated with a change in the level of *PYK1* expression.

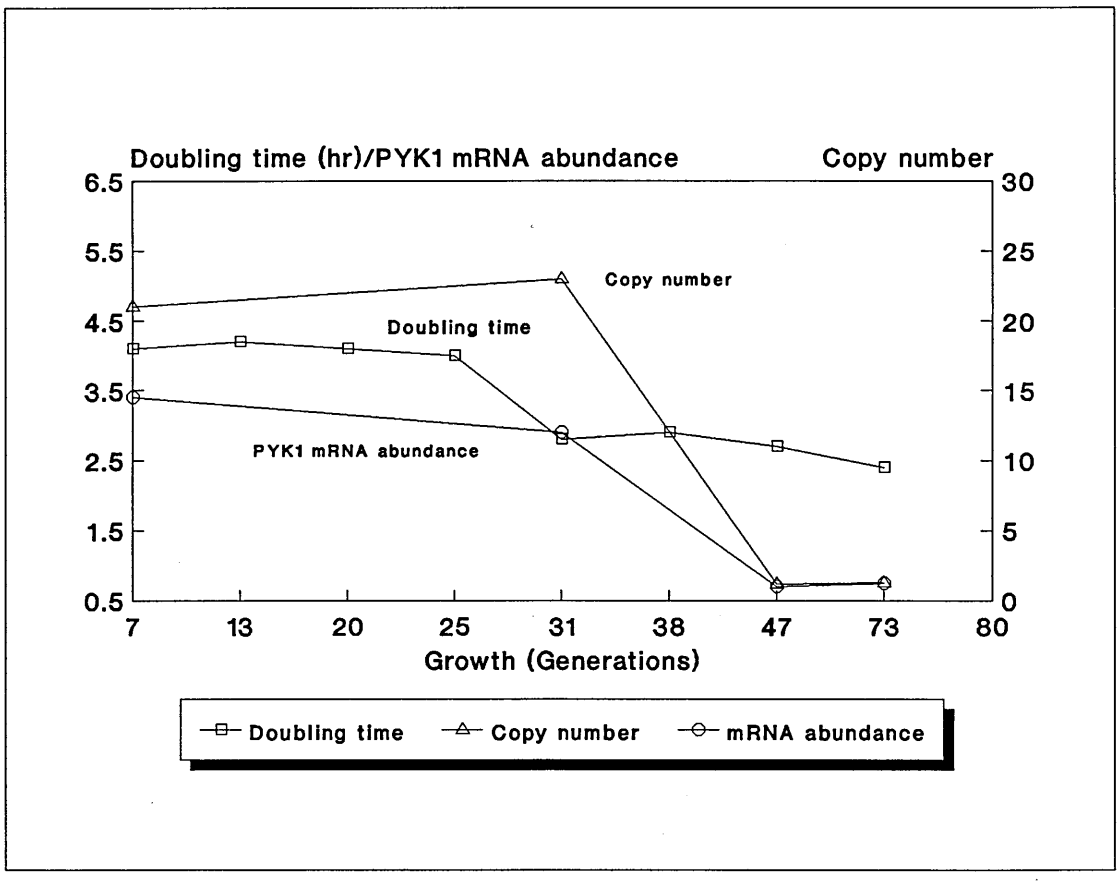
X4003-5B was transformed with pLD1(35), and a transformant which had formed a small colony (and therefore was presumed to have an abnormally slow growth rate) was picked for further analysis (designated [35.9]). It was inoculated into 5ml of selective broth and grown for 3-4 days at 30^{o}C until it had reached stationary phase. 0.5 ml of cells were then added to 200 ml of fresh minimal medium, and the resulting culture grown to the late logarithmic phase of growth. At this stage 0.5-1.0 ml of cells were added to 200 ml of fresh prewarmed minimal broth, and regrown at 30^{o}C. This was continued until the cells had undergone about 80 generations of growth.

The doubling time of the culture during exponential phase was monitored throughout the experiment using the absorbance at 600nm. Initially, the culture had a doubling time of 4.1 hrs but this decreased to 2.4 hrs during the course of the experiment. Fig. 4.5 demonstrates the change in doubling time which was accompanied with prolonged growth of this transformant.

At four time-points, cells were also harvested for DNA and RNA extraction. The DNA was used to calculate plasmid copy number, while the RNA was used to determine the level of the *PYK1* mRNA. Both of

Figure 4.5 Prolonged growth analysis of a pLD1(35) transformant.

The X4003-5B:pLD1(35) transformant, [35.9], was grown in serial batch culture for approximately 75 generations in minimal medium lacking leucine. Doubling times (in hrs) were measured by monitoring the absorbance at 600nm of the culture during exponential growth, *PYK1* mRNA abundance as a percentage of total mRNA, and *PYK1* copy number is expressed per haploid genome.


Doubling time (hr)/PYK1 mRNA abundance
Copy number
6.5
5.5
4.5
3.5
2.5
1.5
0.5
30
25
20
15
10
5
0
Copy number
Doubling time
PYK1 mRNA abundance
7
13
20
25
31
38
47
73
80
Growth (Generations)
Doubling time
Copy number
mRNA abundance

these calculations were carried out by dot-blot hybridisation as described previously (2.2.3.13; 2.2.4.2). The results of this are presented in Fig. 4.5. After about 45 generations of growth, significant decreases were observed both in *PYK1* gene dosage and in the abundance of the *PYK1* mRNA. By 70 generations of growth the copy number of the *PYK1* gene had approached normality, while the abundance of the *PYK1* mRNA had also approached wild-type levels. These changes correlated with an increase in growth rate (Fig. 4.5).

To confirm the existence of both chromosomal and plasmid-encoded *PYK1* sequences throughout the experiment, uncut DNA extracted at each time-point was subjected to Southern blotting and probed for *PYK1*. Fig. 4.6 shows the presence of two bands of the same size in each lane (on further exposure two bands are also evident in lane 3). The intensity of these 2 bands with respect to each other varies at the different time-points. In addition the total level of hybridisation to *PYK1* sequences in each lane varies across the blot. This is partly due to the different amounts of DNA loaded in each lane (Fig. 4.6a.) and also due to the changes in plasmid copy-number (Fig. 4.5). One of the two bands represents the chromosomal copy of *PYK1* and the second band represents uncut plasmid (either relaxed or nicked). In addition it was also possible that hybridisation to other plasmid bands (relaxed or nicked) coincided with hybridisation to the chromosomal copy, and is thus obscured. This makes comparison of the relative levels of plasmid *PYK1* to chromosomal *PYK1* difficult. The essence of this gel was to determine if any plasmid rearrangements had occurred during prolonged growth. These might have formed via recombination with the endogenous 2-micron plasmid during the prolonged growth. Gross rearrangements would have been revealed through additional bands on the southern blot. Additional bands were observed neither with the *PYK1* probe (Fig. 4.6b) nor a *LEU2* probe (not shown).

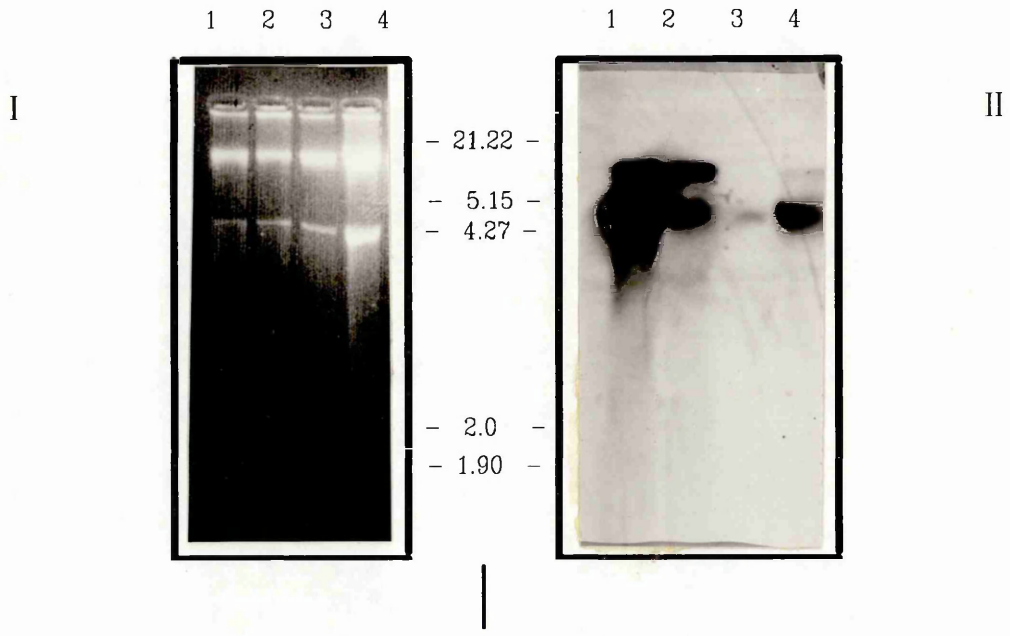
4.2.3 INACTIVATION OF THE *PYK1* GENE ON pLD1(35).

The experiments so far described, clearly show a correlation between increasing growth rates of pLD1(35) transformants with decreasing levels of *PYK1* gene expression. However, the possibility

Figure 4.6 Southern Analysis of DNA isolated from a pLD1(35) transformant.

a: Uncut DNA, isolated at four time-points (1-4; see Fig 4.5) during the growth of [35.9] was subjected to gel electrophoresis and Southern blotted.

b: The Southern filter was probed for *PYK1* sequences using random-prime labelled pSPK2. Only two bands were observed in each lane suggesting that no gross plasmid re-arrangements had occurred. On over-exposure 2 bands are evident in lane 3.



lambda EcoR1/ Hind III

digested size markers

that plasmid sequences other than *PYK1* are mediating this effect could not be ruled out. The *CYC3* and *FUN11* genes are located 5' and 3' to the *PYK1* gene on chromosome 1 (Coleman *et al.*, 1986). Comparing the restriction map of this region of chromosome 1 (Coleman *et al.*, 1986) with that of the genomic insert in pLD1(35) suggests that while the entire *CYC3* gene is probably present in pLD1(35), only part of the *FUN11* gene is present. Therefore, these genes may have contributed at least partially to the growth effects observed in pLD1(35) transformants.

To determine if the *PYK1* gene present on pLD1(35) was indeed mediating the growth effects, this gene was disrupted and the resulting plasmid transformed into yeast. The growth of these pLD1(37) transformants was then analysed.

4.2.3.1. Construction of pLD1(37).

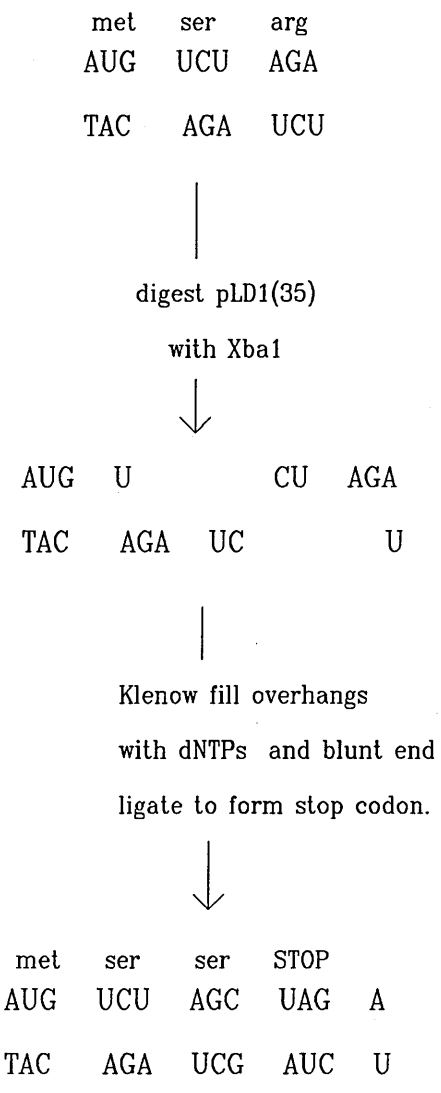
A premature stop codon was introduced near the 5' end of the coding region to disrupt the *PYK1* gene present on pLD1(35). Advantage was taken of the existence at the 5' end of *PYK1* of an *Xba* I site. This is the only *Xba* I site present in pLD1(35) and as shown in Fig. 4.7, it presents a manner in which to introduce a stop codon into the *PYK1* mRNA. pLD1(35) plasmid DNA was digested by *Xba* I and the resulting 5' overhangs were blunt-ended with Klenow and dNTPs. This DNA was religated and transformed into *E.coli*. Mini-plasmid preparations were carried out on 12 transformants, and a series of restriction digests were performed to identify a plasmid which had the *Xba* I site removed. One such clone was obtained and the plasmid labelled pLD1(37). As shown in Fig. 4.7, the manipulations carried out to delete the *Xba* I site and create pLD1(37) result in the creation of a stop codon immediately after the start codon.

4.2.3.2 Transformation of *S.cerevisiae* with pLD1(37)

When either X4003-5B or DBY746 was transformed with pLD1(37), the resulting transformants generally showed stronger growth compared to that seen previously with pLD1(35) transformants (Fig. 4.2c). However, when some of these transformants were grown in minimal liquid culture lacking leucine, some still grew slower than the

Figure 4.7 Construction of pLD1(37)

A stop codon was introduced near the begining of the *PYK1* coding region, borne on the plasmid pLD1(35) to create pLD1(37).



Sequence immediately 3' to the translational start codon of the PYK1 gene.

Sequence after translational start codon of PYK1 in the plasmid pLD137.

TABLE 4.2. Plasmid copy number and *PYK1/pyk1* mRNA levels in pLD1(37) transformants

Strain	Plasmid Copy Number[a]	*pyk1/PYK1* mRNA levels[b]
[37. 1]	45	0.67%
[37. 2]	14	0.75%
[37. 3]	12	0.74%

a: Plasmid copy number was calculated by determining the abundance of the *PYK1/pyk1* sequences by dot-blot analysis of total yeast DNA, and comparing to the abundance of *ACT1* sequences (a single copy gene).

b: The *PYK1/pyk1* mRNA level was determined by dot-blot analyses and comparing the abundance of *PYK1/pyk1* mRNA sequences to the abundance of *ACT1 mRNA.* It is expressed as % of total mRNA.

host-strain. Three pLD1(37) transformants (designated [37.1-3]) were analysed for both their plasmid copy-number and the level of *pyk1/PYK1* mRNA (Table 4.2). *pyk1* mRNA originates from the pLD1(37) plasmid while the chromosomal copy of the *PYK1* gene directs synthesis of *PYK1* mRNA. The probe used in the mRNA abundances was unable to distinguish between these two transcripts. The results of this analysis (Table 4.2) show that in none of the transformants analysed was the abundance of *pyk1/PYK1* mRNA significantly greater than the level observed in the untransformed host (0.6% of total mRNA). However, this was not due to the absence of additional gene copies, as the plasmid copy-number was greater than ten for all three transformants.

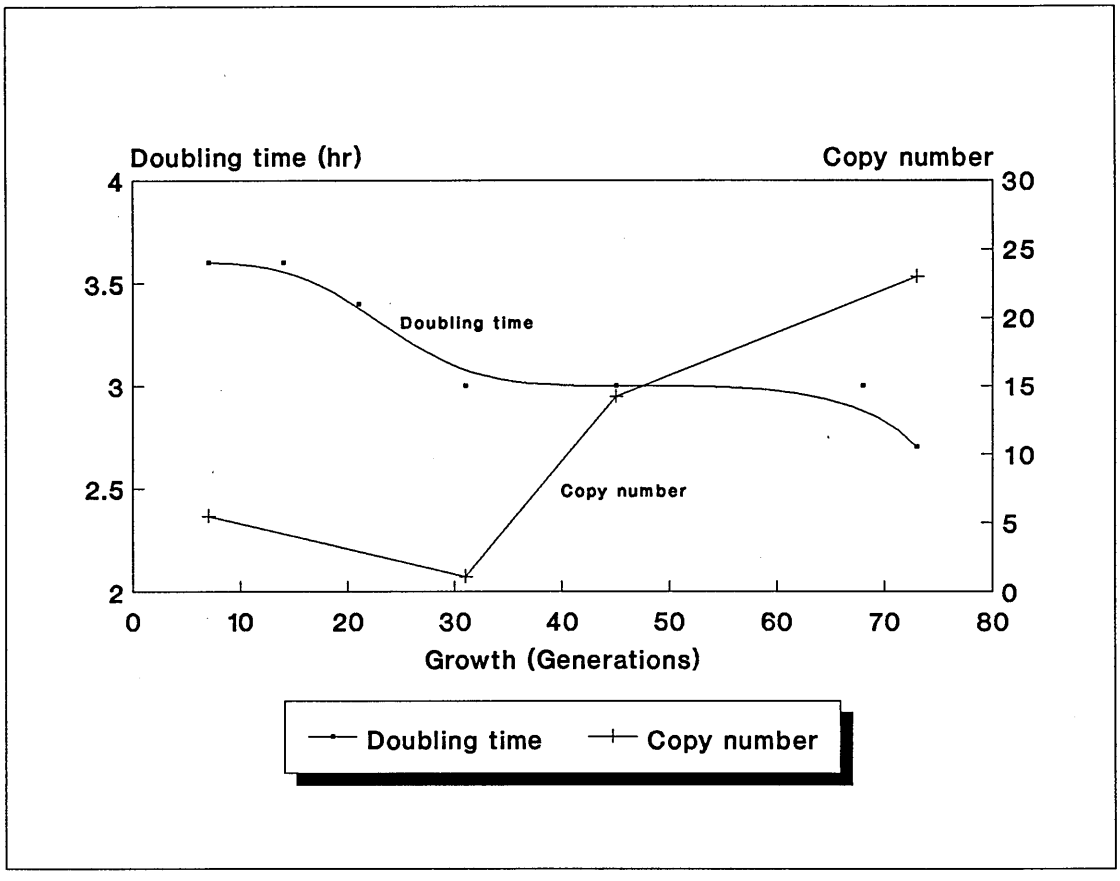
To determine if the correlation between cell growth rate and plasmid copy number had been disrupted in the pLD1(37) transformants, one of the slower growing pLD1(37) transformants was subjected to the prolonged growth carried out on a pLD1(35) transformant previously (4.2.2).

A fresh pLD1(37) transformant of X4003-5B (designated [37.4]) was grown exactly as the pLD1(35) transformant had been previously (4.2.2). The doubling time was monitored throughout, and plasmid copy number and *pyk1/PYK1* mRNA levels determined at four time points as before. There was no significant change in the steady state abundance of the *pyk1* mRNA, much above the level of the untransformed host (0.6%) at any of the four time points checked. However, both the doubling time and the plasmid copy number do vary with number of generations of growth. The results for the copy number and the doubling time are shown graphically in Fig. 4.8.

Like the pLD1(35) transformant, there was again a decrease in doubling time during prolonged growth. In the case of the pLD1(37) transformant, the decrease in doubling time was from 3.6 hr to 2.7 hr. However, in contrast with pLD1(35) this was accompanied by an increase in plasmid copy number. At the beginning of the experiment the culture had an average *pyk1* copy number of between 5 and 6. By the time the culture had undergone 70 generations the *pyk1* copy number had increased 5-fold to over 20. Thus, in the case of the pLD1(37) transformant, the increase in growth rate observed during prolonged growth is accompanied by an increase in the copy number.

Figure 4.8 Prolonged growth analysis of a pLD1(37) transformant.

The X4003-5B:pLD1(37) transformant, [37.4], was grown in serial batch culture for approximately 75 generations in minimal medium lacking leucine. Doubling times (in hrs) were measured by monitoring the absorbance at 600nm of the culture during exponential growth and *pyk1* copy number is expressed per haploid genome.


Doubling time (hr)
Copy number
4
3.5
3
2.5
2
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Doubling time
Copy number
Growth (Generations)
Doubling time
Copy number

Therefore, the inactivation of the *PYK1* gene on pLD1(35) disrupts the deleterious effect of increased *PYK1* copy number upon yeast cell growth. Indeed, it appears there is selective advantage in having increased copies of the pLD1(37) plasmid, as the copy-number increased as the doubling time decreased.

4.2.4 HIGH INTRACELLULAR LEVELS OF PYRUVATE KINASE DO NOT IMPAIR CELL GROWTH.

It was possible that the detrimental effect on cell growth caused by having elevated levels of *PYK1* gene was mediated by having excess pyruvate kinase which could perhaps have effected glycolytic flux. This question was addressed by using a multi-copy vector pMA91(+PYK) which carries a *PGK:PYK* gene fusion in which the *PGK* promoter and 5' untranslated region were fused to the *PYK1* coding sequence and 3' untranslated region (Fig 4.9). pMA91 itself is a vector which has been used to drive the high level expression of other proteins in yeast (Mellor *et al.*, 1985). Like pJDB207 (the parental plasmid of pLD1(35)), it is 2-micron based containing the origin of replication but not all of the 2-micron encoded genes, and contains *the LEU2d* allele for selection in yeast. pMA91(+PYK) contains the *PYK1* coding and 3' untranslated regions inserted at the *BglII* expression site of pMA91. Therefore, in this construct there are no *PYK1* 5' sequences. However, the vector has been shown to complement a *pyk* mutant, inferring that functional pyruvate kinase is synthesised (Murcott *et al.*, 1991). By comparing the phenotype of pMA91(+PYK) transformants with that of pLD1(35) transformants, it was possible to determine if the deleterious effects on cell growth were mediated by the pyruvate kinase protein itself, or whether 5' *PYK1* sequences were also required.

When DBY746 was transformed with pMA91(+PYK), a higher frequency of transformation than that obtained with pLD1(35) was observed. In addition, all of the transformants tested (approximately 8) had a doubling time of less than 2.5 hr. Thus, there was no slow growth effects associated with pMA91(+PYK) transformants, again in contrast to pLD1(35) transformants.

