Messenger RNA Translation in *Saccharomyces cerevisiae*

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by

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Dedicated to mum and dad

for their continued love and support

CONTENTS

ABBREVIATIONS

Chemicals

ATP - adenosine 5'-triphosphate

BSA - bovine serum albumin

dATP - deoxyadenosine 5'-triphosphate

dCTP - deoxycytidine 5'-triphosphate

dGTP - deoxyguanosine 5'-triphosphate

dTTP - deoxythymidine 5'-triphosphate

NTP - nucleoside triphosphate

UTP - uracil 5'-triphosphate

DNA - deoxyribonucleic acid

cDNA - complementary DNA

RNA - ribonucleic acid

dsRNA - double-stranded RNA

mRNA - messenger RNA

mRNP - messenger ribonucleoprotein

DPC - diethylpyrocarbonate

DTT - dithiothreitol

EDTA - ethylenediaminetetraacetic acid (disodium salt)

G3PD - glyceraldehyde-3-phosphate dehydrogenase

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (sodium salt)

MOPS - morphpropanesulfonic acid

PYK - pyruvate kinase

RP1 - ribosomal protein1 (L3)

SDS - sodium dodecyl sulphate

SSC - standard saline citrate

TBE - tris-borate, EDTA

TCA - trichloroacetic acid

TE - tris, EDTA

TK - thymidine kinase

Tris - tris (hydroxymethyl) amino ethane

Measurements

V - volts

kDa - kilo Dalton

bp - base pair

kb - kilo base pair(s)

kcal - kilo calorie

^{o}C - degrees centigrade

x g - gravitational force equal to gravitational acceleration

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)

M - molar (moles per litre)

mM - millimolar (10^{-3}M)

Mol.Wt. - molecular weight

Ci - curie

μCi - microcurie (10^{-6}Ci)

$^{w}/_{v}$ - weight by volume

$^{w}/_{w}$ - weight by weight

$^{v}/_{v}$ - volume by volume

Miscellaneous

log - logarithm

UV - ultraviolet light

Fig. - figure

cpm - counts per minute

HSV - herpes simplex virus

ORF - open reading frame

STNV - satellite tobacco necrosis virus

TCF - total counts bound per filter

ACKNOWLEDGEMENTS

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SUMMARY

The aims of this study were to investigate the effects of mRNA abundance, codon bias, and secondary structure formation upon the translation of the PYK mRNA in yeast. A method has been devised to analyse translation in vivo in the yeast S.cerevisiae. The method involves fractionation of yeast polysomes on sucrose density gradients, followed by analysis of the distribution of specific mRNAs across these polysome gradients. Experiments showed that the PYK gene of S.cerevisiae appears to be subject to dosage compensation at the translational level; the PYK mRNA carries significantly fewer ribosomes when it is present at elevated levels within the yeast cell. Experiments were also devised to analyse the effects of codon bias on the translation of the PYK mRNA. The insertion of 13 non-preferred codons into the N-terminus of the coding region of the PYK gene appeared to have no effect on the translation of this mRNA. However, the ribosome loading of a β-galactosidase coding sequence (codon bias index = -0.05%) was shown to decrease on increasing the abundance of a B-galactosidase/PYK fusion mRNA. This appears to be due to the detrimental effects of poor codon bias, since the translation of an mRNA with poor codon bias (TRP2) was also affected. Therefore, poor codon bias seems to have a significant influence upon the translation of an mRNA only under "extreme circumstances" (for example, when abnormally high levels of an mRNA with extremely poor codon bias are present). Increasing the secondary structure formation at the 5'-end of the PYK mRNA was also shown to decrease its ribosome loading in vivo. This is thought to be due to inhibition of translational initiation on this mRNA.

CHAPTER 1

BACKGROUND

1.1 Introduction

Translation is the process by which an RNA "template" is decoded and translated into an amino acid sequence. It is a very complex process involving the interaction of large numbers of proteins and RNA, and has been shown in recent years, to be very tightly regulated in a number of systems. It now seems clear that, while there are large similarities in the organisation of the eukaryotic and prokaryotic ribosome, in some respects eukaryotic ribosomes behave differently from prokaryotic ribosomes. This thesis deals almost exclusively with eukaryotic translation.

1.2 Components of the Translational system

1.2.1 Ribosomes

Eukaryotic cytoplasmic ribosomes appear to be more complex than those found in prokaryotes: an E.coli ribosome contains 50-55 proteins, whereas the ribosomes in rat-liver cytoplasm contain 70-75 proteins, and those of S.cerevisiae contain approximately 73 proteins (Otaka & Osawa, 1981). Many ribosomal proteins are dispensable in translation. However, their removal reduces the kinetic efficiency of the ribosome and this is reflected in a reduced growth rate (Ehrenberg et al, 1986).