DBY746, 2 fresh pLD1(35) ([35.10] and [35.11]) transformants and a fresh pMA91(+PYK) transformant ([91P.1]) were grown to late

Figure 4.9 Structure of pMA91+PYK plasmid.

pMA91+PYK contains the *PYK1* coding region and 3' untranslated region (*Xba*1-*Hind*III) cloned into the *Bgl*II and *Hind*III sites of pMA91. The fusion at the BglII site was achieved using a linker oligonucleotide with a *Bgl*II compatible end (complementary to the vector) and a *Xba*I compatible end (complementary to the insert). Restriction sites marked are not necessarily unique, and their distance from an arbitrary origin (0.00) are given in kb. While not all restriction sites are depicted, all the *Eco*R1 sites are shown. This plasmid was a generous gift from Dr J. Mellor

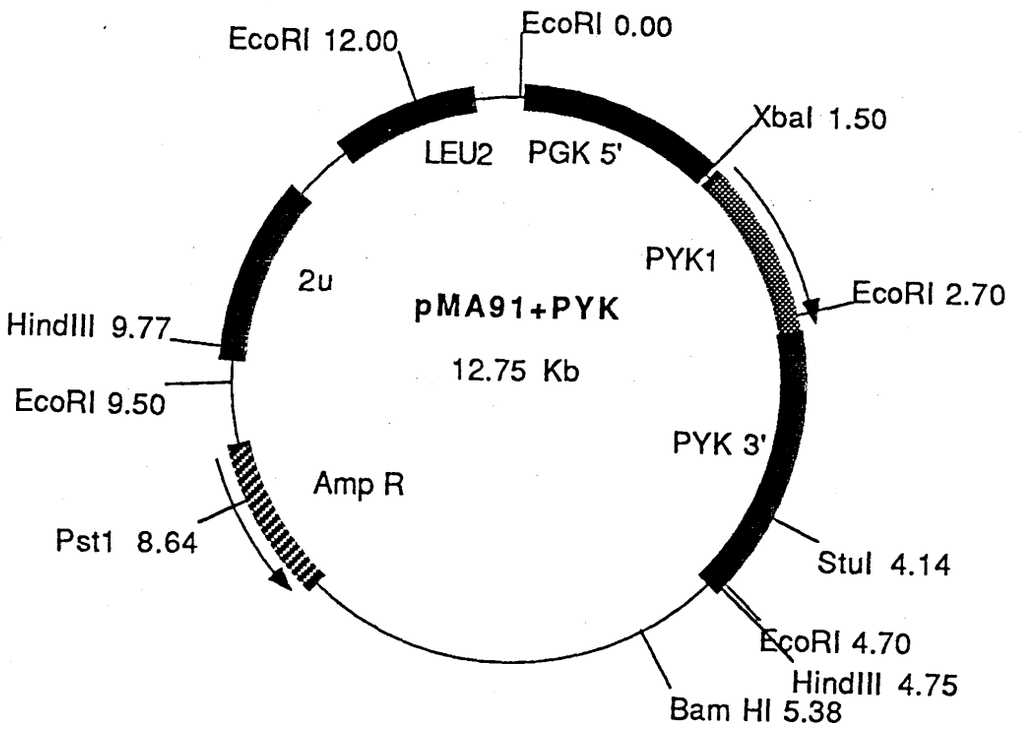

EcoRI 12.00
EcoRI 0.00
Xbal 1.50
LEU2
PGK 5'
PYK1
2u
pMA91+PYK
12.75 Kb
EcoRI 2.70
HindIII 9.77
EcoRI 9.50
PYK 3'
Amp R
Pst1 8.64
StuI 4.14
EcoRI 4.70
HindIII 4.75
Bam HI 5.38

Figure 4.10 Synthesis of high levels of pyruvate kinase.

Total soluble protein extracts from DBY746 (A), two fresh pLD1(35) transformants ([35.10] (B) and [35.11] (C)) and a fresh pMA91+PYK transformant [91.1] (D), were electrophoresed on an 8% polyacrylamide/SDS gel and stained with coomassie blue. The loading for DBY746 is greater than for the transformants. The arrow indicates the pyruvate kinase protein.

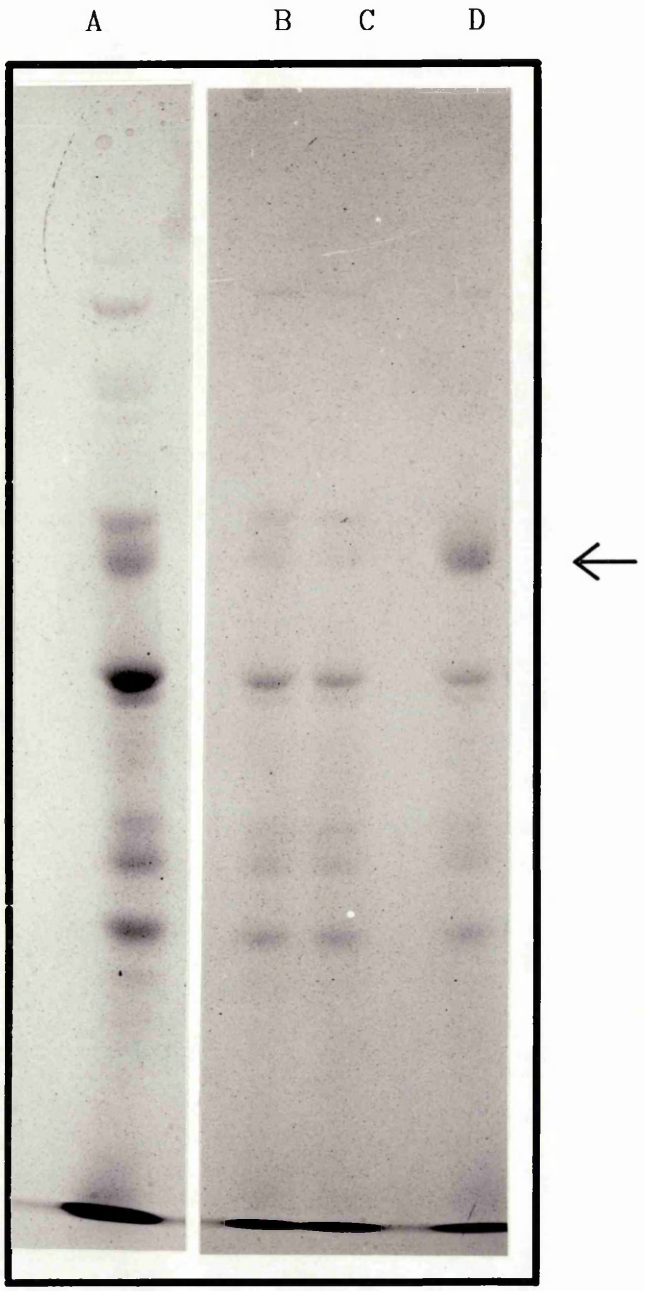

A
B
C
D

TABLE 4.3: *PYK1* mRNA levels in strains studied for levels of pyruvate kinase

Strain	*PYK1* mRNA level[a]
DBY746	0.6%
[35.10]	0.9%
[35.11]	1.6%
[91P.1]	5.5%

a: The *PYK1* mRNA level was determined by dot-blot analyses and comparing the abundance of *PYK1* mRNA sequences to the abundance of *ACT1 mRNA*. It is expressed as % of total mRNA.

exponential phase of growth in 200 ml of minimal media lacking leucine. 100 ml of cells were harvested and used for RNA isolation, while 50 ml of cells were used for protein extraction. The RNA was used to calculate the abundance of the *PYK1* mRNA in each of the transformants. In the case of the pMA91(+PYK) transformant the *PYK1* mRNA originating from the plasmid is modified at the 5' end as it contains the *PGK* 5' leader in place of the *PYK* 5' untranslated leader. These mRNA levels are expressed as percentage of total mRNA in Table 4.3.

Interestingly the pMA91(+PYK) transformant has a high level of *PYK1* mRNA (nearly 10-fold higher than untransformed host) and yet no detrimental effect on cell growth was observed. However, the majority of this mRNA will be *PGK:PYK* mRNA synthesised from the plasmid. Thus, it appears that excess *PGK:PYK* mRNA does not affect cell growth in the manner observed for *PYK1* mRNA.

Protein extracts isolated from each strain were subjected to SDS-PAGE (Fig. 4.10). Only limited over-production of pyruvate kinase was apparent in both of the pLD1(35) transformants. However, massive over-production of pyruvate kinase was observed in the pMA91(+PYK) transformant. Thus, high levels of pyruvate kinase appear not to cause poor growth. This suggests that the deleterious effects of multi-copy plasmids carrying the wild-type *PYK1* gene were not due to the synthesis of excess pyruvate kinase.

4.3. DISCUSSION.

Transformation of *S.cerevisiae* with the multicopy plasmid pLD1(35) gave rise to transformants with different growth rates (Fig. 4.2a). This was observed for two strains of yeast with different genetic backgrounds and thus appears to be caused by the plasmid. The plasmid contains the entire *PYK1* gene on a 6.3kb genomic fragment sub-cloned into pJDB207 (Fig. 4.1). Slow growing transformants carried high levels of the *PYK1* mRNA although there was not a linear relationship between growth rate and doubling time (Fig. 4.4).

There appears to be strong pressure for slow growing pLD1(35) transformants to revert to faster growth (Fig. 4.2b). Previously, it had been shown that when pLD1(35) transformants were stored for a month

at 4°C, *PYK1* mRNA levels decrease (Bettany, 1988). In the case of one particular transformant an 8-fold reduction in *PYK1* mRNA levels was observed. Thus, it appears there is strong selection against high *PYK1* mRNA levels.

In this study, one pLD1(35) transformant which initially grew slowly, was grown for 80 generations by serial batch culture in minimal media lacking leucine. Growth rate, plasmid copy number and *PYK1* mRNA levels were monitored throughout growth (Fig. 4.5). This analysis demonstrated that there is indeed a strong selection for cells with increased growth rate, as the doubling time of the transformant decreased from 4.1hr to 2.4hr. This decrease in doubling time was accompanied by a decrease in plasmid copy number and a decrease in the abundance of the *PYK1* mRNA. Presumably this decrease in the average *PYK1* gene and mRNA levels was mediated by unequal partitioning of this pJDB207-based vector during cell division (Futcher and Cox, 1984) followed by more rapid growth of cells carrying reduced *PYK1* copy numbers. Thus, there appears to be rough correlation between the copy number of pLD1(35) and division time of the yeast cell.

The involvement of sequences on pLD1(35) other than *PYK1* in mediating the growth effect was ruled out following analysis of a pLD1(37) transformant. This plasmid is virtually identical to pLD1(35) with the exception that pLD1(37) contains a premature stop codon near to the translational initiation codon of *PYK1* (Fig. 4.7).

Analyses of several pLD1(37) transformants showed that while they have high plasmid copy numbers, the level of *PYK/pyk* mRNA does not significantly exceed the level observed in the untransformed host. The reason for this is unclear and was not examined further. However, it seems likely that it is either due to an effect on the synthesis of mRNA from the plasmid copy of the *pyk1* gene (ie transcription), or is due to *pyk1* mRNA instability. It is plausible that because the *pyk1* mRNA will have at most one ribosome attached (due to the proximity of the stop codon relative to the translational start codon) it is more exposed to digestion by ribonucleases. However, previous evidence from this laboratory has shown that the introduction of a premature stop codon at the 5' end of the *PYK1* mRNA or the 5' end of a *PYK1:lacZ*

mRNA has no effect on the stability of that mRNA (Purvis *et al.*, 1987c). The stability of the *pyk1* mRNA in the pLD1(37) transformants needs to be measured.

Prolonged growth of a pLD1(37) transformant demonstrates that while the doubling time of the transformant decreases during growth in a manner similar to the pLD1(35) transformant, it is accompanied by an increase in plasmid copy number (Fig. 4.8). Thus when the *pyk1* gene is disrupted, there are no other sequences which are detrimental to cell growth when their dosage is increased. Indeed there appears to be a selective advantage to increase the copy-number of the pLD1(37) plasmid in transformants.

This increase in the copy-number of the plasmid could not have occurred as a consequence of 2-micron amplification, a mechanism utilised by the naturally occurring 2-micron plasmid to increase its abundance (Murray *et al.*, 1987). This method of plasmid amplification depends on the presence of two copies of the 2-micron inverted repeat which are a substrate for the site specific recombination event which is pivotal to plasmid amplification. pJDB207 contains only one of these inverted-repeat sequences. Therefore, the increase in plasmid copy number observed must have occurred through unequal partitioning (as described above) followed by a selection for a higher plasmid copy number which resulted in faster growth. It has previously been noted that pJDB207 carries a *LEU2d* allele which only partially complements the *leu2* mutation due to the presence of a truncated promoter (Janes *et al.*, 1990). To fully complement the *leu2* mutation, multiple copies of the *LEU2d* allele present on the pJDB207 plasmid are required and this perhaps explains the selection for increased plasmid copy number in the case of the pLD1(37) transformant.

Recently, Janes and co-workers (1990) transformed yeast with a pJDB207 based vector which carried the heterologous gene hirudin under the control of the *TDH1* promoter. While hirudin is apparently not toxic to yeast cells, over-expression of this gene resulted in poor growth. Interestingly, the growth rate and plasmid copy number of the transformant are related to the strength of the *TDH1* promoter (four size variants of the promoter were tested). Transformants which contained a plasmid with a powerful promoter displayed slow growth

combined with low plasmid copy number. Conversely, when a plasmid carrying a weak *TDH1* promoter variant was used, transformants displayed a faster growth rate and a higher plasmid copy number. The workers hypothesised that the copy number of the pJDB207-based vector used is influenced not only by the need for multiple copies of the *LEU2d* gene to complement the *leu2* mutation in the host, but is also influenced by the expression level of the plasmid-borne hirudin gene (Janes *et al.*, 1990)

In this study it would appear that the growth rate of pLD1(35) transformants also hinges on the expression of two plasmid encoded genes: *PYK1* and *LEU2d*. While there is a selection to increase the copy number via *LEU2d*, the pressure to decrease the gene dosage of the *PYK1* gene is stronger. Thus, cells with decreased plasmid copy number show a selective advantage. Importantly, in this study the negative effects on growth rate are not caused by the over-expression of a heterologous gene (as demonstrated by Janes *et al.*, 1990) but by a yeast gene.

The fact that more than one aspect of the plasmid has an affect on cell growth might account for the non-linear relationship which was observed between growth rate and *PYK1* mRNA level in pLD1(35) transformants.

Alternatively, mutations could have occurred during growth, either in the genome of the transformant or in the plasmid itself. These could have overcome the growth defects associated with low *LEU2d* plasmid dosage and/or the effects associated with high *PYK1* dosage. Gross plasmid rearrangements were not evident during the prolonged growth of the pLD1(35) transformant (Fig. 4.6). However, small sequence changes would not have been detected in this analysis. This could have been checked by rescuing the pLD1(35) plasmid from yeast cells at the end of the prolonged growth experiment, and transforming this rescued plasmid back into yeast. If resulting transformants grew normally this would suggest that a mutation had occurred in the plasmid which disrupted the original growth effects observed with pLD1(35). Likewise, it is possible that a mutation had occurred in the genome of the transformant during growth and that this imparted a growth advantage which was selected for. Again, this could be checked by

curing the transformant derived cells of plasmid at the end of prolonged growth and re-transforming this strain with pLD1(35). If the strain was mutated then all the resulting transformants would have grown normally.

It would also have been informative to have analysed individual cells within the culture which had been grown for many generations (both the [35.9] and [37.4] cultures). This would have given an indication of clonal variation within the population with respect to doubling time, *PYK1* mRNA level, and plasmid copy number. However, these experiments are technically time consuming and it was decided to concentrate on other aspects of *PYK1* expression in yeast.

The observation that high *PYK1* copy number affects cell growth appears to set this gene apart from most other glycolytic mRNAs. It has become clear that yeast can tolerate extremely high levels of expression from most other glycolytic genes with little apparent effect upon cell growth or viability. These include the *HXK2*, *PGI1*, *TPI1*, *PGK1* and *PGM1* genes (Alber and Kawasaki, 1982; Kawasaki and Fraenkel, 1982; Mellor *et al.*, 1985; Aguielera and Zimmerman, 1986; Schaaf *et al.*, 1989). Interestingly, it has previously been shown that some *pyk1* alleles can confer cell cycle arrest: the cell cycle mutant *cdc19* has been shown to be allelic to *PYK1* (Fraenkel, 1982). Perhaps therefore, the *PYK1* gene has an important role in cell cycle and increasing its dosage causes some imbalance in gene expression which results in poor growth. However, there is no evidence that supports this statement and further experiments are necessary.

It must be emphasised that excess pyruvate kinase itself does not inhibit growth as pMA91(+PYK) transformants grow normally and yet can massively over-produce pyruvate kinase (Fig. 4.10). In this respect the enzyme is similar to hexokinase, phosphoglucose isomerase, triosephospate isomerase, phosphoglycerate kinase and phosphoglycerate mutase (Alber and Kawasaki, 1982; Kawasaki and Fraenkel, 1982; Chen et al., 1984 Mellor *et al.*, 1985; Aguielera and Zimmerman, 1986; Schaaf *et al.*, 1989). This fact that yeast can tolerate high levels of pyruvate kinase suggests that the relatively normal behaviour of pLD1(37) transformants, in which the plasmid-borne *pyk1*

locus carries a nonsense mutation, was not brought about by blocking over-production of pyruvate kinase.

In pMA91(+PYK) transformants, the plasmid directs the synthesis of a *PGK:PYK* mRNA. While yeast appears to tolerate high levels of this fusion mRNA (Table 4.3) without any effect on growth rate, cells grow slowly when the wild-type *PYK1* mRNA is at abnormally high levels cells grow slowly. Interestingly, the pLD1(37) transformant does not show elevated levels of *pyk1* mRNA, and perhaps this is critical to disruption of the growth effects characteristic of pLD1(35) transformants. Thus, it is possible that the detrimental growth effects observed in pLD1(35) transformants are caused by having elevated *PYK1* mRNA levels. However, high levels of a *PGK:PYK1* fusion mRNA has no effect on cell growth. This mRNA comprises the *PYK1* coding and 3' untranslated regions but not the *PYK1* 5' leader. This suggests that the 5' leader of *PYK1* mRNA is required for the slow growth effects.

To test the hypothesis that the *PYK1* 5' leader does mediate the growth effects, it would be informative to introduce this sequence into yeast on a multi-copy vector, without any other *PYK1* sequences. This could be achieved by simply over-expressing the leader sequence on its own, or as part of an alternative gene fusion. If this led to transformants showing poor growth then the *PYK1* 5' leader would appear to be the cause of the detrimental growth effects observed.

To summarise, it has been shown that abnormally high levels of *PYK1* mRNA result in deleterious growth of *S.cerevisiae*. There is a strong selective pressure to decrease *PYK1* copy number to restore normal growth. This selection was disrupted with the introduction of a premature translational stop codon at the beginning of the *PYK1* coding region. However, pyruvate kinase can be present at high levels in yeast with no effect on growth rate. Therefore excess *PYK1* mRNA appears to cause the poor growth phenotype, with the 5' translational leader of *PYK1* essential for this effect. This requires testing. Interestingly, the *PYK1* 5' translational leader is involved in a co-ordinated mode of gene regulation (Chapters 5, 6).

CHAPTER 5

RESULTS (III)

THE YEAST *PYK1* GENE IS SUBJECT TO DOSAGE LIMITATION

5.1. INTRODUCTION

Pyruvate kinase is one of the most abundant glycolytic enzymes (Fraenkel, 1982), and hence the promoter from this gene has been considered as a potential promoter for directing the synthesis of heterologous proteins (Goodey *et al.*, 1987; Kingsman *et al.*, 1985).

In this chapter, the level of over-expression of the *PYK1* gene itself has been studied in yeast. This has involved introduction of multiple copies of the *PYK1* gene into yeast on the multi-copy vector pLD1(35), and an analysis of the increase in both *PYK1* mRNA and protein levels achieved in individual transformants. In addition the translational efficiency of excess *PYK1* mRNA was also tested.

In general, the transformation of a yeast gene back into yeast using a multi-copy plasmid vector leads to the synthesis of large amounts of protein encoded by that gene. When the *PGK1* gene is introduced into yeast on a multi-copy vector, 50% of the soluble cell protein is phosphoglycerate kinase (Mellor *et al.*, 1985). Other glycolytic enzymes have also been over-expressed in yeast when the corresponding gene is present in multi-copy (Schaaf *et al.*, 1989). This high level expression from multi-copy vectors has also provided the basis for cloning strategies for genes whose over-expression provides a selectable phenotype in yeast (Rine *et al.*, 1983).

However, there are a number of well defined cases where the existence of multiple copies of a specific gene in yeast does not result in the expected increase in the intracellular concentration of the appropriate protein. To date, most examples belong to two gene families, those encoding histone and ribosomal proteins (Osley and Hereford, 1981; Warner *et al.*, 1985). The results of experiments presented in this chapter indicate that over-expression of *PYK1* is also limited. This appears to set the *PYK1* gene apart from the other glycolytic genes.

5.2 RESULTS.

5.2.1 GENE DOSAGE EFFECTS LIMIT *PYK1* mRNA ABUNDANCE.

DBY746 was transformed with the multi-copy vector pLD1(35) (Fig. 4.1). Results from chapter 4 have shown that different DBY746:pLD1(35) transformants carry different plasmid copy-numbers and different levels of *PYK1* mRNA. In addition, it was demonstrated that when a pLD1(35) transformant with a high plasmid copy number was grown for a prolonged length of time (even under conditions which select for plasmid containing cells) there is a strong selective pressure for cells with reduced copy number. Thus, it was decided to analyse a range of transformants immediately after transformation. *PYK1* mRNA levels and *PYK1* copy numbers were determined to establish whether the presence of multiple copies of the *PYK1* gene leads to a proportional increase in the level at which the *PYK1* mRNA is present.

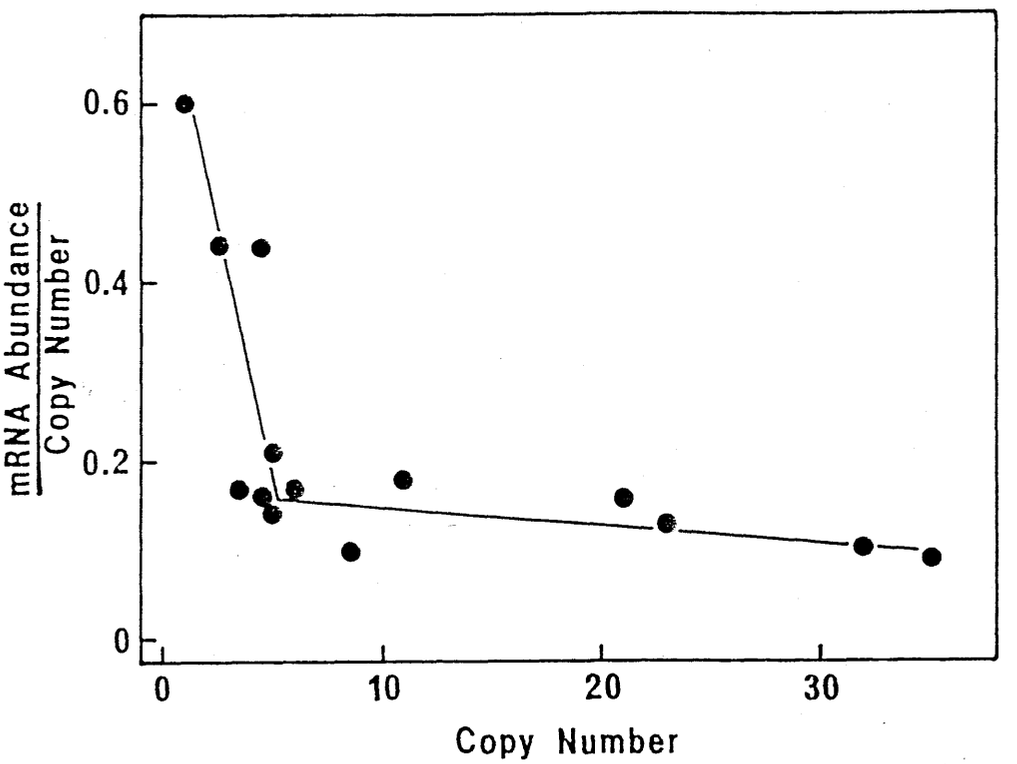
Fresh pLD1(35) transformants were grown in 5ml of minimal media to stationary phase, an inocula sub-cultured into 200 ml of minimal media lacking leucine and the culture grown to an absorbance of between 0.6-0.8. To exclude the possible influence of growth-phase upon plasmid copy-number and mRNA abundance, cells were harvested at the same time for both DNA and RNA extractions.