Each eukaryotic ribosome is composed of two subunits: a large subunit (60S) and a small subunit (40S). In the yeast, S.cerevisiae, the 60S subunit contains approximately 50% RNA (1 molecule each of 28S rRNA, 5.8S rRNA and 5S rRNA)

and approximately 40 proteins. The 40S subunit contains approximately 58% RNA (1 molecule 18S rRNA) and approximately 30 different proteins. The yeast ribosome, once assembled, contains approximately 73 different proteins and sediments at 80S. Magnesium ions are known to be important for the structural and functional properties of the 80S ribosome (Bielka *et al*, 1982).

1.2.2 Translation Factors

At least twelve eukaryotic initiation factors (eIFs) are known to be involved in the initiation of translation. A further three factors (eEFs) are known to be involved in translational elongation in *S.cerevisiae*. Also, one or more release factors (eRFs) are thought to be involved in the termination of translation (Tuite & Plesset, 1986). Table 1.2.2.1 outlines the different translation factors, along with their possible roles.

1.2.3 Transfer RNAs

Transfer RNAs (tRNAs) transport amino acids to the ribosome. Eukaryotic tRNAs range from 76-80 bases in length, and fold to form highly ordered secondary structures (reviewed in Sprinz *et al*, 1985). tRNAs from all organisms contain modified nucleosides (reviewed in Bjork *et al*, 1987); most are not thought to be essential for viability, but are thought to play an important role in the "fine-tuning" of tRNA activity. Not only does the presence of a modified nucleoside improve the efficiency of a tRNA in the decoding event, but it may also influence the fidelity of

FACTOR	SUBUNITS	MOL. WT. (kDa)	NO. POLY-PEPTIDES	NOTES
eIF-1		15	1	stabilises various initiation complexes, possibly including preinitiation complexes
eIF-2	alpha	35-38	1	phosphorylated by kinase activated by haem deficiency, dsRNA or interferon
	beta	48-52	1	phosphorylated by casein kinase II
	gamma	52-56	1	binds met-$tRNA_f$ and mRNA
				involved in ternary complex formation. Binds met-$tRNA_f$ and 40S ribosomal subunits
eIF-2A		-[a]		catalyses: binding met-$tRNA_f$ to ribosomes; removal deacylated tRNA on chain termination from 40S ribosomal subunit
eIF-2B (GEF)		80-85,65-67		
		52-58,37-45	5	catalyses GTP/GDP exchange on eIF-2.GDP
		21-40		
eIF-3		500-700		[40S.eIF-3.eIF-4C] complex stimulates met-$tRNA_f$ binding to 40S ribosomal subunit. Possibly involved in ribosome dissociation preventing 40S/60S reassociation
		36,40,44,47	7-10	
		66,115,150		

FACTOR	SUBUNITS	MOL. WT. (kDa)	NO. POLY-PEPTIDES	NOTES
eIF-4A		44-55	1	stimulate binding of mRNA to 40S ribosomal subunit and cross-link
eIF-4B		80	1	specifically to 5'-cap in presence of ATP
eIF-4C		17.5-22.5	1	accessory to eIF-3 in ribosome dissociation
eIF-4D		14-19	1	stabilises intermediate initiation complexes; responsible for correct alignment of met-$tRNA_f$ on ribosome; stimulates activity eIF-5 and hence 80S initiation complex formation; may be involved in reactions which form first peptide bond
eIF-4E	CBP I	24	1	recognition of 5'-cap by ribosome; may be involved, directly or indirectly, in reactions other than cap recognition [b]
(CBP II)	eIF-4A	50	1	destabilising secondary structure of capped mRNA complexes;
	x	200-220		exhibits activities of both free CBP I and eIF-4A
eIF-5		(100)-160	1	catalyses hydrolysis 40S bound GTP; release eIF-2, eIF-3 from 40S complex leaving [40S.met-$tRNA_f$.mRNA] complex free to react with 60S ribosomal subunit
eIF-6		23-25	1	binds 60S ribosomal subunit; prevents ribosomal subunit reassociation
CBP I		24	1	cross-links specifically to 5'-cap, no requirement for ATP

FACTOR	SUBUNITS	MOL. WT. (kDa)	NO. POLY-PEPTIDES	NOTES
eEF-1	alpha	50-60	1	major component [eEF-1 .GTP.aa-tRNA] complex; brings aa-tRNA to the ribosome
	beta	30	1	commonly found with alpha, important role in eEF-1 catalysed reactions; catalyses GTP/GDP exchange on [eEF-1 .GDP] complexes
	gamma	55	1	found complexed with beta; not thought important for activity (?)
eEF-2		100-110	1	translocation peptidyl-tRNA from A site to P site of ribosome
eEF-3[c]		125	1	definite requirement in every cycle of peptide chain elongation; ribosome-dependent GTPase, ATPase activities; not involved in aa-tRNA binding to ribosome, peptide bond formation or translocation
eRF	2	2 x 55	2	release factor involved in chain termination/ribosome dissociation. Requires presence of GTP

Table 1.2.2.1 Eukaryotic translation factors and their possible roles in protein synthesis (from Moldave, 1985; Qin et al, 1987)

[a] conflicting sizes ascribed; [b] Altmann et al, 1985; [c] only required in yeast

translation. Furthermore, some modified nucleosides may also be involved in maintaining the reading frame (reviewed in Bjork et al, 1987). "Charging" of tRNAs is based on the aminoacylation reaction in which specific amino acids are covalently attached via the action of aminoacyl-tRNA synthetases to tRNAs that have triplet anticodons cognate to the attached amino acids. Since the genetic code is degenerate, some tRNAs with different anticodons will become "charged" with the same amino acids. These are termed isoacceptor tRNAs. The tRNAs form a "pool" in the cytoplasm where they become "charged" by binding an amino acid. Upon binding a tRNA at the ribosomal A site, and following transfer of its amino acid to the peptidyl-tRNA, the "discharged" tRNA leaves the ribosome and re-enters the uncharged tRNA "pool" to become "charged" once more.

1.2.4 Messenger RNAs

The vast majority of yeast messenger RNAs (mRNAs) have a number of features in common:

(1) 5'-cap: The 5'-phosphate moiety of 7-methylGMP is covalently linked to the 5'-phosphate of the first nucleotide of the mRNA to form a 5'-5' phosphodiester bond. Two types of cap structure have been detected in yeast, m^7GpppG and m^7GpppA (Sripati et al, 1976; De Kloet & Andrean, 1976). The situation is more complex in mammalian systems, with other nucleotides being modified with methyl groups (Adams & Cory, 1975). Cap structures are added post-transcriptionally to the 5'-end of mRNA precursors by the

nuclear enzyme mRNA guanylyltransferase (Itoh et al, 1987). Translation of yeast mRNAs seems to be dependent to a great extent on the 5'-cap, since translation in cell-free extracts is inhibited by the addition of cap analogues (Gasior et al, 1979; Tuite et al, 1980).

(2) 5'-untranslated region (5'-UTR): The region between the 5'-cap and the AUG initiator codon is usually termed the 5'-UTR. However, it might be more accurate to refer to this region as the 5'-leader since, in a small proportion of mRNAs, short open reading frames (upstream ORFs) have been discovered 5' to the AUG initiation codon. 5'-UTRs vary in length, but most (94%) are less than 150 bases long (Kozak, 1983).

(3) coding region: In many mRNA coding sequences, a bias is shown in the choice of which of several degenerate triplets are selected to code for a particular amino acid. In highly expressed genes of S.cerevisiae, 25 out of the possible 61 coding triplets are used almost exclusively and these triplets correlate with the most abundant isoacceptor tRNA species (Bennetzen & Hall, 1982). Also, in S.cerevisiae a strong bias exists in the first codon after the AUG initiation codon; A/U/GCU (Ser,Thr,Ala) are used much more frequently than any other triplet (Hamilton et al, 1987). This may well relate to the requirement for a particular sequence environment around the initiation codon as discussed in section 1.3.2.

(4) 3'-UTR and poly(A) tail: The 3'-end of most

eukaryotic mRNAs is polyadenylated. In yeast, a homoadenine sequence, with an average chain length of 50 bases (Mclaughlin et al, 1973) is added approximately 20 bases 3' to the proposed polyadenylation consensus sequence, AAUAAA (Bennetzen & Hall, 1982). In the majority of eukaryotes, histone mRNAs are not polyadenylated (Adesnik & Darnell, 1972). However, all yeast mRNAs so far studied have a poly(A) tail, including histone mRNAs (Fahrner et al, 1980). Also, many aberrant mRNAs are polyadenylated in yeast (Zaret & Sherman, 1982). This has led to the proposal that transcription termination and polyadenylation may be linked in this organism (Zaret & Sherman, 1982). Until very recently the 3'-UTR and the poly(A) tail were only considered important in terms of mRNA stability, but recent studies have suggested that these sequences may also influence translation (Zaret & Sherman, 1984; Purvis et al, 1987b; Kruys et al, 1987).

1.3 The Process of Eukaryotic Translation

1.3.1 Introduction

For the purposes of simplicity, translation can be broadly divided into three major component processes:

(1) Initiation, in which the [80S ribosome.mRNA.Met-tRNA$_f$] complex is formed (Figs. 1.3.1.1 & 1.3.1.2).