Plasmid copy number and *PYK1* mRNA abundance were determined for fourteen different pLD1(35) transformants by dot-blot hybridisation as described previously (2.2.3.13; 2.2.4.2). The actin gene and mRNA were used as internal controls for the *PYK1* copy number and mRNA abundance measurements, respectively. The results of this analysis are summarised in Fig. 5.1.

Transcripts from the single chromosomal *PYK1* locus are present at 0.6% of total mRNA (Purvis *et al.*, 1987a). If a parallel increase in *PYK1* mRNA abundance accompanied an increase in *PYK1* gene dosage, then *PYK1* mRNA should always be present at 0.6% of total mRNA per copy of the *PYK1* gene. As shown in Fig. 5.1 this was found not to be the case. Instead the data reveals a strong dosage effect. The abundance of *PYK1* mRNA per gene copy decreases markedly as more copies of the *PYK1* gene are present. This decrease is biphasic. A

Figure 5.1 Over-expression of *PYK1* is limited at the level of mRNA abundance.

Fourteen DBY746:pLD1(35) transformants were analysed. *PYK1* gene dosage (relative to the actin gene) and *PYK1* mRNA abundance (relative to the actin mRNA and 18S rRNA) were measured in each transformant using cells harvested at the same time from the same culture. The amount of *PYK1* per gene copy is plotted against *PYK1* copy number for each transformant.


mRNA Abundance / Copy Number
0.6
0.4
0.2
0
0
10
20
30
Copy Number

three-fold decrease in mRNA per gene copy occurred when *PYK1* copy number was increased to five, but as the copy number is increased further up to 35 per haploid genome, a further decrease of only 1.6-fold in *PYK1* mRNA per gene copy was observed. Therefore, although some transformants contained relatively high *PYK1* mRNA levels, the build up of *PYK1* mRNA is limited by an undefined gene dosage dependent control mechanism.

5.2.2 ABUNDANCE OF *PGK* mRNA IN pLD1(35) TRANSFORMANTS.

Perhaps the most plausible explanation for the pattern of gene expression presented in Fig. 5.1, is that as the level of the *PYK1* gene increases, there is saturation of a factor required for *PYK1* transcription. Thus, as this factor becomes depleted the level of transcription at each *PYK1* gene is reduced. If this is indeed the case, then the biphasic nature of Fig. 5.1 would suggest that not only one transcriptional factor becomes limiting. Instead, several transcriptional activators could become limiting for multiple *PYK1* genes as would appear to be the case for *ADH2* (Irani *et al.*, 1987).

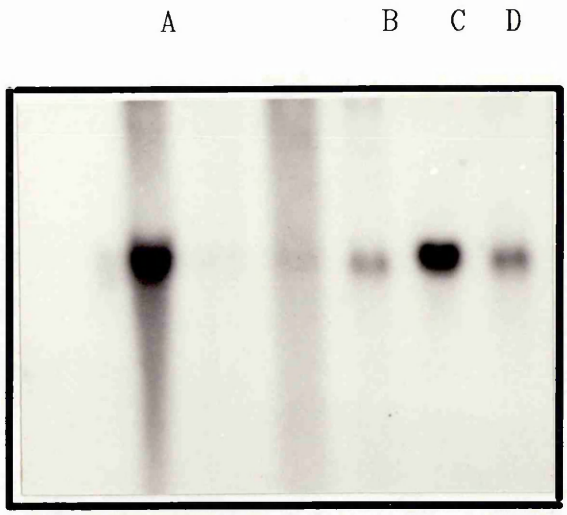
It has become apparent from recent studies that there are several *cis*-acting sites which are important in controlling the transcription of *PYK1* (Purvis *et al.*, 1987a; Buchman *et al.*, 1988 Nishizawa *et al.*, 1989). Also, several *trans*-acting factors have been identified which either bind to the promoter sequences of *PYK1 in vitro* (Buchman *et al.*, 1988; Chambers *et al.*, 1990) or mediate transcription of the *PYK1* gene *in vivo* (Nishizawa *et al.*, 1990). Two of the DNA-binding proteins shown to bind the *PYK1* promoter *in vitro* (ABF1 and RAP1), have also been shown to bind to the promoters of other glycolytic promoters including *PGK* (Chambers *et al.*, 1990). As pointed out in 1.3.2, the promoter of the *PYK1* gene appears in many respects to be similar to the promoter of *PGK*. Furthermore, both genes appear to have sequences within the coding region which are required for full transcriptional activity.

Bearing this in mind it was decided to determine if the level of the *PGK* mRNA was affected by an increased *PYK1* gene dosage. This was carried out by determining the abundance of the *PGK* mRNA in three pLD1(35) transformants which had previously been shown to

Figure 5.2 Northern analysis of the *PGK* mRNA in pLD(1)35 transformants.

RNA isolated from DBY746 (A) and three slow growing pLD1(35) transformants ([35.6-8] (B-D)) was subjected to Northern analysis. The filter was probed sequentially for the *PYK1* mRNA (Fig 4.4) and for actin mRNA (II). The filter was then re-analysed for the *PGK* mRNA (I). At each stage the amount of bound radioactivity in each band was determined using an AMBIS 2D-Radioimaging System. Note that the amount of total RNA loaded in each lane differs for each sample.

I



II

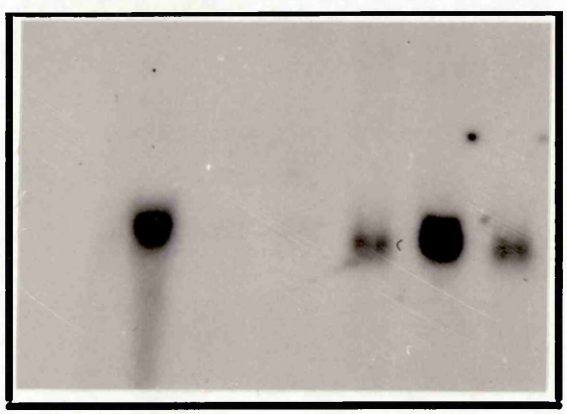


TABLE 5.1: ABUNDANCE OF THE *PYK1* AND *PGK* mRNA LEVELS IN pLD1(35) TRANSFORMANTS.

STRAIN	*PYK1* mRNA LEVEL[a]	*PGK* mRNA LEVEL[b]
DBY746	0.6	1.00
[35.6]	2.7	0.41
[35.7]	1.8	0.57
[35.8]	1.6	0.63

a: *PYK1* mRNA levels are expressed as percentage of total mRNA (using *ACT1* mRNA as loading control) and were determined by Northern analyses, followed by quantitation using the AMBIS β-scanner.

b: *PGK* mRNA levels are expressed relative to the level of DBY746 (using *ACT1* mRNA as loading control) and were determined by Northern analyses, followed by quantitation using the AMBIS β-scanner.

contain elevated levels of *PYK1* mRNA. RNA from these three transformants together with RNA isolated from the host DBY746 was subjected to Northern blotting and the same filter analysed sequentially for the *PYK1* mRNA, *ACT1* mRNA, and *PGK* mRNA. After each individual hybridisation, the amount of hybridised probe was quantified using the AMBIS ß-scanner. Table 5.1 presents the abundance of the *PYK1* mRNA and *PGK* mRNA in each of the three pLD1(35) transformants and the untransformed host. Northern analyses showing the abundance of the *PYK1* and *ACT* mRNA have been presented previously (Fig. 4.2) while Fig. 5.2 presents the Northern analysis of the *PGK* mRNA. The analysis of the *ACT1* mRNA is also incorporated into Fig. 5.2 to serve as loading control.

These results indicate that there is a decrease in the level of the *PGK* mRNA in the pLD1(35) transformants compared to the level present in the untransformed host. Interestingly, this decrease appears to depend on the level of the *PYK1* mRNA. The higher the level of the *PYK1* mRNA, the greater the decrease in *PGK* mRNA levels. However, the decrease in the level of the *PGK* mRNA in the transformants studied was not as great as the elevation in *PYK1* mRNA levels was. For example, in [35.6] there was approximately a five-fold increase in the level of the *PYK1* mRNA abundance but only a two-fold decrease in the abundance of the *PGK* mRNA.

5.2.3 OVER-EXPRESSION OF *PYK1* IS LIMITED AT THE POST-TRANSCRIPTIONAL LEVEL.

To ascertain whether *PYK1* over-expression was also limited at a post-transcriptional level, the amount of pyruvate kinase protein produced by yeast containing elevated levels of *PYK1* mRNA levels was determined. Ideally, the most accurate method for determining the amount of pyruvate kinase protein present in a yeast culture would be to subject yeast cell extracts to western blotting. Unfortunately, no pyruvate kinase anti-body was available and instead it was decided to assay the activity of pyruvate kinase in yeast transformants.

Several assays for pyruvate kinase (which converts phosphoenolpyruvate and ADP to pyruvate and ATP) have been

described, but the majority are indirect. For example the most commonly used assay monitors the level of pyruvate produced by pyruvate kinase activity through coupling the reaction to a lactate dehydrogenase assay (Bucher and Pfleiderer, 1955). The main disadvantage with this method is the potential for interference from other metabolites or enzymes in the crude cell extracts used here.

To overcome this potential problem, the direct pyruvate kinase assay described by Kaslow and Garrisson (1983) was used. This method measures the rate of conversion of labelled substrate ([^{14}C]-Phosphoenol-pyruvate) to [^{14}C]-pyruvate. Following the reaction, samples are subjected to hydroxyapatite chromatography to remove unreacted [^{14}C]-PEP, and the amount of [^{14}C]-pyruvate is then determined by scintillation counting.

Several refinements to the published method (Kaslow and Garrison, 1983) were required to allow analyses of yeast extracts. This section describes the controls which were carried out to test the validity of this assay system which is described in detail in 2.2.6.4.

5.2.3.1 Development of a direct pyruvate kinase assay for analysing yeast extracts.

The effectiveness of the pyruvate kinase assay described by Kaslow and Garrison (1983) depends on the ability to remove unreacted [^{14}C]-PEP by hydroxyapatite chromatography at the end of the assay to permit the accurate quantitation of [^{14}C]-pyruvate. It was decided therefore to confirm that hydroxyapatite could achieve this before the assay conditions were optimised for yeast extracts. In their protocol, Kaslow and Garrison (1983) employed the use of HA-ultrogel (beaded hydroxyapatite-agarose gel), a suspension which has better flow properties than hydroxyapatite, and as such can be used in column form. Unfortunately HA-ultrogel (or any similar suspension) is no longer commercially available and unmodified hydroxyapatite had to be used instead. Due to its poor flow properties, hydroxyapatite was not used in column form, but in batch form. Importantly, [^{14}C]-PEP was effectively removed from the reaction mix by this approach. This was shown using a mixture which contained all the constituents of a typical reaction mix minus pyruvate kinase. By scintillation counting, it was

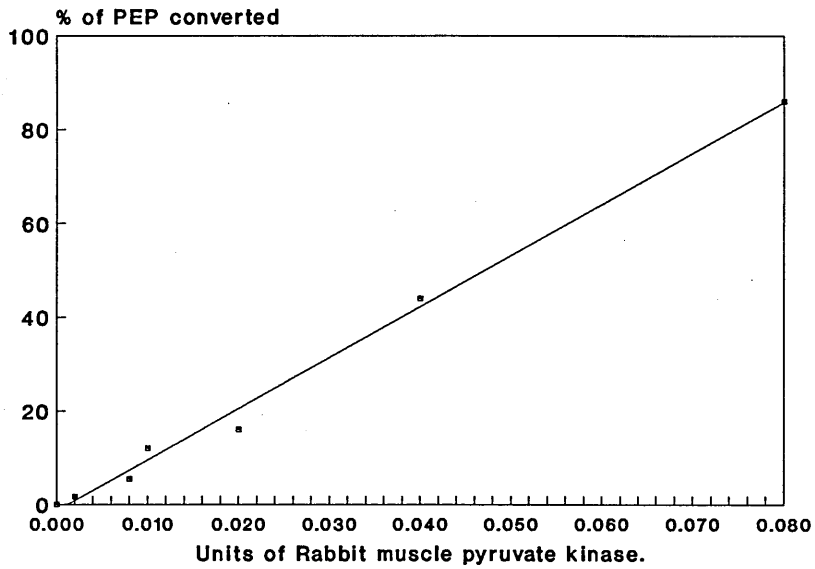
Figure 5.3a Assay of rabbit muscle pyruvate kinase.

Increasing units of rabbit muscle pyruvate kinase were assayed for their PYK activity using the modified assay method described (5.2.3.1). Reactions were carried out at 37^{o}C for 40 mins and terminated by the addition of EDTA. The percentage of PEP converted to pyruvate was determined for each sample after purification of the reaction mix through hydroxyapatite, and is plotted against theoretical units of rabbit muscle pyruvate kinase. Each point on the graph is the average of two readings.

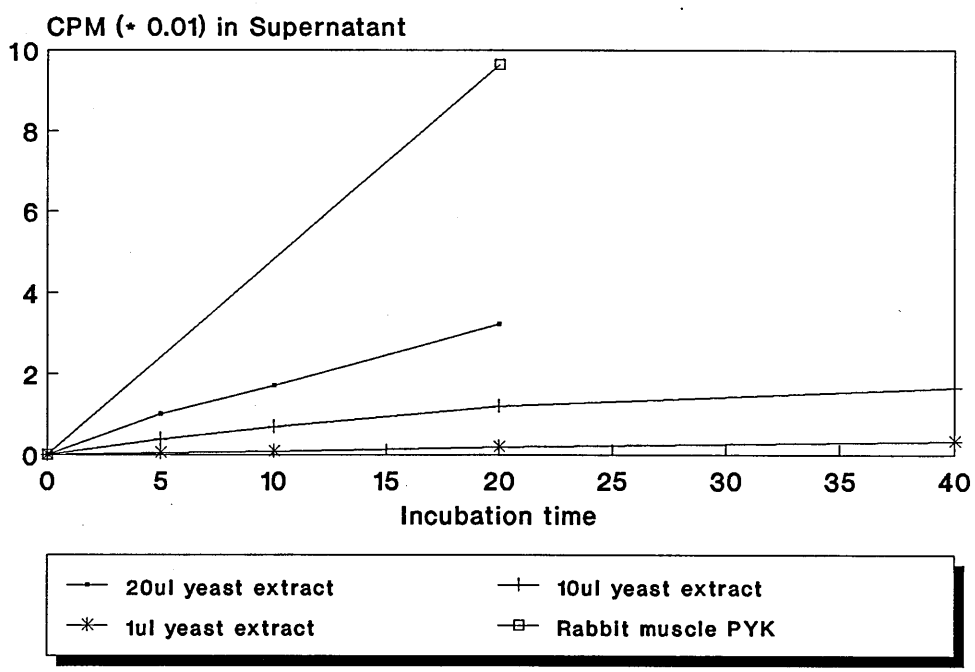
Figure 5.3b Assay of yeast extracts for pyruvate kinase activity.

1µl, 10µl and 20µl of DBY746 yeast extract were analysed for their pyruvate kinase activity using the method described (5.2.3.1). Reactions were carried out at 30^{o}C, and duplicate samples taken after 0, 5, 10, 20 and 40 mins, and the amount of [^{14}C]-PEP converted to [^{14}C]-pyruvate determined. The amount of pyruvate generated in each is expressed on the graph as CPM (*10^{-2}) in supernatant and is plotted against incubation time in mins.

A


% of PEP converted
100
80
60
40
20
0
0.000
0.010
0.020
0.030
0.040
0.050
0.060
0.070
0.080
Units of Rabbit muscle pyruvate kinase.

B


CPM (* 0.01) in Supernatant
10
8
6
4
2
0
0
5
10
15
20
25
30
35
40
Incubation time
20ul yeast extract
10ul yeast extract
1ul yeast extract
Rabbit muscle PYK

determined that less than 1% of the [^{14}C]-PEP added to the mix remained in solution after purification with hydroxyapatite. To account for this residual level of unbound [^{14}C]-PEP, a similar negative control was always carried out alongside samples being tested.

The reaction mixture used here differs slightly from that described by Kaslow and Garrison (1983) as it contains Fructose-1,6-diphosphate, a positive activator of pyruvate kinase. Fig. 5.3a demonstrates that this assay efficiently monitors rabbit muscle pyruvate kinase activity.

Before analysing pLD1(35) transformants for their pyruvate kinase activity, several controls were conducted on untransformed DBY746 yeast extracts. To determine a suitable concentration of yeast extract to be analysed, different concentrations and volumes of yeast extracts were tested for their pyruvate kinase activity over a time-course. The concentrations found to give linear results over the time-course used are described in 2.2.6.4. The results of such an assay are shown in Fig. 5.3b, demonstrating the linearity achieved.

To exclude the possibility that no enzyme present in yeast extract other than pyruvate kinase acts on the [^{14}C]-PEP, yeast extracts were incubated with a reaction mix which lacked the substrate ADP. No activity was observed in this control, suggesting that in the assay system, only pyruvate kinase activity is being recorded.

Despite the presence of PMSF (a protease inhibitor), the pyruvate kinase activity in yeast extracts, decreased markedly upon storage at -20^{o}C. To overcome this problem, pyruvate kinase assays on yeast extracts had to be carried out immediately after the extracts had been prepared. Subsequently, it was discovered that yeast pyruvate kinase is cold sensitive (Murcott *et al.*, 1991) and this will have contributed to the observed instability of yeast pyruvate kinase in this study.

5.2.3.2. Pyruvate kinase activity in pLD1(35) transformants.

Protein extracts were prepared from a range of pLD1(35) transformants which had been grown to an absorbance at 600 nm of between 0.4-0.6 in minimal media and the level of pyruvate kinase activity determined. At the same time, cells were harvested for RNA

TABLE 5.2: Pyruvate kinase activity in cells containing elevated levels of *PYK1* mRNA.

Expt	Strain	Abundance of *PYK1* mRNA[a]	S.A. of pyruvate kinase[b]	Ratio enzyme/mRNA[c]
1	746	0.6	4.8	8.0
	[35.20]	3.1	15.9	5.1
2	746	0.6	6.5	10.9
	[35.21]	1.1	11.7	10.6
	[35.22]	3.3	13.7	4.2
3	[35.23]	0.8	10.7	13.2
	[35.24]	1.1	14.6	13.9

a : The abundance of the *PYK1* mRNA relative to control mRNAs was measured by dot-blotting and is expressed as % of total mRNA.

b : The specific activity of pyruvate kinase activity in protein extracts is expressed as nmoles of pyruvate produced per min per µg of protein. Each assay is based on eight measurements.

c : Ratio of specific activity of pyruvate kinase (b) and *PYK1* mRNA abundance (a).

isolation to determine the abundance of the *PYK1* mRNA (by dot-blot hybridisations).

10mls of yeast culture were used to prepare extracts for analyses of pyruvate kinase activity. The concentration of protein in these extracts were determined by using the Bradford assay.

Data are only comparable for strains which were analysed for pyruvate kinase activity at the same time. This was due to variations in both the reaction mix used in each analysis, and in the batch of hydroxyapatite used. Thus, the experiment was repeated three times in an effort to establish the relationship between intracellular mRNA and protein levels for *PYK1*. The results of the analyses are summarised in Table 5.2.

Despite variations in pyruvate kinase specific activity between experiments, an overall trend is clear from the data within individual experiments. Comparison of DBY746 with [35.20] in experiment 1, and of DBY746 with [35.22] in experiment 2, reveals that the ratio of pyruvate kinase specific activity to *PYK1* mRNA, decreases as the abundance of the mRNA increases. However, as shown in experiments 2 and 3, this dosage effect only manifests itself when the *PYK1* mRNA level is increased above 1.1% of total mRNA.

Thus, it appears that in addition to limitation at the level of *PYK1* mRNA abundance, *PYK1* over-expression is also limited at a post-transcriptional level. This could be mediated in either of two ways; the efficiency of *PYK1* mRNA translation is reduced when the level of *PYK1* mRNA is increased, or excess pyruvate kinase is rapidly degraded.

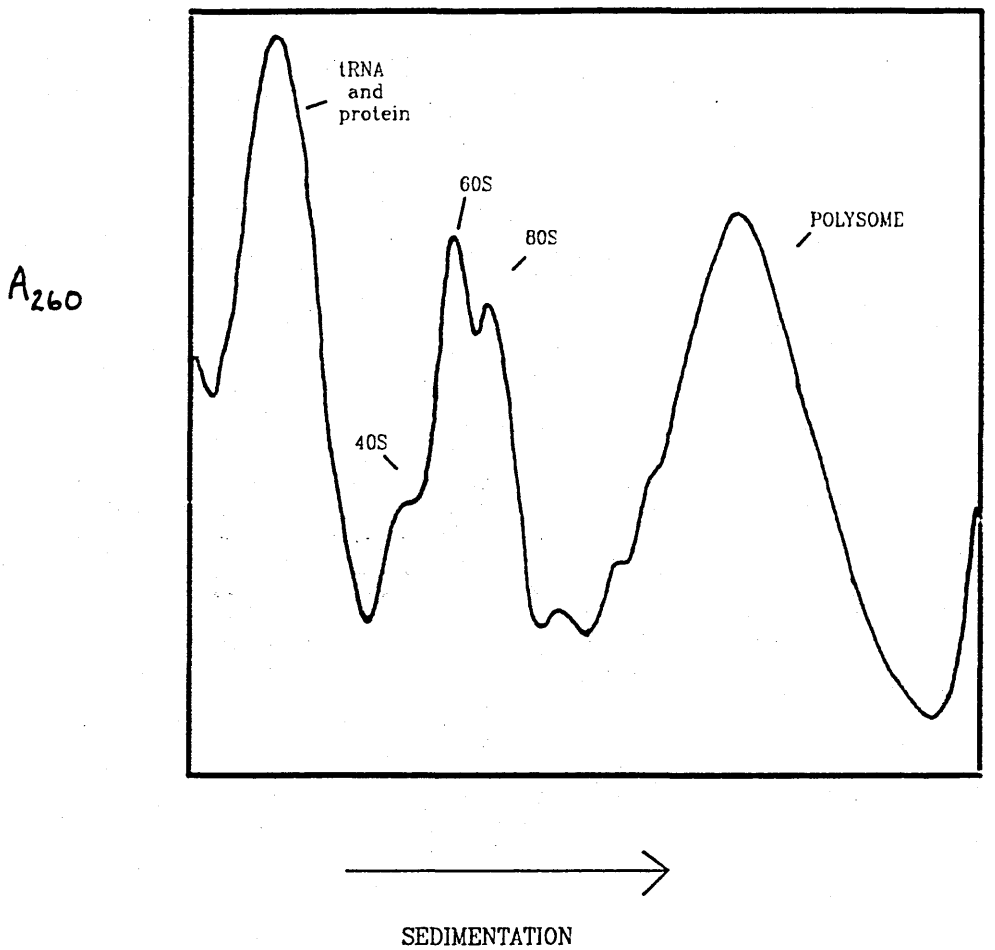
5.2.4 TRANSLATIONAL EFFICIENCY OF *PYK1* mRNA IN pLD1(35) TRANSFORMANTS.

Previously, it has been shown in this laboratory that the level of *PYK1* mRNA translation is reduced as the level of *PYK1* mRNA is increased in pLD1(35) transformants (Bettany, 1988). However, this had only been shown clearly for a single pLD1(35) transformant, and it was decided to confirm this observation.

The translation of *PYK1* mRNA *in vivo* was assessed by determining its distribution across yeast polysomes fractionated by

Figure 5.4 Absorbance profile at 260nm of a DBY746 polysome gradient.

Polysomal RNA isolated from DBY746 was layered onto a 38 ml 10-50%(w/w) sucrose gradient and centrifuged at 25,500 rpm for 2hr 50 min using a Beckman SW28 rotor. The gradient was fractionated through a spectrophotemeter to obtain the A_{260} profile presented. The arrow represents the direction of sedimentation, with mRNAs heavily bound with ribosomes in the polysome fraction, to the right of the picture. To the left of the picture the peaks representing the 80S, 60S, and 40S ribosomal subunits are shown.


A_{260}
tRNA
and
protein
60S
80S
POLYSOME
40S
SEDIMENTATION

sucrose density gradient fractionation (see 2.2.5 for method). The A_{260} profile of such an analysis on DBY746, is presented in Fig. 5.4 which demonstrates the separation of mRNA on the basis of its ribosome loading. To determine the ribosome loading on a particular mRNA, fractions from such a gradient were dot-blotted and analysed for the mRNA of interest. The amount of bound probe in each fraction was then quantified by scintillation counting.

Two pLD1(35) transformants which formed small colonies on the initial transformation plate were cultured as described (2.2.5.1) to an absorbance at 600nm of between 0.4-0.6 for preparation of polysomes. The transformants were studied as soon as possible to combat any reduction in plasmid copy number.

As this method requires the use of rich media to prepare cells for analysis there is no selective pressure for plasmid-containing cells. Thus, the percentage of *LEU+* cells (and therefore plasmid- containing) was determined at the point of harvesting. For both of the transformants, a dilution of cells were plated onto a YPG plate and grown for three days. 100 colonies from each plate were then transferred to a plate lacking leucine to test if they still contained plasmid and all grew. Hence, plasmid-containing cells represented greater than 99% of the total number of cells for each culture.

The abundance of the *PYK1* mRNA in each of the two transformants was determined relative to the *ACT1* mRNA by dot-blot hybridisation. For [35.29], the abundance of *PYK1* mRNA was 6.3% of total mRNA, while in [35.30] the level was equal to 4.7% of total mRNA. Thus, the level of *PYK1* mRNA in these transformants was well within in the range at which post-transcriptional repression of *PYK1* expression is observed (Table 5.2).

Polysome gradients were prepared and analysed for the distribution of the *PYK1* and *Rp1* mRNAs. The results are presented in Fig. 5.5 and summarised in Table 5.3. The *Rp1* mRNA was used to control for any differences in the physiological state of the cells at time of harvesting, or through minor variations in the sucrose gradients used for preparation of each individual polysome profile. Comparison of the distribution of this mRNA shows that there are minor

Figure 5.5 Distribution of the *PYK1* and *Rp1* mRNAs across polysome gradients prepared from [35.29] and [35.30].

Polysome gradients were prepared from two pLD1(35) transformants, [35.29] and [35.30], which contained *PYK1* mRNA levels equal to 6.3 and 4.9% of total mRNA respectively. The gradients were fractionated into 45 and 38 aliquots respectively and then equal volumes of each aliquot subjected to dot-blot analysis. Filters were then probed separately for the *PYK1* and *Rp1* mRNAs. The amount of radioactivity in each dot was determined by cutting out each individual dot and subjecting it to scintillation counting.

The results of this are plotted as % of total cpm in a given fraction against fraction number. The fractions are numbered from the bottom of the gradent. Thus, fraction 1 corresponds to the fraction containing mRNAs with the highest number of ribosomes. Conversely, higher numbered fractions contain mRNAs with lower numbers of ribosomes attached.

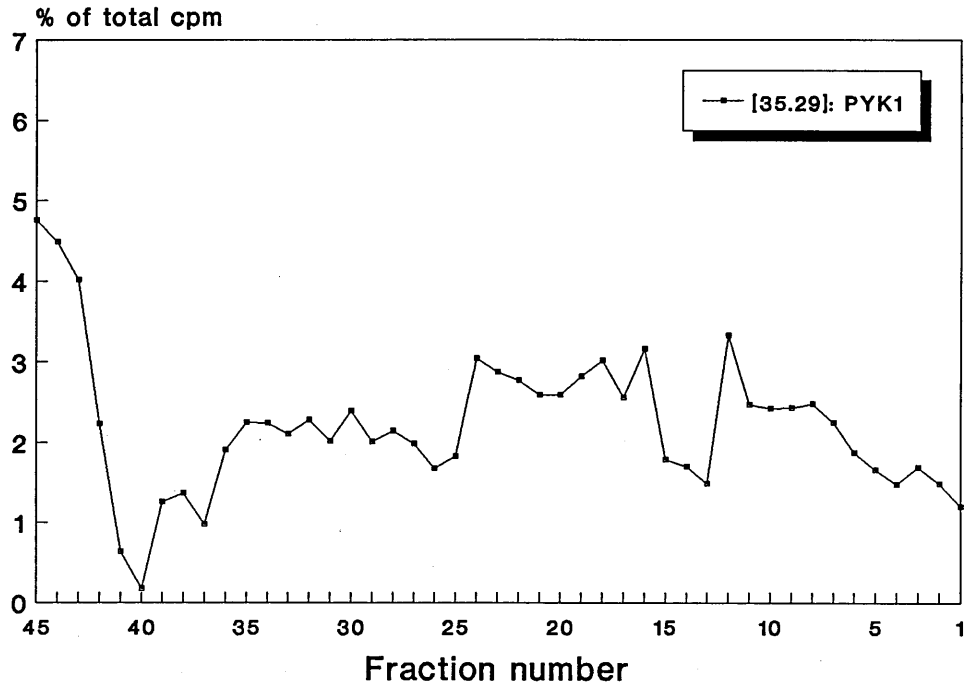
(A) Fractions from polysome gradient [35.29] analysed for *PYK1*.

(B) Fractions from polysome gradient [35.29] analysed for *Rp1*.

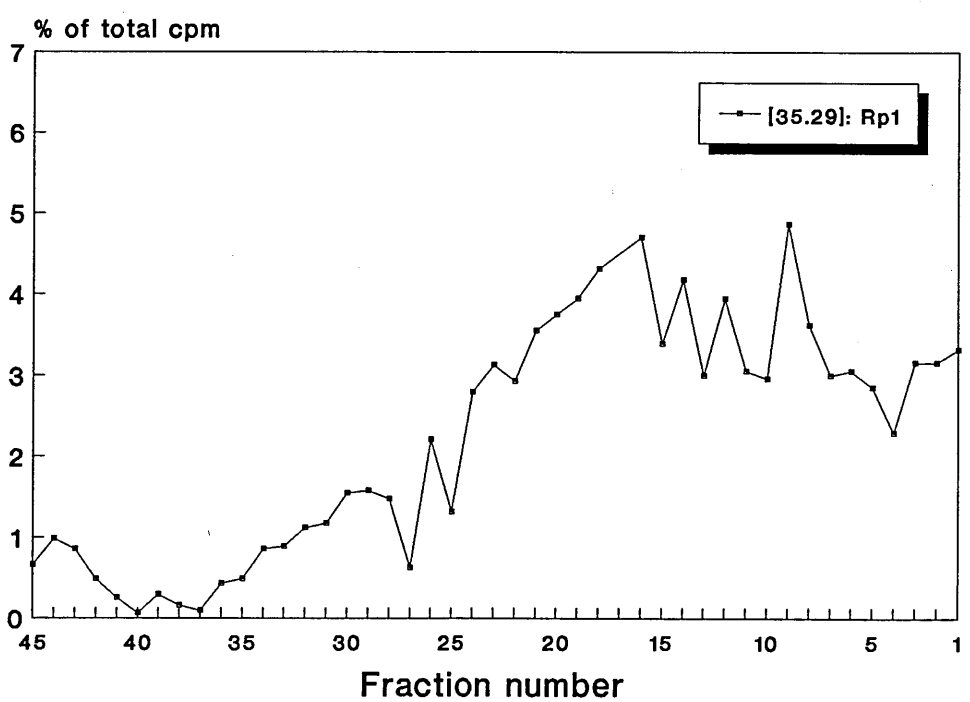
(C) Fractions from polysome gradient [35.30] analysed for *PYK1*.

(D) Fractions from polysome gradient [35.30] analysed for *Rp1*.

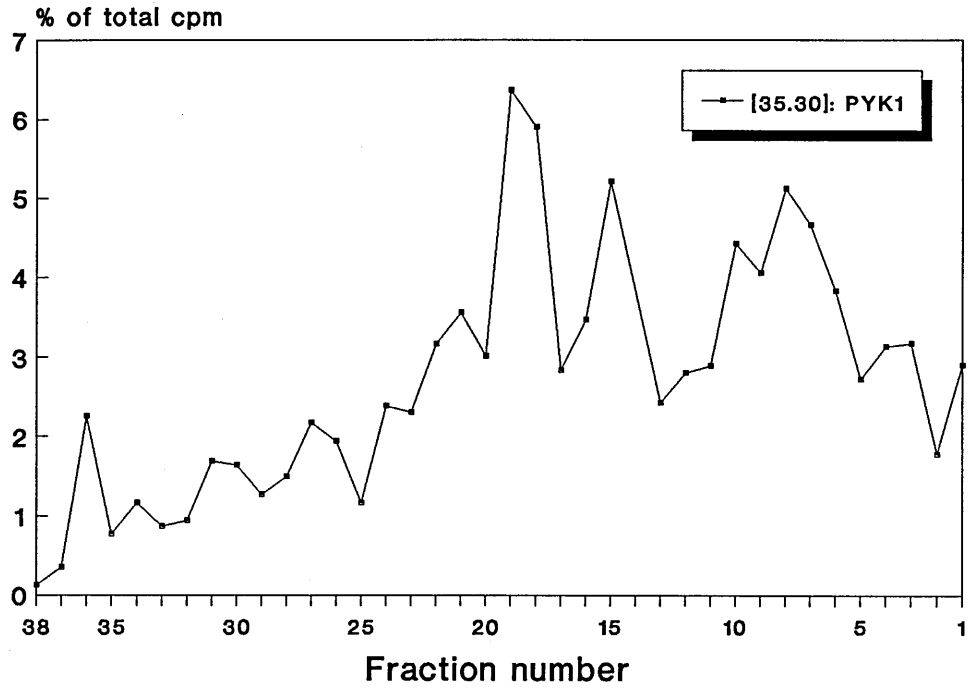
A


% of total cpm
7
6
5
4
3
2
1
0
[35.29]: PYK1
45
40
35
30
25
20
15
10
5
1
Fraction number

B


% of total cpm
7
6
5
4
3
2
1
0
[35.29]: Rp1
45
40
35
30
25
20
15
10
5
1
Fraction number

C


% of total cpm
[35.30]: PYK1
0
1
2
3
4
5
6
7
38
35
30
25
20
15
10
5
1
Fraction number

D

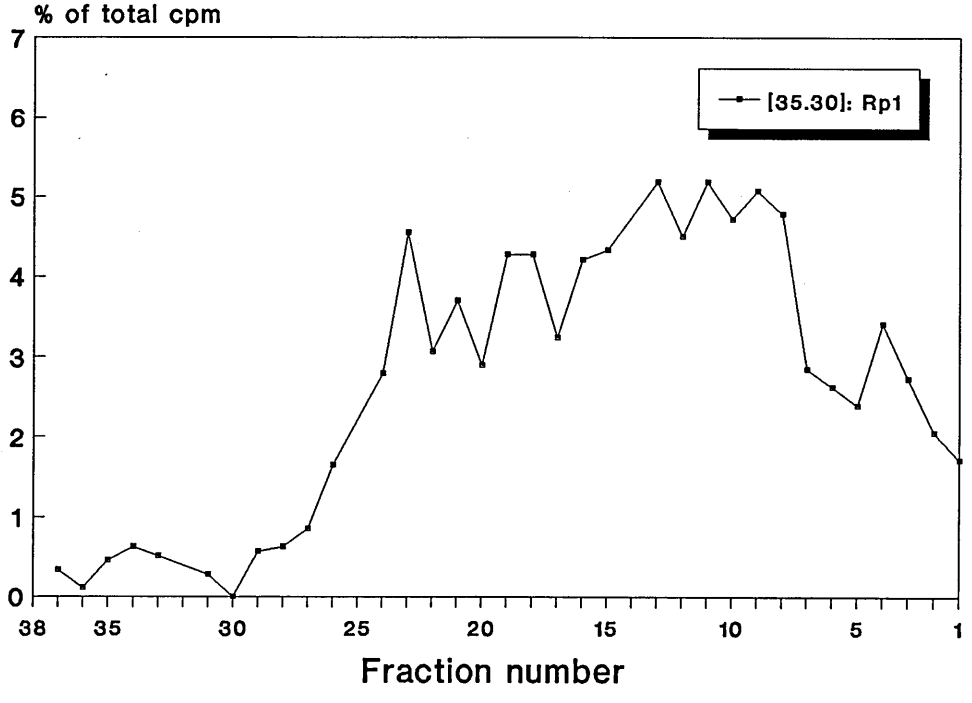

% of total cpm
[35.30]: Rp1
0
1
2
3
4
5
6
7
38
35
30
25
20
15
10
5
1
Fraction number

TABLE 5.3 : Distribution of the *Rp1* and *PYK1* mRNAs across polysome gradients prepared from DBY746, [35.29] and [35.30].

Strain	*PYK* mRNA level	mRNA analysed	polysome fraction (% total cpm)			
			0-2	2-6	6-15	>15
746	0.6	*Rp1*	36	29	31	4
[35.29]	6.3	*Rp1*	22	31	25	22
[35.30]	4.7	*Rp1*	33	28	28	11
746	0.6	*PYK1*	13	27	55	5
[35.29]	6.3	*PYK1*	51	22	14	13
[35.30]	4.7	*PYK1*	42	32	10	15

Polysome gradients from DBY746 and two pLD1(35) transformants were fractionated and probed separately for *PYK1* and *Rp1* mRNAs (Fig 5.5). The distribution of each mRNA is described as the percentage of total cpm in fractions bearing 0-2, 2-6, 6-15 and >15 ribosomes. The fractions of each gradient which contain mRNAs which bear these numbers of ribosomes, was determined from each gradient's A_{260} trace (not shown). The information on the DBY746 gradient is taken from Bettany (1988).

difference between the polysome profiles of the two transformants and the untransformed host DBY746 (Table 5.3).

The distribution of the *PYK1* mRNA across these gradients demonstrates that the number of ribosomes on the *PYK1* mRNA decreases as its abundance increases. In DBY746 the *PYK1* mRNA is predominantly associated with 6-15 ribosomes, while in both of the two pLD1(35) transformants the majority of *PYK1* mRNA carries only 0-2 ribosomes (Table 5.3). Assuming there is no significant increase in the rate of translational elongation on the *PYK1* mRNA as its abundance increases, these results suggest there is an inhibition of *PYK1* mRNA translation in the pLD1(35) transformants resulting in a decrease in ribosome loading. Therefore, *PYK1* expression appears to be limited at the level of translation.

5.3. DISCUSSION.

Data presented in this chapter demonstrate that the over-expression of *PYK1* from a multi-copy plasmid is limited at two levels. Firstly, an increase in the copy number of the *PYK1* gene does not cause a parallel elevation in the abundance of the *PYK1* mRNA. Secondly, the translation of the *PYK1* mRNA is less efficient when it is present at levels significantly above that of the wild-type cell. This sets the *PYK1* gene apart from some of the other glycolytic genes which can be over-expressed at extremely high levels in yeast (Alber and Kawasaki, 1982; Kawasaki and Fraenkel, 1982; Mellor *et al.*, 1985; Aguilera and Zimmerman, 1986; Schaaf *et al.*, 1989)

Fig. 5.1 illustrates the gene dosage effect which occurs at the level of *PYK1* mRNA abundance. When the *PYK1* copy number is increased to five, a three-fold decrease in mRNA per gene copy occurs, while when the copy number is increased further to 35 per haploid genome, a further decrease of only 1.6-fold in *PYK1* mRNA per gene copy was observed. Interestingly, in Chapter 3 it was demonstrated that when *PYK1:lacZ* reporter gene constructs were integrated into the chromosome, there was a decrease in the level of induction of the *PYK1* locus with glucose suggesting that dosage effects were also occurring. This would suggest that the dosage effect is not therefore a plasmid artefact.

The biphasic nature of the dosage response at the mRNA level (Fig. 5.1) implies there are at least two mechanisms operating to limit high *PYK1* mRNA levels. This could be mediated by a combination of transcriptional and post-transcriptional mechanisms. Precedents for this already exist in yeast; for example the cessation of histone biosynthesis during the cell cycle at the end of S-phase is achieved by a reduction in histone gene transcription and by increased histone mRNA turnover (Hereford *et al.*, 1981; Marzluff and Pandey, 1988). The efficiency with which the *PYK1* mRNA is translated has been shown to decrease as its abundance increases. This results in lower ribosome loading on the *PYK1* mRNA, and could in theory result in decreased mRNA stability due to increased exposure to exo-ribonucleases. However, it has previously been shown that changes in the translation of *PYK1* mRNA does not affect the stability of the mRNA (Purvis *et al.*, 1987c; Brown *et al.*, 1988). This would suggest that if any change in the stability of the *PYK1* mRNA had occurred, it was not caused by the decrease in its ribosome loading. Nevertheless, a specific regulatory mechanism may exist in yeast to degrade excess *PYK1* mRNA. This could be tested by analysing the stability of the *PYK1* mRNA in pLD1(35) transformants.

Transcriptional dosage effects on the *PYK1* gene could be mediated either by saturation of a positive transcriptional factor or by activation of a repressor of *PYK1* transcription. Analyses of the *PYK1* promoter has identified distinct *cis*-acting sequences which repress or activate *PYK1* transcription. There are at least two UAS elements and a URS element important in controlling *PYK1* transcription (Nishizawa *et al.*, 1989). Furthermore there is an element in the *PYK1* coding region required for optimal transcripion of *PYK1* (3.2.3.4; Purvis *et al.*, 1987b). Thus, the potential exists for *PYK1* to be subject to either of the transcriptional effects described above, exists.

Two positive transcriptional factors have been identified which bind to the 5' promoter region of *PYK1 in vitro*. RAP1 has been shown to bind to the UAS1 of *PYK1*, and is known to be important in dictating *PYK1* transcription (Buchman and Kornberg, 1989). ABF1 has also been shown to bind at a site in the 5' promoter but outside a sequence identified as a UAS (Chambers *et al.*, 1990). The importance of ABF1 binding in directing transcription of *PYK1* has not been established. It

is unlikely however, that either of these two factors become limiting as the *PYK1* gene dosage is increased. These are highly abundant DNA binding proteins, which bind the 5' promoter regions of a multitude of genes and in addition, bind regions of DNA not involved in transcription eg. at ARSs and telomeres (Buchman and Kornberg, 1990).

Recently, a genetic analysis has shown that *gal11* mutants have reduced levels of *PYK1* mRNA (Nishizawa *et al.*, 1990). However, this can be alleviated if the distance between the *PYK1* TATA box and the RPG box is reduced in a *gal11* mutant, suggesting that the GAL11 protein functions to allow contact between the TATA box factor and the RAP1 protein to activate transcription (Nishizawa *et al.*, 1990). Thus, GAL11 appears to play a positive role in the transcription of *PYK1*. As the *gal11* mutation effects the expression of other genes (Nishizawa *et al.*, 1990), it would be interesting to determine the abundance of these genes mRNA, in strains of yeast with elevated levels of *PYK1* mRNA. If there was a decrease in the level of the mRNA synthesised by these *gal11* affected genes, it would suggest that the GAL11 protein contributes to the *PYK1* gene dosage effect observed in Fig. 5.1. The level of the *PGK* mRNA has been shown in this study to decrease as the abundance of the *PYK1* mRNA increases (Table 5.2). It is not known whether *PGK* transcription also requires GAL11. This could easily be checked by determining the abundance of the *PGK* mRNA in both the *gal11* mutant and in an isogenic *GAL11* strain.

There are several characteristics which are common to both the *PGK* and *PYK1* promoters. Both bind RAP1 and ABF1 (Chambers *et al.*, 1990). Both depend on "CT" blocks and DAS elements for optimal transcription. There does not however appear to be a URS element in the *PGK* promoter as there is in *PYK1*. Thus, while there are certain similarities between these promoters there are also important difference. Furthermore, it has previously been shown that *PGK* can be over-expressed in yeast to such an extent that *PGK* protein contributes 50% of total cell protein (Mellor *et al.*, 1985). Therefore, there does not appear to be dosage effects operating on *PGK* as there are on *PYK*1. Thus, on the one hand it appears that *PGK* can be over-expressed in yeast, yet in the presence of excess *PYK1* gene dosage the level of the *PGK* mRNA is reduced suggesting it too is subject to the same dosage

limitation as *PYK1*. This apparent anomaly could be explained if excess *PYK1* gene dosage activates a transcriptional repressor which represses *PYK1* and to a lesser extent *PGK* transcription. Alternatively, a positive transcriptional factor required by both genes may bind with higher affinity to the *PYK1* promoter than to the *PGK* promoter.

An explanation for this phenomenon may be forthcoming with the characterisation of mutants which can overcome the dosage limitation.

PYK1 over-expression has also been shown to be limited post-transcriptionally. There are two lines of evidence to support this. Firstly, large increase in *PYK1* mRNA levels (to 3.0% of total mRNA) did not yield an equivalent increase in enzyme activity (Table 5.2). This could be caused by either rapid degradation of excess enzyme *in vivo*, or decreased rates of enzyme synthesis (or both).

Direct measurements of pyruvate kinase degradation *in vivo* have not been performed. However, it has been shown earlier in this study, that excess pyruvate kinase can be tolerated by yeast when the coding region is fused to the *PGK* promoter (Fig. 4.10). This would suggest that excess pyruvate kinase is not rapidly degraded.

Polysome analyses demonstrated that excess *PYK1* mRNA has a lower ribosome loading than normal (Table 5.3). Analyses of several other pLD1(35) transformants with different *PYK1* mRNA levels also showed a similar trend: as the *PYK1* mRNA levels increase the number of ribosomes attached to that mRNA decrease (Bettany, 1988; Moore *et al.*, 1990a). Therefore, it appears that the rate of translational initiation on *PYK1* mRNA decreases as the level of that mRNA increases. This decreased rate of pyruvate kinase synthesis is consistent with the pyruvate kinase assay data described above.

Thus, *PYK1* joins the select group of identified genes which are subject to translational control in *S.cerevisiae*. The mechanism of this translational control is analysed and discussed in greater detail in the following chapter.

As stated in 5.1, the majority of yeast genes which are subject to dosage compensation belong to either of two gene families: either ribosomal proteins or histone genes. Each of these gene families are co-ordinately expressed such that the relevant proteins are

synthesised at the appropriate time and in the correct molar ratios. The mechanisms involved have been shown to act at transcriptional, post-transcriptional and post-translational levels (Osley and Hereford, 1981; Pearson *et al.*, 1982; Warner *et al.*, 1985; Maicas *et al.*, 1988). In this study, *PYK1* was shown to be subject to dosage limitation at both the transcriptional and post-transcriptional levels.

Interestingly, the yeast plasma membrane ATPase (*PMA1*) is also subject to these dosage limitation effects. When *PMA1* was introduced into yeast on a multi-copy vector, only limited over-expression was observed due to a limitation at the mRNA level and at the level of protein synthesis and/or degradation (Eraso *et al.*, 1987). Furthermore, the expression of the *PMA1* gene on a multi-copy vector was detrimental to cell growth, and there was a selection for cells with reduced plasmid-copy number (Eraso *et al.*, 1987) in a manner analogous to that demonstrated for *PYK1* in Chapter 4. The physiological relevance of why both *PYK1* and *PMA1* cannot be over-expressed in yeast is not yet known.

PYK1 is not the only yeast glycolytic gene which is subject to gene dosage effects. Recently it was reported that pyruvate decarboxylase is subject to auto-regulation and dosage compensation through an apparently different mechanism than *PYK1* (Seeboth *et al.*, 1990; Hohmann and Cederberg, 1990).

The structural gene which encodes pyruvate decarboxylase is *PDC1*. Other genes (first identified as *pdc* mutants), have also shown to be important in regulating the expression of *PDC1*: *PDC2, PDC3,* and *PDC4*. Interestingly, (considering the discovery that *PYK1* expression is limited at the translational level) the *PDC2* gene is believed to act at the post-transcriptional level, since *pdc2* mutants contain *PDC1* mRNA levels at the normal level but have reduced pyruvate decarboxylase activities (Schmitt *et al.*, 1983). It would be interesting to determine if *PDC2* is important in regulating the expression of *PYK1* at the translational level. It has been suggested that the *PDC3* is involved in post-translational modifications (Seeboth *et al.*, 1990), while the *PDC4* gene is involved in transcriptional regulation of *PDC1* (Hohmann and Cederberg, 1990).

When the *PDC1* gene is deleted, pyruvate decarboxylase activity is still 60-70% of the wild-type level (Seeboth *et al.*, 1990; Hohmann and Cederberg, 1990). This is because in *pdc1* deletion mutants, a second pyruvate decarboxylase encoding gene becomes activated. This gene, *PDC5*, is not transcribed in a *PDC1* genetic background (Hohmann and Cederberg, 1990). Interestingly, it has also been demonstrated that in a *pdc1* deletion mutant, the level of expression from a reporter gene fused to the *PDC1* promoter and the level of truncated *pdc1* mRNA are increased (Seeboth *et al.*, 1990). This was shown for two different reporter genes, either integrated into the genome, or encoded on an autonomously replicating plasmid (Seeboth *et al.*, 1990). These observations have led to the supposition that there is feed-back regulation on the *PDC1* and *PDC5* promoters to increase transcription from these promoters when the level of *PDC1* protein is decreased (Seeboth *et al.*, 1990).

Considering the results generated in this thesis, it is conceivable that *PYK1* feed-back regulates its expression to ensure the gene is not over-expressed in a manner analogous to *PDC1*, which uses feed-back regulation to ensure the gene is not under-expressed. However, this remains to be tested experimentally..

To summarise the findings of this chapter, it has been shown that over-expression of the *PYK1* gene is limited both at the level of mRNA abundance and at the level of mRNA translation. The effect at the level of mRNA abundance is at least partly (if not completely) due to transcriptional limitation, as the level of the *PGK* mRNA was shown to decrease in the presence of increasing *PYK1* gene dosage. The effect on *PYK1* translation was demonstrated by both pyruvate kinase assays and by polysome analyses. The translational repression of *PYK1* mRNA only comes into effect when the level of *PYK1* mRNA is greater than 1.1% of total mRNA and appears to operate at the level of translational initiation.

CHAPTER 6

RESULTS (IV)

TRANSLATIONAL REGULATION OF *PYK1* EXPRESSION

6.1 INTRODUCTION

In the previous chapter, evidence was presented which demonstrated that the expression of the yeast *PYK1* gene is both transcriptionally and translationally repressed when the abundance of that gene is elevated. In *S.cerevisiae*, it is not uncommon for the regulation of gene expression to be mediated at the transcriptional level (Verdier, 1990). Indeed experiments in Chapter 3 demonstrate transcriptional regulation of *PYK1* and other glycolytic genes in response to carbon source. In contrast, only a handful of yeast genes have been identified which are subject to translational regulation. Bearing this in mind, it was decided to investigate the translational regulation of *PYK1* in more detail.

In particular it was of interest to: (1) define the sequences of the *PYK1* mRNA which are necessary for translational repression; (2) to gain an insight of how the regulation operates and (3) to determine if any other glycolytic genes are subject to this mode of control. By answering these questions it was hoped that a better understanding would be gained of how and why *PYK1* is subject to this apparently unusual mode of control.

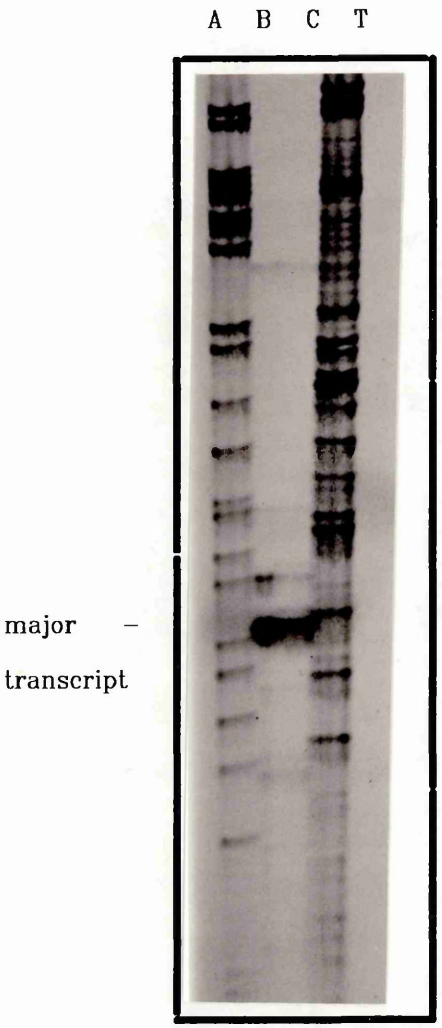
6.2. RESULTS.

6.2.1 5' MAPPING OF THE PLASMID-ENCODED *PYK1* mRNA.

Translational repression of *PYK1* mRNA has only been observed in DBY746:pLD1(35) transformants which contain levels of *PYK1* mRNA above 1.1% of total mRNA (Chapter 5; Bettany, 1988; Moore *et al.*, 1990a). Polysome analysis has demonstrated that there is a general decrease in the number of ribosomes attached to *PYK1* mRNA when the abundance of the mRNA is increased. However, it is possible that only *PYK1* mRNA originating from the pLD1(35) plasmid is subject to lower ribosome loading. pLD1(35) has been shown to complement the *pyk* alleles *cdc19* and *pyk 1.5* (Bettany *et al.*, 1989), and pyruvate kinase can be over-produced to a limited degree in pLD1(35) transformants (Table 5.3). Therefore, there are no premature stop codons in the *PYK1* coding sequence resident on the pLD1(35) plasmid.

Figure 6.1 Primer extension analysis of plasmid encoded *PYK1* mRNA.

RNA isolated from [35.6] (B) DBY746 (C) was subjected to primer extension analysis to determine the 5' transcriptional start site of the *PYK1* mRNA. While DBY746 contains a *PYK1* mRNA abundance of 0.6% of total mRNA, [35.6] was calculated to carry a *PYK1* mRNA abundance of 2.7% of total mRNA. Thus, the majority of *PYK1* mRNA in [35.6] will have been synthesised from the plasmid locus. The resulting primer extension products from each RNA preparation are presented. Adenine (A) and Thymidine (T) tract sequencing ladders of known length were used as size markers.


A B C T
major
transcript

However, it is plausible that possible differences in the length (and thus the sequence and structure) of the 5' untranslated leaders of the mRNAs encoded by the plasmid and chromosomal copies of *PYK1* could have an effect on translational initiation rates of these mRNAs. Such differences have been observed when other genes are expressed from plasmids. For example, chromosomally encoded *PFK2* mRNA has a 5' untranslated leader of 77 nt, while plasmid-encoded *PFK2* mRNA has two start sites 25 and 57 nt upstream from the start codon (Heinsch *et al.*, 1989). This disparity in leader length is presumably caused by plasmid sequences affecting the transcriptional initiation site(s). Hence, to determine if the plasmid-encoded *PYK1* directed the synthesis of an mRNA with an aberrant 5' end, the 5' end of this mRNA was compared with that of the chromosomally derived *PYK1* mRNA.

Approximately 10µg of RNA isolated from both DBY746 and a pLD1(35) transformant were subjected to the primer extension method described in 2.2.2.4 and the products analysed on an 8% denaturing urea:polyacrylamide gel (Fig. 6.1). The pLD1(35) transformant used for this analysis ([35.6]) has previously been shown to have a *PYK1* mRNA abundance of 2.7% of total mRNA (Table 4.1), which is approximately five times higher than the level of *PYK1* mRNA observed in the untransformed host. Thus, the majority of *PYK1* mRNA in [35.6] will have been synthesised from the plasmid locus. The oligonucleotide used for primer extension on the *PYK1* mRNA is described in Table 2.2 and hybridises between +3 and +24 nt with respect to the *PYK1* translational initiation codon.

As illustrated previously (Fig. 3.5), *PYK1* uses one major transcriptional initiation site in DBY746, which maps to 28 nt upstream of the *PYK1* initiation codon (Fig. 6.1). The same major *PYK1* transcriptional start site is evident for [35.6] suggesting that there is no difference in the length of *PYK1* mRNA transcribed either from the chromosome or from the pLD1(35) plasmid (Fig. 6.1). Furthermore, on further exposure of the gel presented in Fig. 6.1, the same three minor transcript start sites previously shown to be used by the chromosomal copy of *PYK1* (Fig. 3.5) are also present in [35.6]. Thus, the translational repression of excess *PYK1* mRNA is not caused by

differences in the length of the 5' untranslated leader of the plasmid encoded *PYK1* mRNA.

6.2.2 THE 5' UNTRANSLATED REGION OF THE *PYK1* mRNA IS REQUIRED FOR TRANSLATIONAL REGULATION.

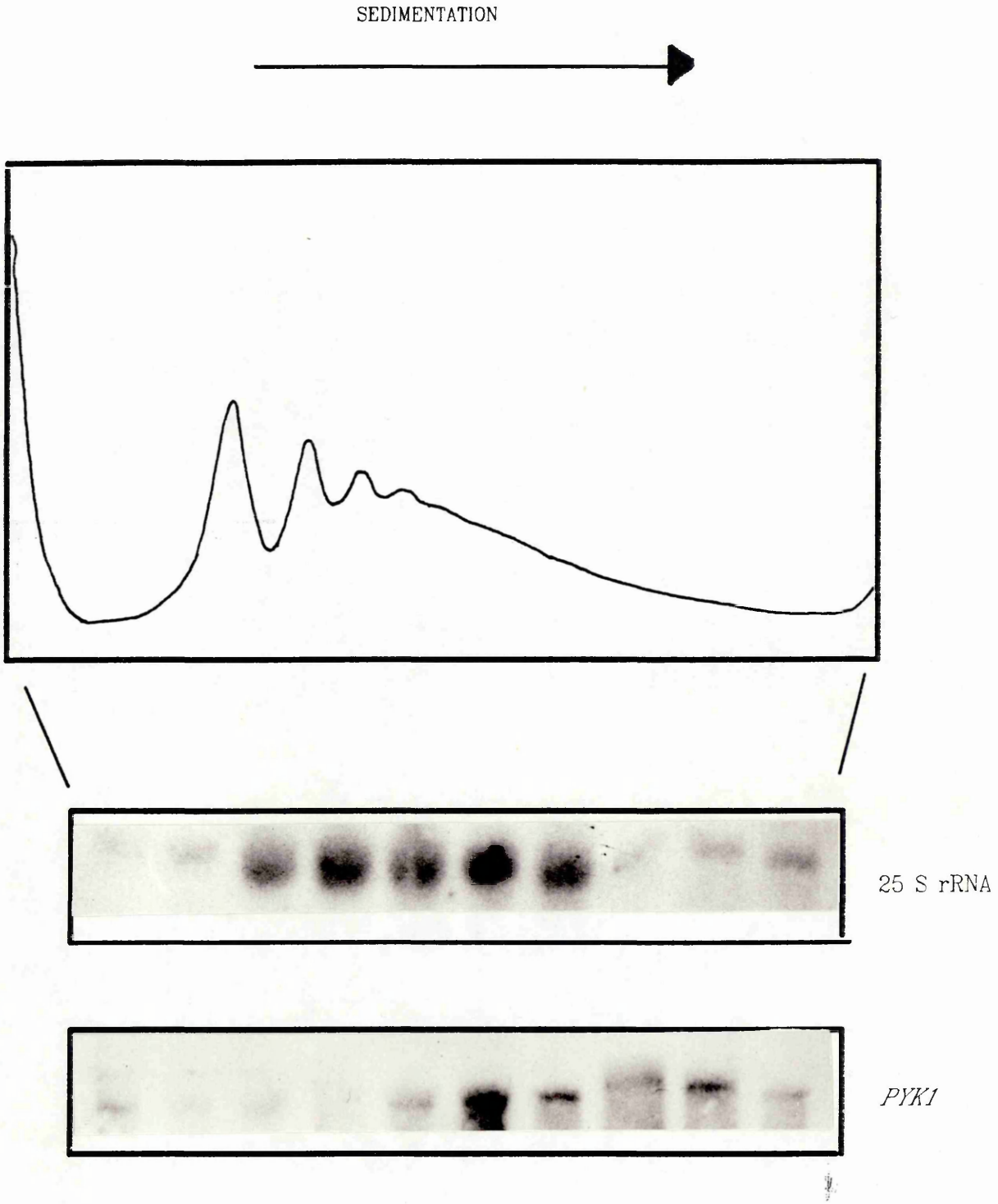
The evidence from the polysome analyses of pLD1(35) transformants carrying elevated levels of *PYK1* mRNA, suggested that translational control of *PYK1* was operating at the level of translational initiation (Chapter 5). Integral to the process of eukaryotic translational initiation is the 5' untranslated leader which serves as the platform to allow the scanning 40S ribosomal sub-unit to locate the AUG initiation codon (Kozak, 1983). Therefore, it was determined whether substitution of the *PYK1* 5' untranslated leader with an alternative sequence resulted in the loss of translational regulation.

Advantage was taken of a multi-copy plasmid made in Jane Mellor's laboratory (Biochemistry Department, Oxford) pMA91(+PYK) (see Fig. 4.9 or Fig. 6.3 for plasmid map). This plasmid carries the *PYK1* coding and 3' untranslated regions fused to the *PGK* promoter and 5' untranslated leader sequence. The *PYK1* sequence in this construct, which starts at -4 with respect to the start codon, excludes only the first 25 nt of the 5' untranslated leader of the *PYK1* mRNA.

Results presented in chapter 4 demonstrated that pyruvate kinase can be synthesised at high levels in yeast carrying the pMA91(+PYK) plasmid (Fig. 4.10). SDS-PAGE showed high levels of pyruvate kinase present in soluble protein extracts of a pMA91(+PYK) transformant. In contrast, there was no indication of high levels of pyruvate kinase in two pLD1(35) transformants analysed in a similar manner (Fig. 4.10). This would suggest that the *PGK:PYK1* gene fusion resident on the pMA91(+PYK) plasmid is not subject either to the transcriptional dosage effects or to the translational repression suffered by *PYK1*. However, it was possible that the over-production of pyruvate kinase in the pMA91(+PYK) transformant was due solely to the release of the transcriptional dosage effects associated with the *PYK1* promoter (Chapter 5). Certainly in the transformants analysed on the SDS-PAGE gel, the level of *PYK1* mRNA (including *PGK:PYK1* mRNA) in the pMA91(+PYK) transformant was substantially higher (5.5% of total

Figure 6.2 Excess *PGK:PYK1* mRNA is translated efficiently

A polysome gradient was prepared from the pMA91(+PYK) transformant [91.2] (*PGK/PYK1* mRNA abundance of 4.1% of total mRNA). Equal proportions of twelve fractions taken across the gradient, were subjected to Northern blotting and probed for 25S rRNA and *PYK1* sequences. The A_{260} profile of the gradient, and the direction of sedimentation are shown (arrow).


SEDIMENTATION
25 S rRNA
PYK1

mRNA) than in either of the two pLD1(35) transformants analysed (0.9% and 1.6% of total mRNA).

Thus, it was decided to determine how efficiently *PYK1* sequences were translated in a pMA91(+PYK) transformant. The transformant studied [91.2] had a *PYK1* mRNA abundance of 4.1% of total mRNA which implies that the majority of the *PYK1* mRNA in this transformant is derived from the *PGK:PYK1* fusion. A polysome gradient was prepared from this transformant as described in 2.2.5., and the gradient aliquoted into 10 equal fractions. RNA was extracted from each fraction and the distribution of the 25S rRNA and *PYK1* sequences analysed by Northern blotting. The results of this are shown in Fig. 6.2 together with the A_{260} profile of the gradient.

The distribution of the *25S* rRNA across this gradient indicates that the majority of mRNAs are being translated by 3-10 ribosomes (Fig. 6.2). In comparison, the *PYK1* mRNAs are predominantly associated with a greater number of ribosomes (Fig. 6.2). This implies that the *PGK:PYK1* mRNA is efficiently translated even when it's abundance is at a level at which *PYK1* mRNA would be poorly translated. Thus, the deletion of the 5' untranslated leader releases the *PYK1* mRNA from translational repression indicating that the 5' proximal 25 nucleotides of the *PYK1* mRNA are required in *cis* for translational control.

6.2.3. EXCESS PYRUVATE KINASE DOES NOT CAUSE TRANSLATIONAL REPRESSION OF *PYK1* mRNA.

Since the 5' untranslated region is integral to the translational regulation of *PYK1*, the mechanism is mediated at least partly via translational initiation. This is consistent with the ribosome loading on the *PYK1* mRNA in pLD1(35) transformants with elevated *PYK1* mRNA levels (Fig. 5.5).

Inhibition of translational initiation of *PYK1* mRNA could occur in either of two ways. Firstly, it could be effected through feedback inhibition of *PYK1* mRNA translation by increased intracellular levels of pyruvate kinase. This could occur via a direct interaction between pyruvate kinase and its mRNA, or indirectly through a secondary RNA-binding molecule. Alternatively, an mRNA-specific translation factor may be required for each round of initiation on the *PYK1* mRNA. If such a

factor was present in limiting concentrations, the average rate of initiation per *PYK1* mRNA molecule would decrease as the concentration of the mRNA increased.

To distinguish between these possibilities, it was decided to determine how efficiently the chromosomal (wild-type) copy of the *PYK1* mRNA is translated in DBY746:pMA91(+PYK) transformants, since these transformants synthesise high levels of pyruvate kinase from the *PGK:PYK1* fusion mRNA. If the translational inhibition of the *PYK1* mRNA is mediated by excess pyruvate kinase, then it would be anticipated that the *PYK1* mRNA originating from the chromosome in pMA91(+PYK) transformants would be translationally repressed.

6.2.3.1. Strategy for the differential probing of *PYK1* and *PGK:PYK1* mRNAs.

To analyse specifically the *PYK1* mRNA in a population containing both the *PGK:PYK* and *PYK1* mRNAs, advantage was taken of the sequence differences which exist at the 5' end of each of these mRNAs. An oligonucleotide complementary to the *PYK1* 5' untranslated region (PYK5') was synthesised and tested for its ability to differentiate between the two mRNAs.

A Southern blot of *Eco* R1 digested pMA91(+PYK) and pLD1(35) plasmid was prepared. pMA91(+PYK) (Fig. 6.3a) is digested into five *Eco* R1 fragments of 4.8, 2.7, 2.5, 2.0, and 0.8 kb with the 2.7kb band containing the *PGK:PYK* gene fusion (Fig. 6.3). pLD1(35) (Fig. 6.3a) is digested into five *Eco* R1 fragments of 4.25, 3.5, 2.0, 1.9, 0.8 kb (Fig. 6.3b) with the 5' untranslated leader of the *PYK1* gene contained within the 1.9kb fragment. This Southern blot was probed with the (PYK5') oligonucleotide (Fig. 6.3c). This analysis demonstrates that the oligonucleotide binds only to the 1.9kb *Eco* RI fragment from pLD1(35). It did not hybridise to the 2.7 kb *Eco* RI fragment of pMA91(+PYK) which carries the *PGK:PYK* fusion. Thus, the oligonucleotide (PYK5') specifically detects the wild-type *PYK1* gene and not the *PGK:PYK1* gene.

As a control, the same Southern blot was then probed with an oligonucleotide specific for the *PGK* 5' untranslated region. The

Figure 6.3 Differential probing of the *PYK1* and *PGK:PYK1* mRNA.

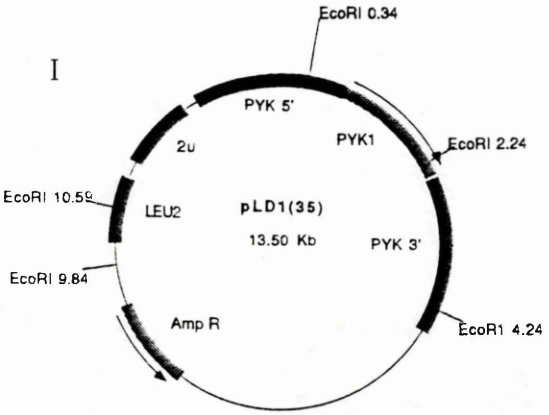
I: Plasmid maps of the vectors pMA91(+PYK) and pLD1(35). Only the positions of the *Eco* RI sites are depicted.

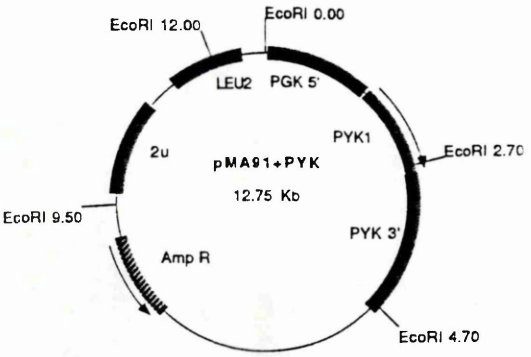
II: pLD1(35) (B) and pMA91+PYK (C) were digested with *Eco* R1 and the digest subjected to electrophoresis. Lamda/*Hind* III (A) standards are included as size markers. Less digested pMA91+PYK than digested pMA91(+PYK) was electrophoresed. Thus, not all the pMA91(+PYK) digest fragments are visible. The gel was southern blotted onto a Hybond N filter and analysed as described below.

III: The filter was analysed firstly for the *PYK* 5' untranslated sequence, using the (PYK5') oligonucleotide. A single band is evident, corresponding to the 5' leader sequence prsent in pLD1(35). No sequences in pMA91(+PYK) were detected.

IV: As a control, the filter was then analysed for the *PGK 5'* untranslated leader sequence. An oligonucleotide which contained the *PGK* leader sequence was used as a probe. This hybridised to appropriate *Eco* R1 fragment in pMA91(+PYK).

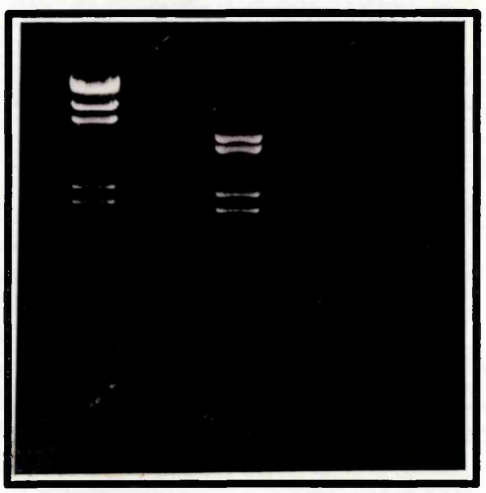
I



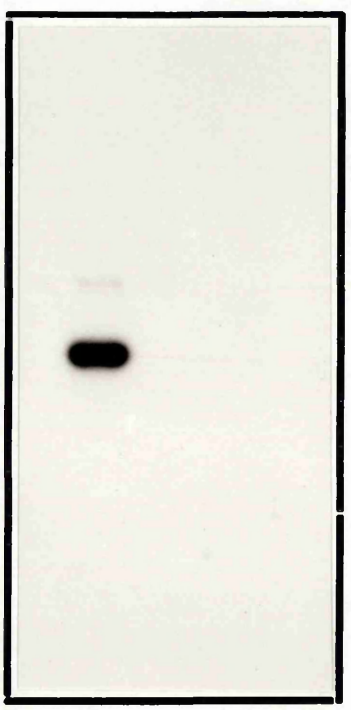


A B C

II



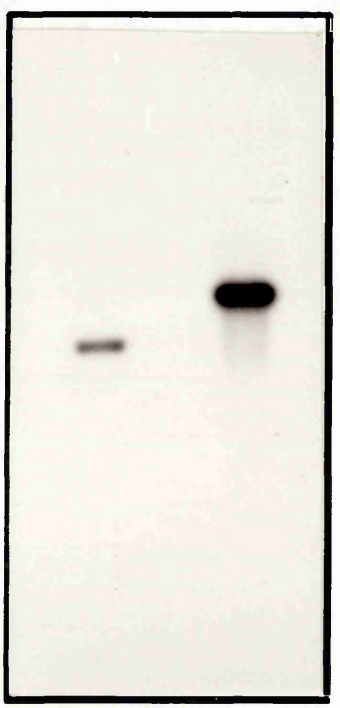
III



– 2.7 kb –

– 1.9 kb –

IV



resulting autoradiogram (Fig. 6.3d) shows specific hybridisation to the 2.7 kb band of pMA91(+PYK).

6.2.3.2 Polysomal distribution of the *PYK1* and *PYK1:PGK* mRNAs.

Polysome gradients were prepared from two DBY746:pMA91(+PYK) transformants ([91.3, 91.4]) and were aliquoted into 8 and 10 equal fractions respectively. RNA was purified from each fraction by phenol:chloroform extraction, and an equal proportion from each was Northern blotted. Each of the two Northern filters prepared (one for each of the transformants) were analysed for the *ACT1*, *PYK1* (total) and *PYK1* (chromosomal) mRNAs using random-primed pSPACT9, random-primed pSPK2 and end-labelled (PYK5'), respectively. The probings were conducted sequentially, with hybridised probe being removed before analysis with a second or third probe. After each individual probing the amount of hybridised probe was quantified using the AMBIS β-scanner. The results of the Northern blot are presented in Fig. 6.4a, and the distribution of the mRNAs in each fraction presented graphically in Fig. 6.4b.

Each of the two pMA91(+PYK) transformants show similar polysomal distributions for the mRNAs analysed for. Total *PYK1* mRNA is heavily loaded with ribosomes in both transformants as is the *ACT1* mRNA. This confirms that the *PYK1:PGK* mRNA is well translated.

Analysis of the wild-type *PYK1* mRNA reveals that it is also well translated in pMA91(+PYK) transformants (Fig. 6.4). Although the ribosome loading on this mRNA is slightly less than for the *PYK1:PGK* in each transformant (Fig. 6.4b), it is associated with the heavily ribosome-bound mRNAs. Thus, the translation of the *PYK1* mRNA is not affected in pMA91(+PYK) transformants, implying that the translational repression is not mediated directly or indirectly by excess pyruvate kinase.

Figure 6.4 (a) Distrbution of mRNAs across polysome gradients prepared from [91.3] and [91.4]

Polysome gradients were prepared from two pMA91(+PYK1) transformants [91.3] and [91.4]. Equal proportions of fractions taken across each gradient (8 and 10 respectively), were subjected to Northern blotting and analysed sequentially for the distribution of total *PYK1 mRNA* (including plasmid and chromosomally encoded mRNA), chromosomal *PYK1* mRNA and the *ACT1* mRNA. Before analysis for a second or third mRNA, the filter was scanned by the AMBIS β-scanner in order to quantify the amount of hbridisation in each lane. The filter was then stripped of probe and reprobed for the next mRNA of interest.

(I) Fractions from [91.3] probed for total *PYK1* mRNA.

(II) Fractions from [91.3] probed for chromosomal *PYK1* mRNA.

(III) Fractions from [91.3] probed for *ACT1* mRNA.

(IV) Fractions from [91.4] probed for total *PYK1* mRNA.

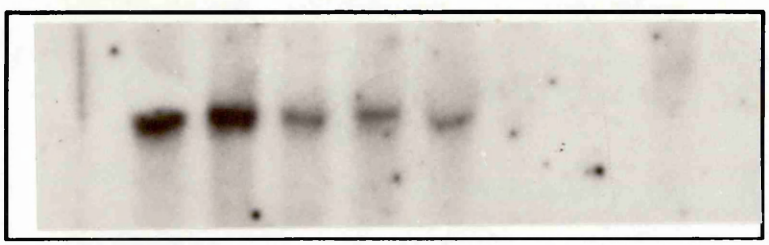
(V) Fractions from [91.4] probed for chromosoml *PYK1* mRNA.

(VI) Fractions from [91.4] probed for *ACT1* mRNA.

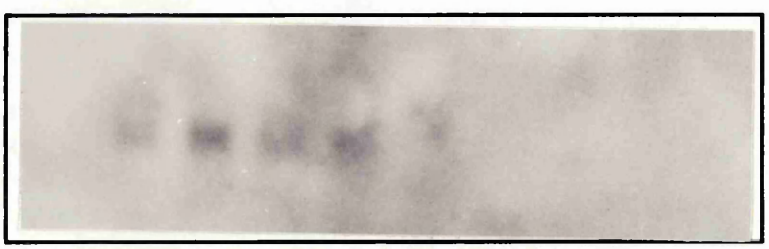
The direction of sedimentation is from right to left on the picture. Thus, fractions which are towards the left of the picture are from the bottom of the gradient and contain mRNAs which are heavily loaded with ribosomes. Conversely, fractions which are towards the right of the gradient are from the top of the gradient and contain mRNAs which are lightly loaded with ribosomes.

I

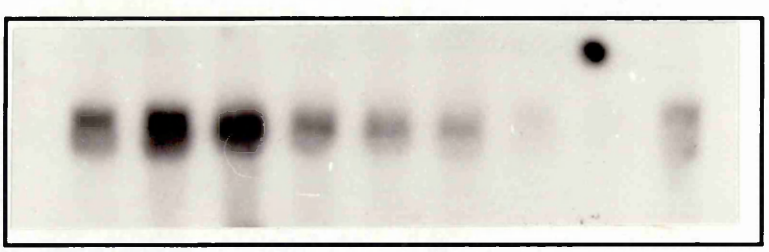
II



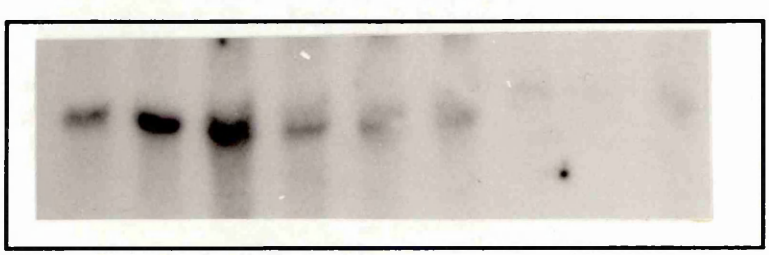
III



IV



V



VI

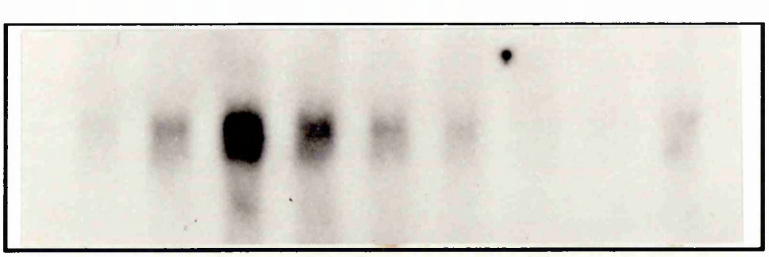


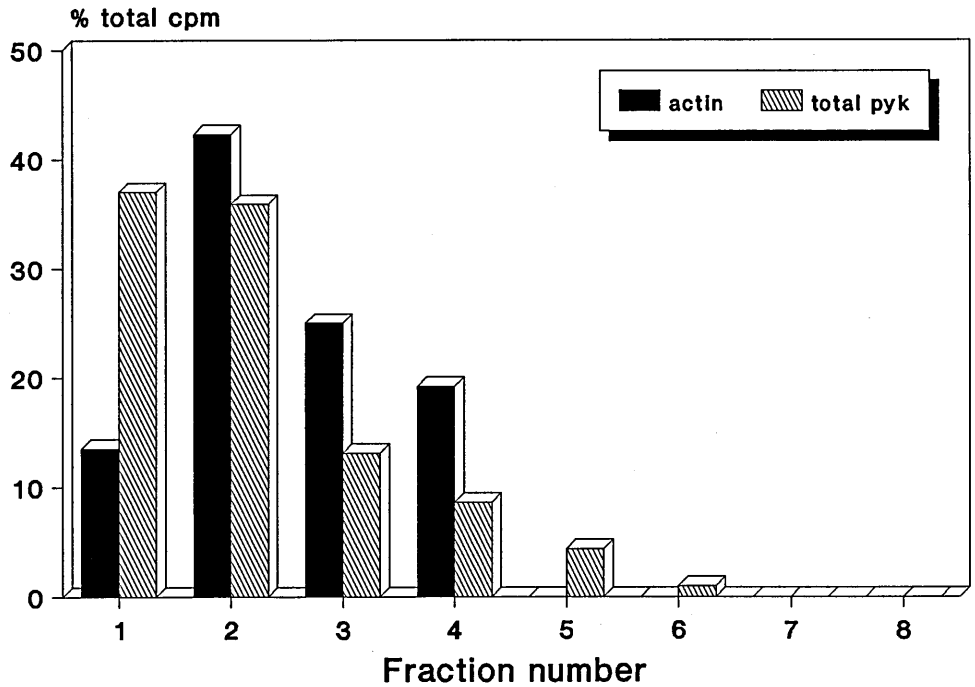
Figure 6.4 (b) The translation of the chromosomal *PYK1* mRNA is not repressed in pMA91(+PYK) transformants.

The information gleaned from the analysis described in (a) is graphically presented.
In each graph, the percentage of total cpm (Y-axis) in a particular fraction (X-axis) for a particular mRNA is plotted. The total cpm equals the sum of the cpm in each fraction for a particular mRNA species. The fractions are numbered from the bottom of the gradient. Thus, fraction 1 contains the mRNA species which are heavily bound with ribosomes.

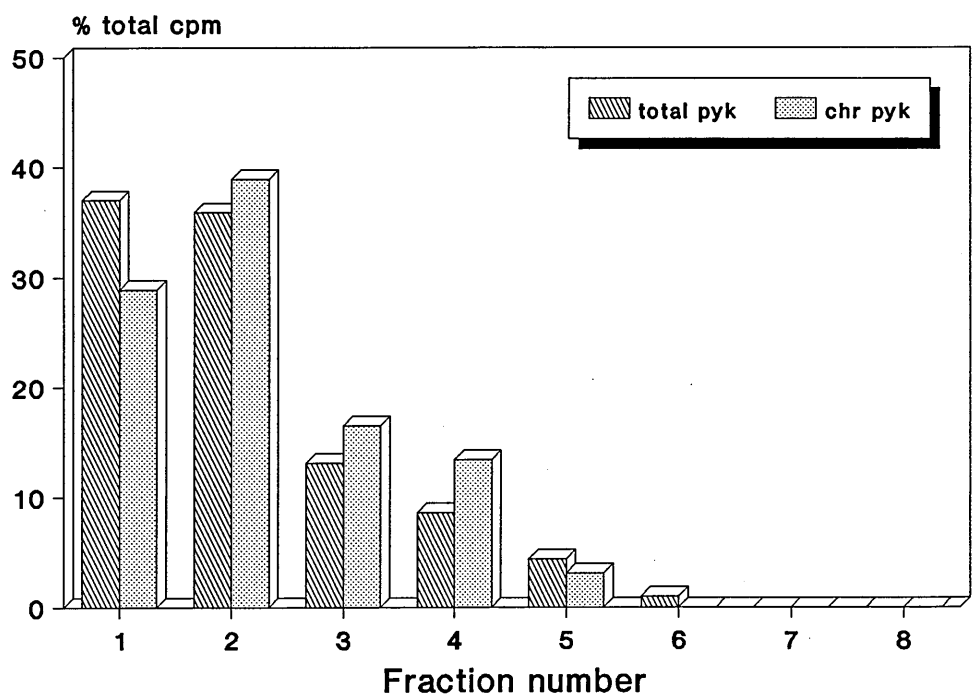
(A) Distribution of the *ACT1* mRNA and total *PYK1* mRNA across the [91.3] polysome gradient.
(B) Distribution of the chromosomal *PYK1* mRNA and total *PYK1* mRNA across the [91.3] polysome gradient.

(C) Distribution of the *ACT1* mRNA and total *PYK1* mRNA across the [91.4] polysome gradient.
(D) Distribution of the chromosomal *PYK1* mRNA and total *PYK1* mRNA across the [91.4] polysome gradient.

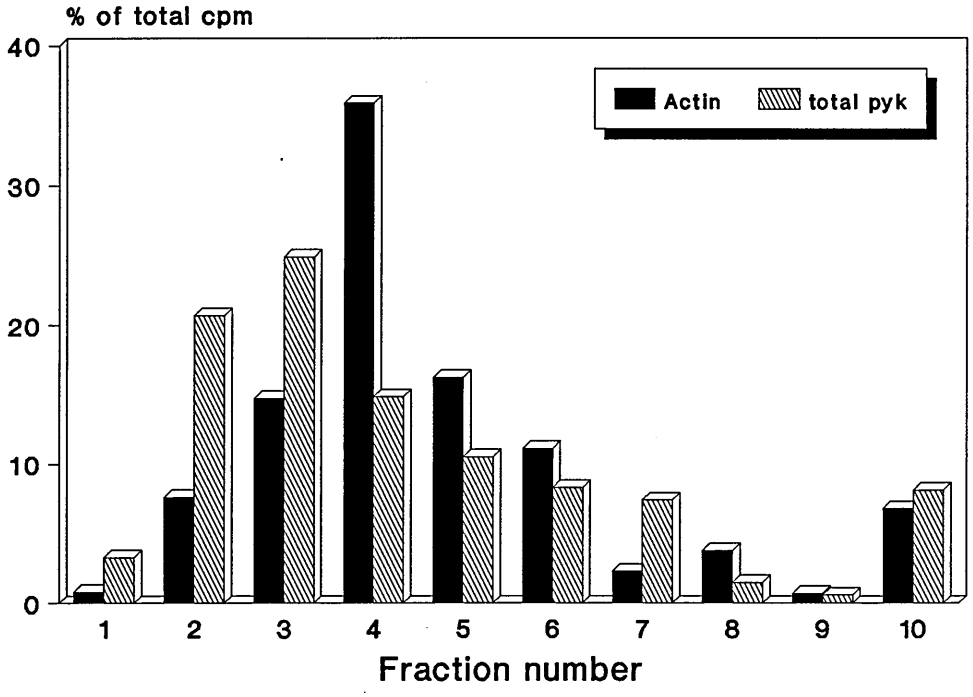
A


% total cpm
50
40
30
20
10
0
actin
total pyk
1
2
3
4
5
6
7
8
Fraction number

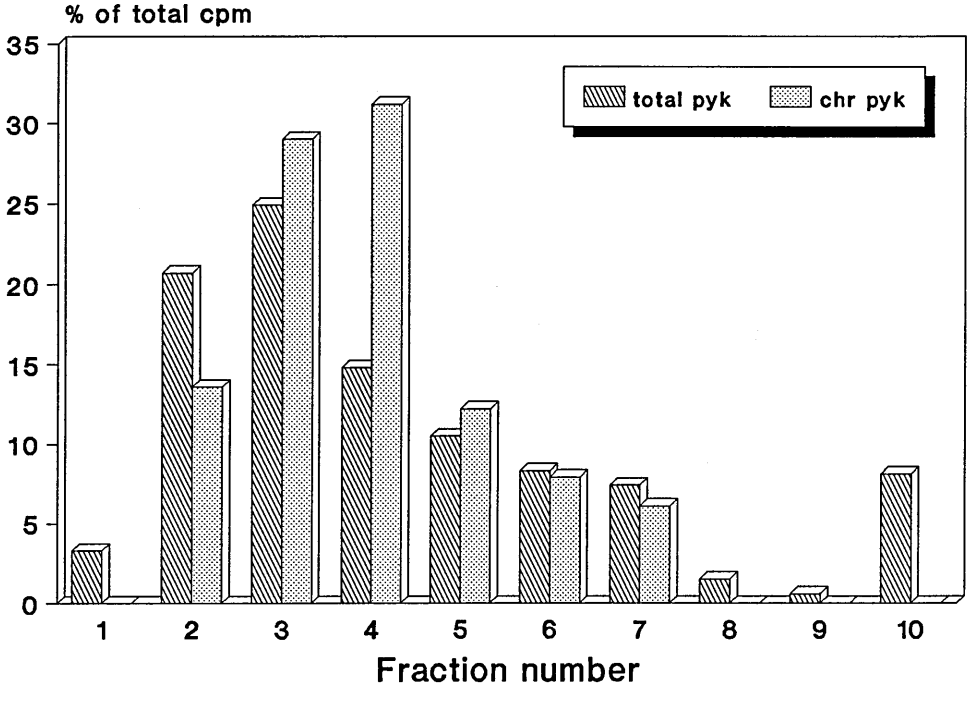
B


% total cpm
50
40
30
20
10
0
total pyk
chr pyk
1
2
3
4
5
6
7
8
Fraction number

C


% of total cpm
40
30
20
10
0
Actin
total pyk
1
2
3
4
5
6
7
8
9
10
Fraction number

D


% of total cpm
35
30
25
20
15
10
5
0
total pyk
chr pyk
1
2
3
4
5
6
7
8
9
10
Fraction number

6.2.4 THE EFFECT OF ELEVATED *PYK1* mRNA LEVELS ON THE TRANSLATION OF OTHER GLYCOLYTIC mRNAs.

To determine whether any of the glycolytic genes in addition to *PYK1* are translationally repressed in the presence of high *PYK1* mRNA levels, a series of polysome gradients were analysed for their distribution of several glycolytic mRNAs: *PFK1; PFK2; TDH; PGK.* Since the *TDH* mRNA was analysed using an oligonucleotide complementary to all three species of *TDH* mRNA (*I, II, III*) this probe will determine an average distribution of all three mRNAs across polysome gradients. However, since the majority of *TDH* mRNA is comprised of *TDH II,* any changes in the distribution of the other two mRNAs will be masked to some degree by the abundant *TDH II* mRNA.

At least two polysome gradients prepared from separate DBY746:pLD1(35) transformants with elevated *PYK1* mRNA levels, were examined for the distribution of each glycolytic mRNA. In addition, the distribution of the mRNAs across a polysome gradient prepared from DBY746 was also determined. These gradients had previously been examined for the distribution of the *PYK1* and *TCM1* mRNAs by dot blot analyses. The results of this, together with the results of the subsequent analyses of the other glycolytic mRNAs are summarised in Fig. 6.5.

For the purposes of clarity, rather than presenting an individual graph for each analysis (ie. twenty graphs), the results are presented in bar-charts. These show the percentage of each mRNA tested that is associated with 0-2, 3-6, 7-15 and greater than 15 ribosomes in each of the strains examined. The abundance of the *PYK1* mRNA in each strain is denoted beside each series of bar-charts.

Of the glycolytic mRNAs examined, in addition to *PYK1*, there is only a substantial shift in the ribosome loading of the *PFK2* mRNA, as the abundance of the *PYK1* mRNA is increased. This shift results in a reduction in ribosome loading and is most dramatically observed when comparing the host strain with [35.31] which has a *PYK1* mRNA abundance equal to 5.9% of the total mRNA (Fig. 6.5b). This is re-iterated in Fig. 6.6, in which the *PFK2, PYK1* and *Rp1* mRNA distributions across these two gradients are presented graphically.

Figure 6.5 The effect of elevated *PYK1* mRNA levels on the translation of other glycolytic gene mRNAs.

Polysome gradients were prepared from DBY746 and three DBY746:pLD1(35) transformants, which contained different levels of *PYK1* mRNA (DBY746 = 0.6% of total mRNA; [35.30] = 4.8%; [35.31] = 5.9%; [35.29] = 6.3%).

Each polysome gradient was fractionated into about 48 fractions starting from the bottom. Fractions from each of the four gradients were used to prepare a series of identical dot blot filters. The filters were probed separately for (A) *PYK1,* (B) *Rp1,* (C) *PFK1,* (D) *PFK2,* (E) *TDH* and (F) *PGK.* In the case of (A) and (B), all four of the gradients were analysed. For (D-F), the host strain gradient, and two others were analysed.

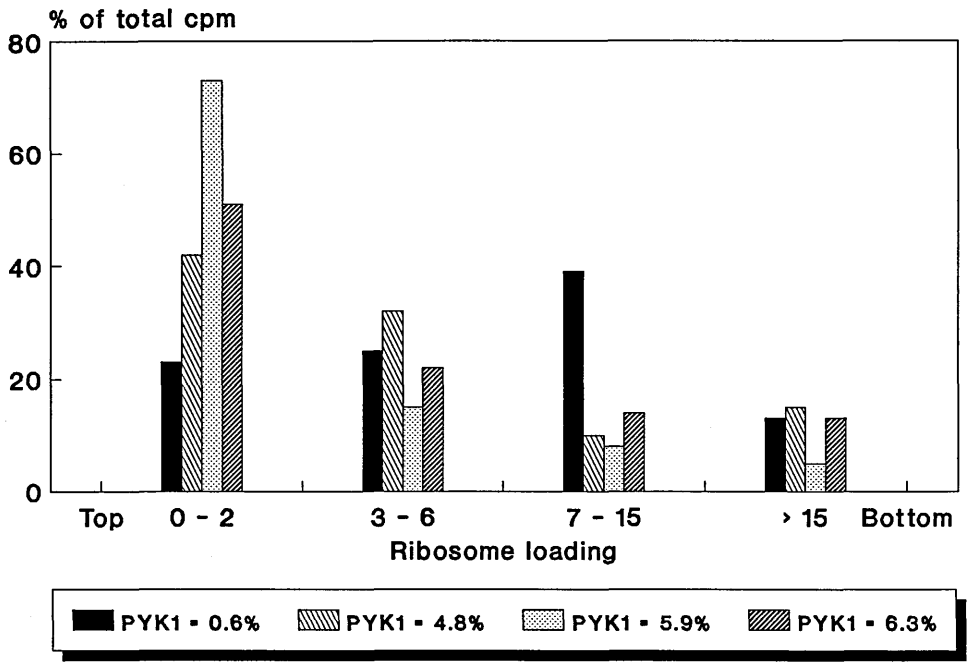
The radioactivity bound to each fraction was determined by scintillation counting and is expressed as % of total cpm. Continuous A_{260} nm profiles were obtained prior to fractionation of each gradient and these were used to divide each polysome gradient into four regions (0-2 ribosomes/mRNA, 3-6, 7-15 and > 15). For clarity, for each mRNA the % of total cpm in each region of the gradient is presented. Thus, in each bar chart, the results from the probing of 4 (or 3) separate polysome gradients for a single mRNA are presented.

The *Rp1* mRNA (B) distribution serves as a control mRNA to highlight any sedimentation differences between gradients. Most notably, the [35.31] gradient (*PYK1* = 5.9%) has in general a lower ribosome loading than the other gradients, and the [35.29] gradient has a higher ribosome loading. These differences, although minor, are important when interpretating the polysome distribution of the other glycolytic mRNAs.

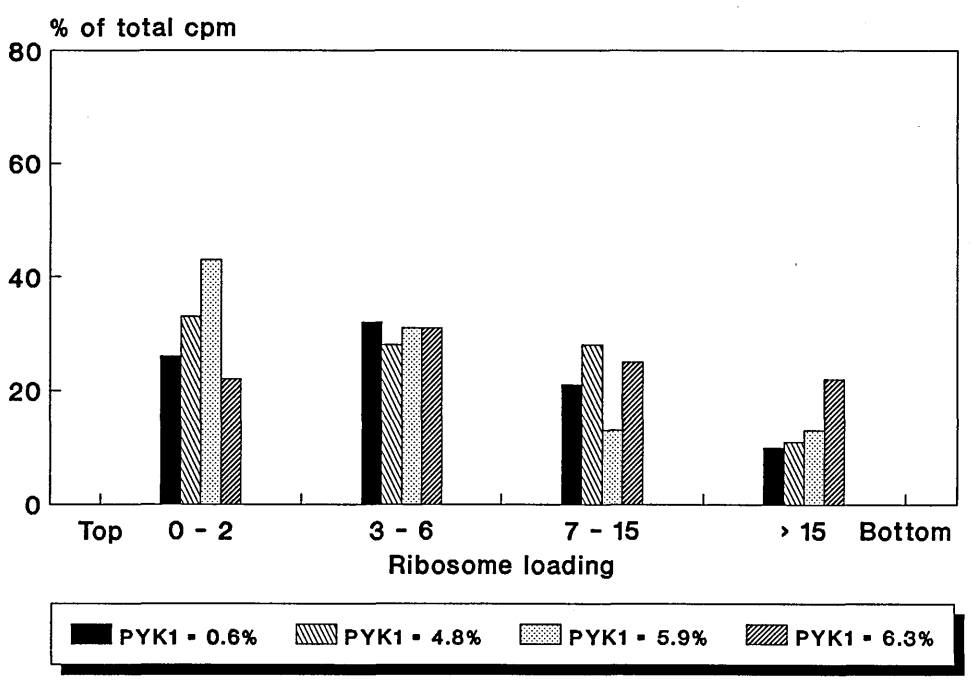
The distribution of the *PYK1* mRNA (A), across the polysome gradients clearly shifts towards the top of the gradient as the abundance of the *PYK1* mRNA is increased. Likewise, there is a decrease in the ribosome loading on the *PFK2* mRNA as the abundance of the *PYK1* mRNA is increased (D). In contrast, there appears to be no substantial shift in the ribosome loading of the other glycolytic mRNAs tested (C, E and F).

Note: the information on the *PYK1, Rp1* and *TDH* analyses of gradient DBY746, and the *PYK1, Rp1* analyses of [35.31] is taken from Bettany, 1988.

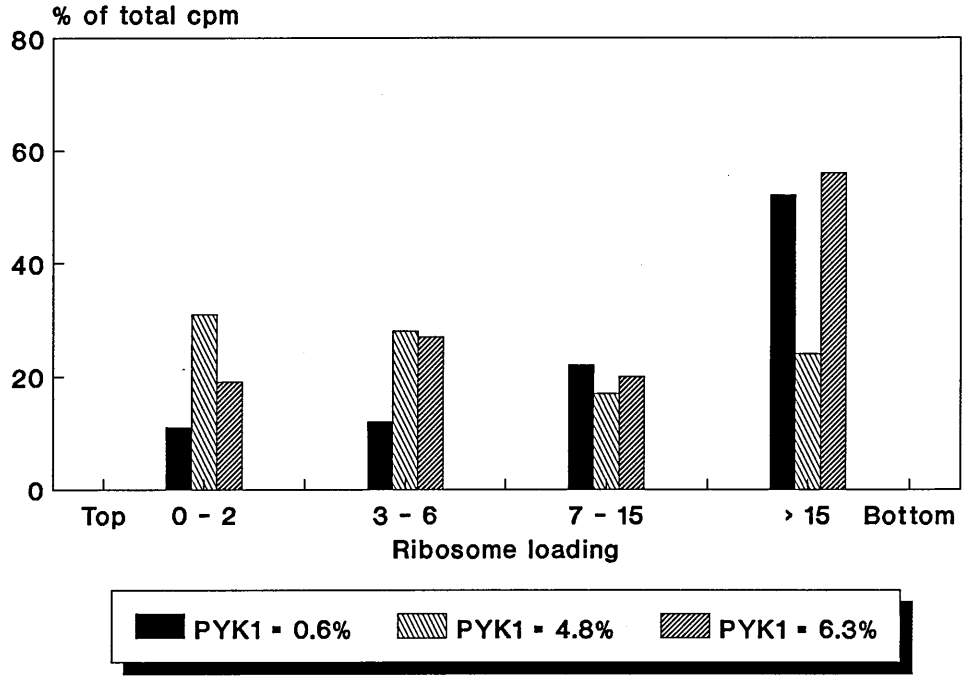
A: PYK1 mRNA distribution



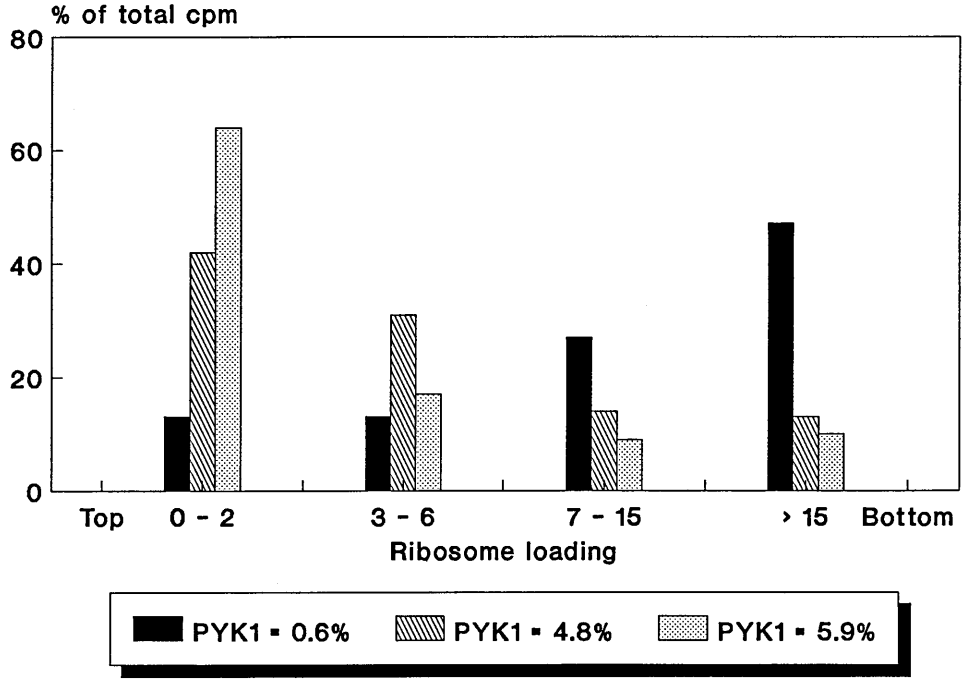
B: Rp1 mRNA distribution



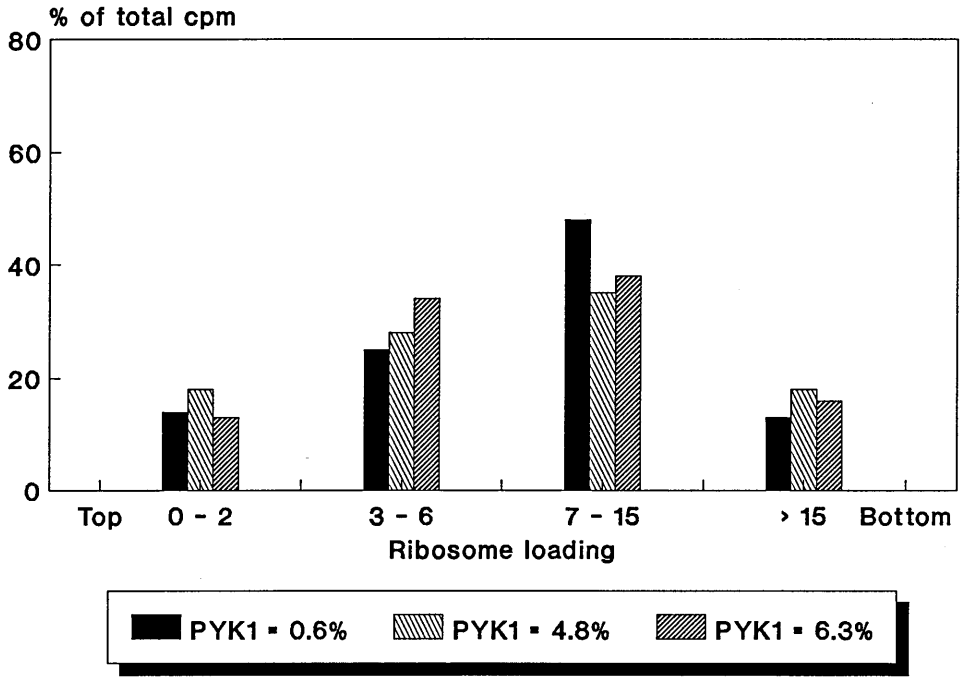
C: PFK1 mRNA distribution



D: PFK2 mRNA distribution



E: TDH(I-III) mRNA distribution



F: PGK mRNA distribution

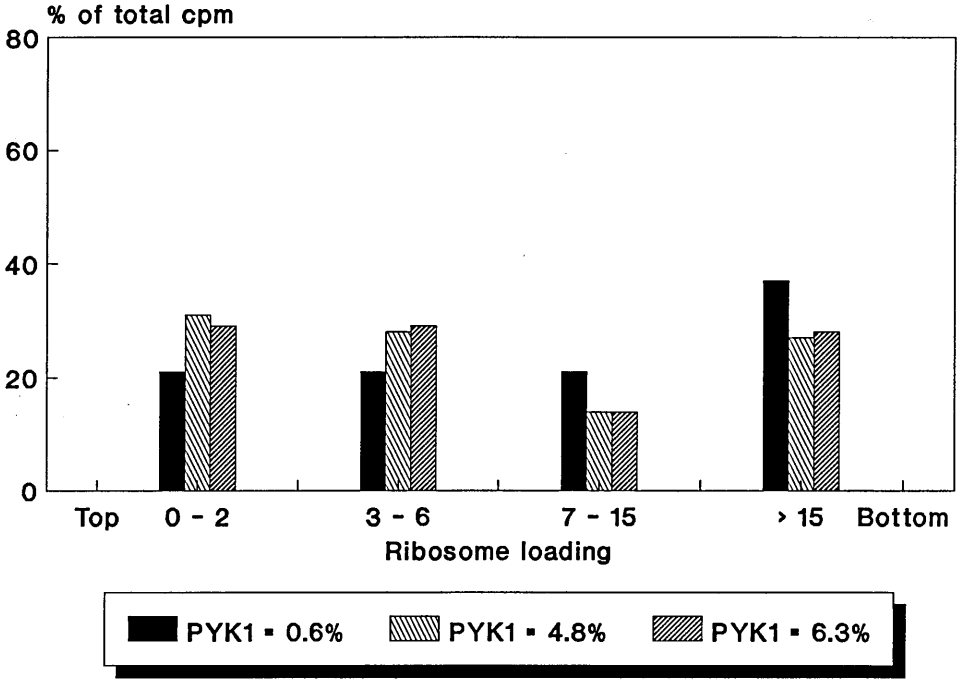
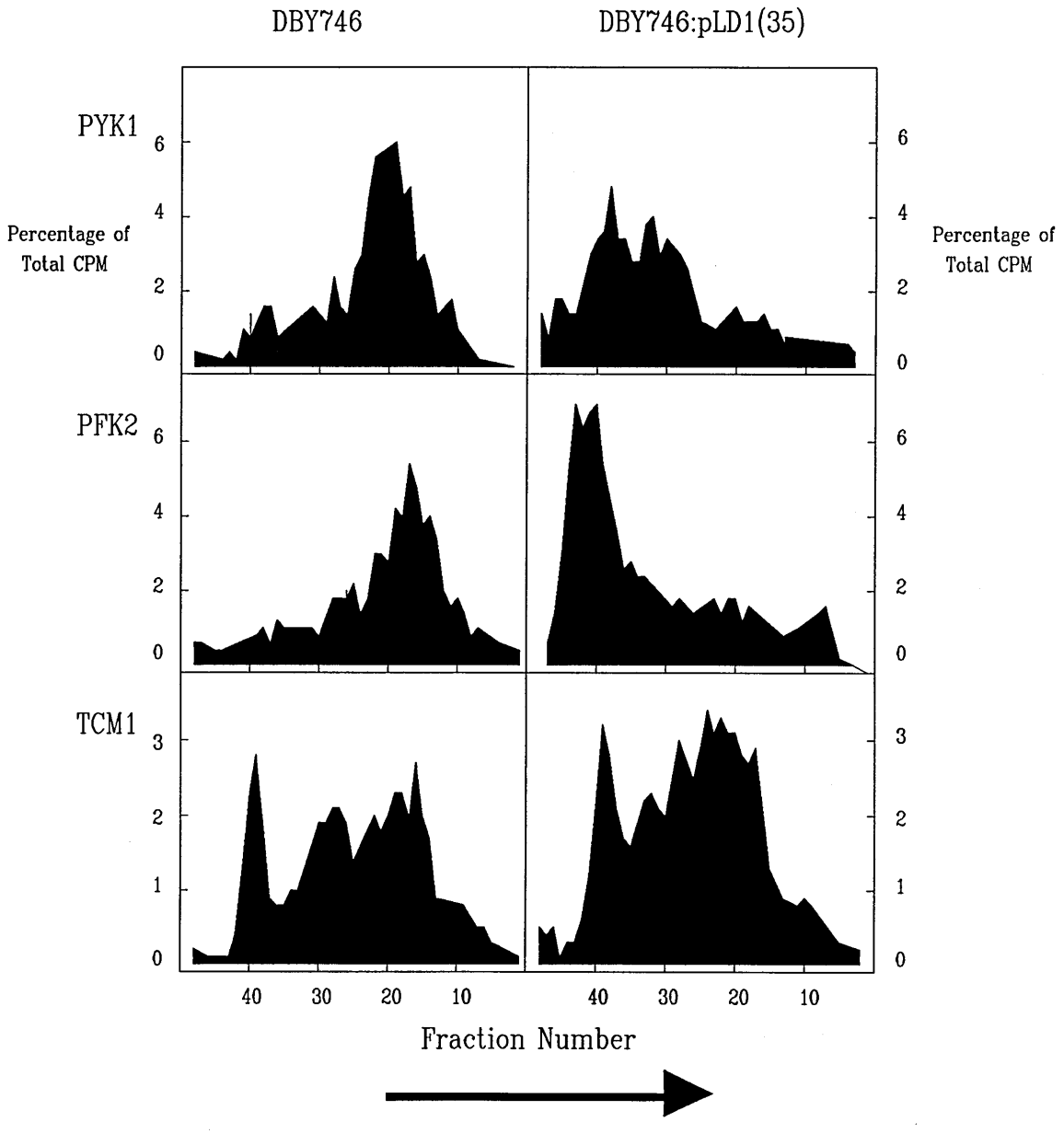


Figure 6.6 Excess *PYK1* mRNA inhibits the translation of both the *PYK1* and *PFK2* mRNAs.

The distribution of the *PYK1* and *PFK2* mRNAs are compared with that of the control *TCM1* mRNA are compared across polysome gradients prepared from DBY746 (*PYK1* mRNA = 0.6% of total mRNA) and the DBY746:pLD(1)35 transformant [35.31] (*PYK1* mRNA = 5.9% of total mRNA). The percentage of total cpm in each fraction is plotted against fraction number for each denoted mRNA. The arrow shows the direction of sedimentation, with the top of the gradient to the left, and heavy polysomes to the right of each box.


DBY746
DBY746:pLD1(35)
PYK1
PFK2
TCM1
Percentage of Total CPM
Percentage of Total CPM
Fraction Number
40
30
20
10

In contrast there is no significant shift in the ribosome loading of the *PFK1*, *TDH*, or *PGK* mRNAs when *PYK1* mRNA levels are elevated (Fig. 6.5). It would appear therefore that only a select group of glycolytic mRNAs are subject to the translational repression observed in the presence of high *PYK1* mRNA.

6.3. DISCUSSION.

Pivotal to the process of translational initiation is the 5' untranslated leader of an mRNA. The length, structure and sequence of the leader sequence can each influence the efficiency of mRNA translation (Kozak, 1989a). Indeed, most examples of translational control thus far discovered operate at the level of translational initiation via the 5' untranslated leader. The importance of the *PYK1* 5' leader in mediating translational control of *PYK1* was addressed in this chapter.

The 5' untranslated leader of *PYK1* was shown to be 28 nt long in this study (Fig. 3.5) and analysis with the computer program FOLD, shows that it does not form any strong secondary structures which could inhibit translation (Bettany *et al.*, 1989). The same transcriptional start sites are used by the *PYK1* gene carried on pLD1(35) (Fig. 6.1). Therefore, the inhibition of *PYK1* mRNA translation observed in pLD1(35) transformants is not caused by the presence of an abnormally long or short 5' untranslated leader.

As the average length of 5' untranslated leaders in yeast is 52 nt (Cigan and Donahue, 1987), the *PYK1* mRNA has a relatively short leader length. It has been shown in *S.cerevisiae* that mRNAs with short 5' leaders are poorly translated, although this has only been demonstrated when the leader length is reduced to below 27 nt. For example, when the length of the *PGK* 5' untranslated leader is reduced from 45 to 27 nt, no detrimental effect on translation was apparent, although a further decrease to 22 nt resulted in a 2-fold decrease in translation (Van den Heuvel *et al.*, 1989). In the case of *TCM1*, which normally has a 5' untranslated leader of 22nt any decrease in leader length reduces translational initiation. Remarkably however, it has been demonstrated for this particular mRNA that translation can still

proceed, albeit at a reduced rate, without any 5' leader sequence at all (Maicas *et al.*, 1990). It is therefore improbable that the *PYK1* mRNA will be translated inefficiently on account of its short 5' untranslated leader. In fact, it has been demonstrated in this study that the *PYK1* mRNA is efficiently translated in the DBY746 host when there is only one copy of the *PYK1* gene (Fig 6.5).

However, as stated in 1.2.5.2, the inefficiency of translation imparted by the presence of a short 5' leader is exaggerated in higher eukaryotic cells subjected to an osmotic stress (Kozak, 1988). In this study, it has been demonstrated that DBY746:pLD1(35) transformants grow considerably slower than the untransformed host (Chapter 4). Thus, these transformants are obviously subject to some form of growth stress. Perhaps this stress causes a detrimental effect on protein synthesis in yeast in a manner analogous to that observed in stressed mammalian cells. This could result in inefficient translation of mRNAs with short leaders including *PYK1*, and might therefore explain the translational effects observed with this mRNA in pLD1(35) transformants. If this was true, it would be anticipated that other mRNAs with short 5' untranslated leaders would also succumb to translational repression in slow-growing pLD1(35) transformants. One of the control mRNAs analysed on the polysome gradients was *TCM1*, which has a 5' leader of 22nt (Maicas *et al.*, 1990). Since there was no shift in the ribosome loading of this mRNA, it would appear that the translational effects on the *PYK1* mRNA were not caused by a stress effect.

When a *PGK:PYK1* gene fusion is over-expressed in yeast the translational effect characteristic of the wild-type *PYK1* mRNA is not observed (Fig. 6.2). The *PGK:PYK1* mRNA is efficiently translated in pMA91(+PYK) transformants suggesting that *PYK1* sequences not present in the fusion mRNA are required for translational regulation. The 25 nt proximal to the 5' end of the *PYK1* mRNA, which are not present in the *PGK:PYK1* mRNA (Fig 6.7), are therefore necessary for the translational regulation of *PYK1*.

Scrutiny of the *PYK1* 5' untranslated leader does not reveal any upstream AUG codons, which may be important in mediating translational control. This therefore sets this regulatory mechanism

apart from the two best characterised examples of translational control in *S.cerevisiae*; *CPA1* and *GCN4*. The regulation of both these mRNAs is mediated by the presence of uORFs in their 5' leaders (Hinnebusch, 1988). There are however, several examples of translational regulation mechanisms which do not require the presence of upstream AUG codons in other eukaryotes. During *Xenopus* development, the synthesis of ribosomal proteins is known to be regulated at the translational level (Mariottini and Amaldi, 1990). In the case of the S19 ribosomal protein mRNA, it has been demonstrated that the 35nt long 5' untranslated leader is sufficient to confer translational regulation. This leader sequence does not contain any AUG codons and when fused to a reporter gene it can impart developmental translational regulation (Mariottini and Amaldi, 1990).

While it was established in this study that the intact *PYK1* 5' untranslated leader is neccessary for translational regulation, it was not determined if it was sufficient to confer regulation.

Attempts were made to answer this question by transforming the pLD1(35) plasmid into two strains which carried different *PYK1:lacZ* fusions integrated at the *ura3* locus (Chapter 3). One of the *PYK1:LacZ* constructs directs the synthesis of an mRNA which contains only the *PYK1* 5' leader fused to *lacZ*, while the other has the *PYK1* 5' leader and about 850 bp of *PYK1* coding region fused to *lacZ*. If the 5' leader is sufficient for translational regulation then the presence of high levels of *PYK1* mRNA (upon introduction of pLD1(35)) would reduce the translational efficiency of both *PYK1:LacZ* mRNAs. If more *PYK1* sequences are required, then the translation of only the longer *PYK:lacZ* mRNA might be affected by high *PYK1* mRNA levels which carried only the 5' leader sequences from *PYK1*. Unfortunately efforts to determine this were thwarted by the inability to obtain pLD1(35) transformants of either of the *PYK1:LacZ* integrants, despite several attempts at the transformation. Due to time restrictions, further attempts were not possible.

As noted in chapter 4, the frequency of transformation of DBY746 with pLD1(35) is relatively low and transformants take longer to form colonies (4-6 weeks) compared with other plasmids. This frequency

appears to be lowered further when transforming DBY746 strains which contain integrated copies of *PYK1:lacZ* fusions.

The relative levels of expression directed by each of the two *PYK1:lacZ* strains described above, indirectly appears to implicate sequences within the coding region as being important in optimal translation of *PYK1*. This evidence is drawn from results presented in chapter 3 and is presented in Table 6.1.

With the introduction of *PYK1* coding region, the efficiency of translation of the *PYK1:lacZ* fusion mRNAs appears to be greater. This is reflected in a four-fold difference in the amount of ß-galactosidase synthesised per mRNA for each of the gene fusions (see Table 6.1). However, it is possible that this difference is not due to differences in translation, but to differing stabilities or activities of the *PYK1:lacZ* fusion proteins synthesised by each construct. Effects at the level of translation could be tested by polysome analyses. If indeed there are sequences within the *PYK1* coding region which are required for optimal translation, they might also be involved in the translational control of the *PYK1* mRNA over-expression.

Several genes that are regulated at the level of translation are subject to auto-regulation. In *E.coli*, the expression of specific ribosomal protein genes, bacteriophage genes and amino acyl tRNA synthetase genes is controlled through feed-back inhibition of the translational initiation by the interaction of the protein product with the translational initiation region (Kearney and Nomura, 1987; Tuerk *et al.*, 1990; Springer *et al.*, 1989). In contrast, the translational regulation of *PYK1* does not appear to be subject to feed-back inhibition either directly or indirectly by excess pyruvate kinase. This was evident from the finding that in DBY746:pMA91(+PYK) transformants (which express pyruvate kinase at high levels) there is no translational inhibition of the chromosomally-encoded *PYK1* mRNA (Fig. 6.4).

Alternatively, translational repression of the *PYK1* mRNA in the presence of high levels of the mRNA, might occur through the saturation of a positive translational factor required for *PYK1* mRNA translation. The results presented in Fig. 6.5 suggest that the *TCM1*, *TDH1-3*, *PFK1*, and *PGK* mRNAs do not require this factor for translation, since their ribosome loading is unaffected by the presence

TABLE 6.1 **Translational efficiency of *PYK1:lacZ* fusion constructs in yeast.**

Strain[a]	*PYK1:lacZ* mRNA[b]	β-gal activity[c]	Ratio[d]
lacZ(DAS-)	0.14	805	5750
lacZ(DAS+)	1.01	22200	22000

a: The *lacZ*(DAS-) strain carries one integrated copy of the *PYK1:lacZ* fusion which contains the 5' *PYK1* untranslated leader sequence fused to the *lacZ* coding region.
The *lacZ*(DAS+) strain carries two integrated copies of the *PYK1:LacZ* fusion which contains the *PYK1* 5' leader sequence and 850 bp of *PYK1* coding region fused to the *lacZ* coding region.
b: The abundance of the *PYK1:lacZ* mRNA relative to the *ACT1* mRNA when strains are grown on glucose, was determined by Northern analyses and subsequent quantitation using the AMBIS β-scanner.
c: The units of β-galactosidase activity are proportional to the increase in O-nitrophenol per minute per yeast cell.
d: Ratio of c/b (β-galactosidase activity/*PYK1:lacZ* mRNA).

of elevated *PYK1* mRNA levels. Previous evidence has demonstrated that the translation of *ACT1* mRNA is also un-affected, suggesting that any translational factor which might become limiting in the presence of high *PYK1* mRNA levels is not a general translational factor (Bettany, 1988; Moore *et al.*, 1990a). However, there is a dramatic reduction in *PFK2* translation in DBY746 strains carrying elevated *PYK1* mRNA levels suggesting that this mRNA is translationally co-regulated with *PYK1*. Perhaps both the *PYK1* and *PFK2* mRNAs require the action of the same specific factor for their translation. Interestingly, attempts to over-produce both pyruvate kinase and phosphofructokinase in the same yeast cell through the introduction of a multi-copy plasmid carrying the *PYK1*, *PFK1*, and *PFK2* genes were unsuccessful (Schaaf *et al.*, 1989). Furthermore, it is also noteworthy that both the *PFK2* and *PYK1* genes were shown in chapter 3 (Fig. 3.4) to be relatively highly induced at the mRNA level in the presence of glucose. Also, both pyruvate kinase and phosphofructokinase are the only glycolytic enzymes used solely for glycolysis and not in gluconeogenesis.

Comparison of the 5' untranslated leader sequences of those mRNAs whose translation is affected by high *PYK1* mRNA levels, and those not affected (Fig. 6.7), does not reveal any obvious motif for translational regulation. If such a sequence exists in the *PYK1* 5' leader, it does not seem to reside near the start codon, since both the *PYK1* and *PGK:PYK1* mRNAs have the same sequence surrounding the AUG (Fig. 6.7). Interestingly, in both the *PYK1* and *PFK2* mRNAs, the 5' proximal nucleotide is a C residue, whereas in those mRNAs shown to be unaffected by excess *PYK1* mRNA, the 5' nucleotide is an A residue. The relevance of this difference could be established by mutating the C residue at the 5' end of the *PYK1* mRNA to an A residue and testing whether the mRNA is still subject to translational regulation.

A discriminating sequence may become more apparent when all of the glycolytic mRNAs have been analysed for their translatability in the presence of high *PYK1* mRNA levels and their sequences compared. Furthermore, as stated above it has not been ascertained whether sequences within the coding region are required for the tanslational regulation of *PYK1* or *PFK2*. Also, it is possible that sequences within the *PYK1* 3' untranslated region act in concert with the *PYK1* 5' leader

Figure 6.7 Sequences of the 5' untranslated regions of mRNAs analysed on polysome gradients.

Leader sequences of mRNAs subject to translational regulation.

PYK1 CAAUCAAAACAAAUAAAACAUCAUCACA **AUG**

PFK2 CUUUCUUAUACCUCAUUUGAACAAUAGAACUAGAUUUAGAGACUAG UUUAGCAUUGGCCAAGAACUAACCAUACGCA **AUG**

Leader sequences of mRNAs unaffected by *PYK1* translational regulation.

PGK:PYK AGGAAGUAAUUAUCUACUUUUUACAACAAAUAUAAAAACCAAAAGA UCCACA **AUG**

PFK1 AAGAAUAACAAUAUAGGGAGAGAAAUUUUUCUAUUUUUAAUUUCGA AACAGGUACCAAAAAAUCUAAGUUCACUUUAGCACUAUUGGGGAAA GCUUUUAUAUAAAAAAUCUGAAACAAAAUCAUAUCAAAG **AUG**

TDH2 ACACCAAGAACUUAGUUUCGAAUAAACACACAUAAAUAAACAAAA **AUG**

PGK AGGAAGUAAUUAUCUACUUUUUACAACAAAUAUAAAAACA **AUG**

TCM1 ACAGUUACUACAACAAUCAAUG **AUG**

sequence. Once the sequence mediating the translational regulation is discovered, it would be informative to carry out RNA:protein binding studies in an attempt to isolate the translational factor(s) which mediate translational regulation of *PYK1* and *PFK2*. However, such analyses might be complicated by a possible requirement for the formation of a pre-initiation complex, before the factor is able to bind.

What can be deduced clearly from Fig. 6.7, is that the 5' untranslated leader of the *PFK1* mRNA is substantially different than that of the *PFK2* mRNA and this may explain their differing responses to the translational control. That both genes are subject to different levels of regulation is somewhat surprising when one considers that they encode a sub-unit (each required in equimolar amounts) of phosphofructokinase. However, it has been shown that *pfk1/PFK2* or *PFK1/pfk2* mutants can still utilise glucose, suggesting that the sub-units are not necessarily required in equimolar amounts (Heinisch, 1986). Also, experiments presented in Chapter 3 demonstrated that with respect to glucose induction, while both genes are transcriptionally activated, *PFK2* mRNA appears to be induced to a slightly higher level.

In conclusion, the results presented in this chapter have answered several questions pertaining to the translational regulation of the *PYK1* mRNA. In addition, several new questions have been raised.

Firstly, it was demonstrated that the translational regulation of *PYK1* observed in transformants carrying multiple copies of a *PYK1*-encoding plasmid is not due to the plasmid encoded *PYK1* mRNA having an aberrant 5' untranslated leader.

Secondly, it has been established that the *PYK1* 5' leader sequence is essential for translational regulation. However, it has not been determined whether sequences within the coding region or 3' untranslated are required in tandem with the 5' untranslated leader.

Thirdly, translational regulation of *PYK1* is not mediated directly or indirectly by the presence of excess pyruvate kinase.

Finally, while the translation of *PFK2* in addition to *PYK1* is affected by the abundance of *PYK1* mRNA, the translation of *TDH*, *PGK*, and *PFK1* remains unaffected. It remains to be determined whether any of the other glycolytic mRNAs are translationally co-regulated with *PYK*.

CHAPTER 7

CONCLUSIONS

The work carried described in this thesis revolves around the general theme of glycolytic gene expression in the yeast *Saccharomyces cerevisiae*. Particular attention was paid to the study of yeast transformants carrying multiple copies of the *PYK1* gene, which led to the discovery of a co-ordinated mode of glycolytic gene regulation at the level of translation. Also, the relative inducibility of each glycolytic mRNA in response to glucose was established.

Multiple copies of the *PYK1* gene were introduced into yeast, by transforming with the multicopy vector pLD1(35) (Fig. 4.1). Transformants with different colony sizes and growth rates were obtained. Colony size and growth rate were shown to correlate inversely with the level of *PYK1* expression. Transformants which formed small colonies (t_d = 3-5 hrs under conditions which select for plasmid-containing cells) generally contained *PYK1* mRNA levels greater than 1.5% of total mRNA. The untransformed host has *PYK1* mRNA abundance of 0.6% of total mRNA (Purvis *et al.*, 1987a). Conversely, transformants which grew faster (t_d = 2-3 hrs) and formed larger colonies, contained *PYK1* mRNA levels less than 1.5% of total mRNA (Fig. 4.2; Table 4.1).

During growth on minimal media lacking leucine, slow growing pLD1(35) transformants reverted rapidly to faster growth. The analysis of one transformant grown in serial batch culture for 75 generations, demonstrated that this decrease in doubling time, correlates with a decrease in plasmid copy number and *PYK1* mRNA abundance (Fig. 4.5).

This relationship between plasmid copy-number and growth rate was disturbed with the introduction of a premature stop codon immediately after the translational start codon of the plasmid-borne *PYK1* gene (plasmid pLD1(37); Fig. 4.7). Transformants with a high copy number of pLD1(37) did not grow any slower than those with a lower copy number. Indeed, during the prolonged growth of a pLD1(37) transformant, the selection for cells with increased growth rates, was accompanied by an increase in plasmid copy number (Fig. 4.8). This

was presumably caused by selection for an increased dosage of the *leu2d* allele carried on the vector, which is required in high dosage to obtain full complementation of *leu2* mutants. Therefore, the detrimental growth effects were mediated by high *PYK1* gene dosages and were dependent upon *PYK1* expression. It was noticeable that in all the pLD1(37) transformants analysed, there was no substantial increase in the level of *PYK/pyk* mRNA levels, despite the presence of up to forty-five copies of the plasmid (Table 4.2).

Analysis of pMA91+PYK transformants demonstrated that yeast can tolerate high levels of pyruvate kinase, without any detrimental effect on yeast cell growth. In this multicopy plasmid, *PYK1* expression is directed by the yeast *PGK* promoter. Hence, while the detrimental effects on growth rate imparted by high *PYK1* gene dosage require *PYK1* expression, it would appear that they are not mediated by high levels of the gene product, pyruvate kinase.

Closer analyses of a range of pLD1(35) transformants revealed that *PYK1* over-expression is limited at the level of mRNA abundance and at the level of translation.

Determination of plasmid copy number and *PYK1* mRNA abundance in fourteen pLD1(35) transformants demonstrated that as the *PYK1* copy number is increased, there is not a corresponding increase in the level of the *PYK1* mRNA. This limitation at the level of mRNA abundance is biphasic, and could thus be mediated at the level of transcription and/or at the level of mRNA stability (Fig. 5.1). The observation that the abundance of the *PGK* mRNA decreases as the level of the *PYK1* mRNA is elevated (Table 5.1), suggests that the limitation is mediated at least partly at the transcriptional level. Several *cis*-acting sequences within the 5' promoter of *PYK1* have been mapped previously (Nishizawa *et al.*, 1989). Also, in this study it has been confirmed that a *cis*-acting element within the *PYK1* coding region is required for the maximal expression of a *PYK1* reporter construct, integrated at the *ura3* locus (Fig 3.9). Perhaps the

saturation of *trans*-acting factors which bind these *cis*-acting sequences occurs as the level of the *PYK1* gene is increased.

The evidence for translational control of *PYK1* over-expression is two-fold. Firstly, when the abundance of the *PYK1* mRNA is elevated above 1.1% of total mRNA, there is not an analogous increase in the activity of pyruvate kinase (Table 5.2). Secondly, polysome analyses of several pLD1(35) transformants showed that as the level of *PYK1* mRNA is increased in pLD1(35) transformants, there is a decrease in the ribosomal loading of that mRNA (Fig. 5.5). This was shown not to be caused by the plasmid-borne *PYK1* mRNA having an aberrant 5' end (Fig. 6.1).

This decrease in ribosome loading on the *PYK1* mRNA could be imparted either by a decrease in translational initiation, or by an increase in the rate of translational elongation. Since the 5' untranslated leader is needed for regulation (Fig. 6.2) and the activity of pyruvate kinase is lower than expected (Table 5.2) it is the rate of translational initiation on the *PYK1* mRNA which is affected. It has not been determined whether sequences other than the 5' leader are required for regulation. It is feasible that sequences in the coding region or in the 3' untranslated region are required in concert with the 5' untranslated leader.

Translational regulation of *PYK1* over-expression, could be controlled in a number of ways. Feedback inhibition by excess pyruvate kinase could act directly or indirectly upon *PYK1* mRNA translation. Alternatively, a positive acting translational factor may be required for each round of *PYK1* mRNA translation. The demonstration that the chromosomal copy of *PYK1* mRNA is unaffected in the presence of excess pyruvate kinase (in pMA91+PYK transformants; Fig. 6.4), rules out the former possibility. This suggests that the *PYK1* mRNA requires a positive translation factor, which becomes limiting when *PYK1* mRNA levels are increased above 1.1% of total mRNA (Table 5.2)

The examination of the translational efficiency of other glycolytic mRNAs in the presence of high *PYK1* mRNA levels in pLD1(35) transformants, demonstrated that while the ribosome loading on the *PFK1, TDH* and *PGK* mRNAs remains unaffected, the loading on *PFK2* is markedly lower than in the untransformed host (Fig. 6.5). Thus, the *PFK2* and *PYK1* mRNAs may share a regulatory factor for translational initiation. Further experiments are required to determine if the translation of any other mRNA is affected by high *PYK1* mRNA levels.

The fact that the translation of *PFK2* mRNA is reduced in pLD1(35) transformants may explain the poor growth rates of these transformants. If this is the case, then it would be of interest to determine if the pLD1(35) transformants grow as poorly on a non-fermentative carbon source where expression of *PYK1* and *PFK2* is lower (Fig 3.4).

Since pyruvate kinase and phosphofructokinase are the catalyse irreversible steps in glycolysis, it is of particular interest that the *PYK1* and *PFK2* mRNAs are translationally co-regulated. Unlike the other glycolytic enzymes, pyruvate kinase and phosphofructokinase are used exclusively during glycolysis and are not required for gluconeogenesis (Fraenkel, 1982). Thus, there is no obvious requirement for these enzymes when cells are grown on non-fermentable carbon sources.

However, both genes are expressed, at least at the mRNA level, on the non-fermentable carbon source lactate (Chapter 3). This became apparent after the abundance of all the glycolytic mRNAs was compared during growth on lactate or glucose (Fig. 3.4). In this experiment, the abundance of each mRNA was measured using the same RNA preparations and was determined relative to the level of the actin mRNA. Interestingly, unlike certain glycolytic mRNAs (eg. *TDH, TPI, HXK*), the abundance of the *PFK2* and *PYK1* mRNA increased when cells were grown on glucose rather than on lactate. Indeed, when the glycolytic mRNAs are ordered with respect to their position in the glycolytic pathway, two peaks of induction on glucose occur at *PFK2* and *PYK1* (2.3 and 3.9-fold respectively). This relatively high level of

induction at the mRNA level may be important with regard to the postulated roles in controlling glycolytic flux of phosphofructokinase and pyruvate kinase (Muirhead, 1983).

Recent experiments, not presented in this thesis, have demonstrated that this increase in mRNA level, is not caused by a change in the rate of mRNA degradation (Moore *et al.*, 1991b). Thus, the fluctuations in the mRNA levels would appear to be mediated at the transcriptional level. In the case of *PYK1*, this increased rate of transcription does not result in the use of an alternative transcriptional start point (Fig 3.5).

Further experiments carried out on the regulation of *PYK1*, suggested that there is also a translational activation of *PYK1* expression in response to glucose. This was demonstrated by analysing the expression of *PYK1:lacZ* constructs integrated at the *ura3* locus of DBY746, during growth on lactate or glucose. While there was about a three-fold increase in the level of the *PYK1:lacZ* mRNA on glucose, there was nearly a nine-fold increase in ß-galactosidase activity (Table 3.5).

This increase in the translation of *PYK1:lacZ* mRNA may be the result of a general increase in the rate of protein synthesis when yeast are grown on glucose (Maicas *et al.*, 1990). However, it has been reported that certain mitochondrial mRNAs are translationally activated when yeast are shifted from growth on glucose to growth on non-fermentable carbon sources (Fox *et al.*, 1990). Conversely, a sub-set of glycolytic mRNAs might be translationally activated in response to glucose.

If the translation of *PYK1* mRNA is specifically activated on a fermentable carbon source such as glucose, it could be mediated by a translational repressor which binds the mRNA during growth on a non-fermentative carbon source but does not bind during growth on glucose.

Alternatively, a positive factor might promote *PYK1* translation during growth on a fermentable carbon source. If such a positive factor existed in limiting concentrations, it would dictate the level of the *PYK1* mRNA translation and as such could control the level of pyruvate kinase synthesised in response to changing environmental conditions. This would be advantageous to the yeast cell by preventing the synthesis of redundant pyruvate kinase when yeast cells are grown on a gluconeogenic carbon source. Also, when glucose became available, the yeast could activate translation of the *PYK1* mRNA and thus be able to metabolise more efficiently the glucose substrate through the glycolytic pathway. This would explain the earlier observation, that when yeast is switched from a gluconeogenic growth medium to a glycolytic medium, pyruvate kinase is the first glycolytic enzyme to demonstrate increased activity (Maitra and Lobo, 1971).

The existence of such a discriminatory translational factor necessary for *PYK1* mRNA translation gains credence when one considers that the translation of *PYK1* is limited when abnormally high levels of the mRNA are present in yeast (Fig. 5.5). As mentioned previously, under these conditions the translation of the *PFK2* mRNA is also affected (Fig. 6.5) and suggests that these two mRNAs are translationally co-regulated. Bearing this in mind, it would be informative to determine if the translation of the *PFK2* mRNA is activated in a manner analogous to *PYK1* mRNA when yeast is grown on glucose rather than lactate. The expression of both these glycolytic-specific genes might be specifically induced at the transcriptional and translational levels in response to glucose. This awaits confirmation by comparing the polysomal distribution of the *PYK1* and *PFK2* mRNAs during growth on fermentable and non-fermentable carbon sources.

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