(2) Elongation, during which ribosomes migrate along the mRNA synthesising the encoded polypeptide (Fig. 1.3.1.2). The number of elongating ribosomes found on an mRNA at any given time varies, these structures are termed

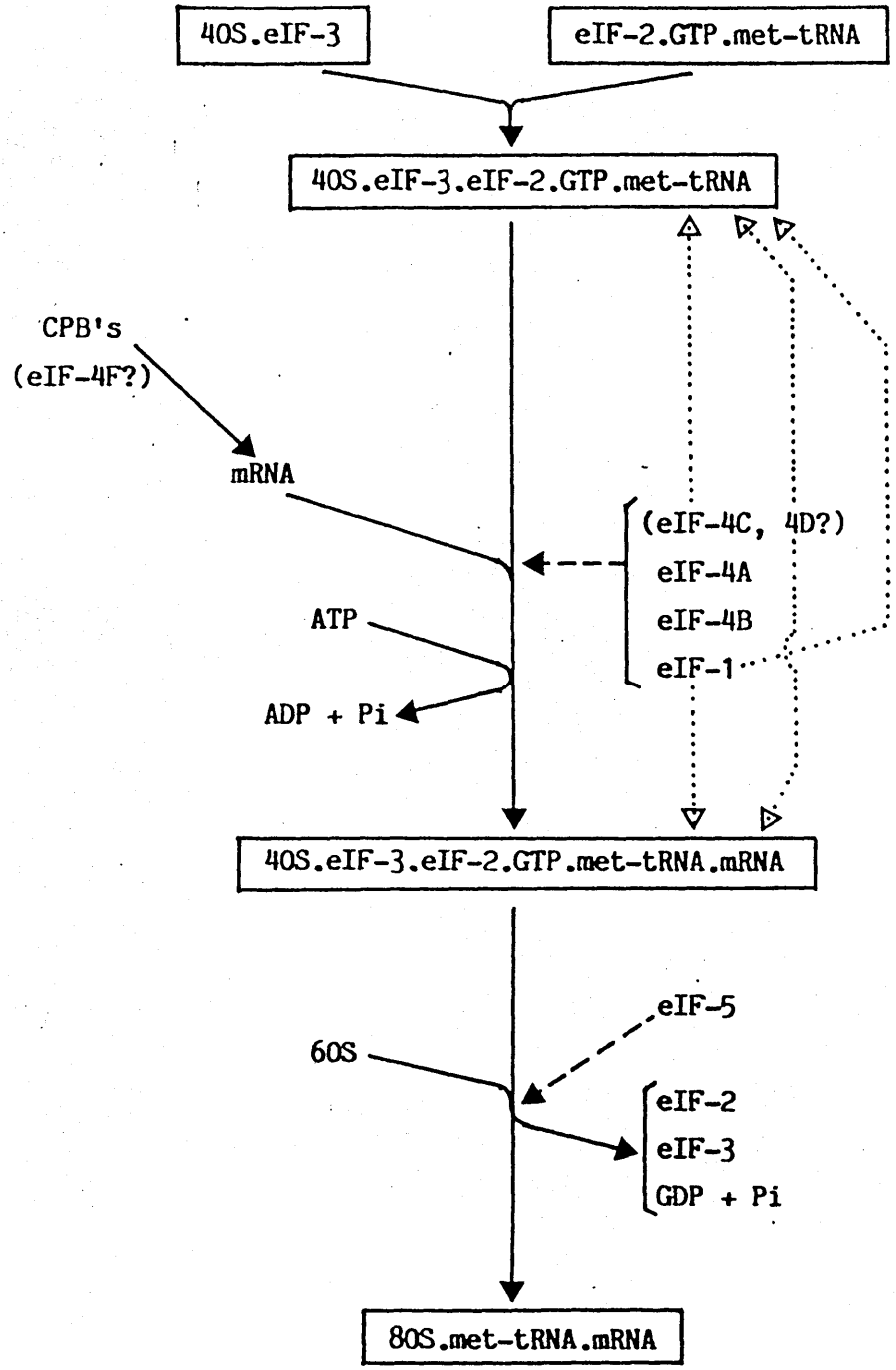


Figure 1.3.1.1 Involvement of Factors and Co-Factors in Eukaryotic Translation Initiation

The boxes show major intermediates formed during the initiation process. Reactions (⟶); catalysing effect (- - -▸); stabilising effect (·····▸).

polysomes or polyribosomes .

(3) Termination, in which the newly synthesised polypeptide is released from the ribosome, and the ribosomal subunits dissociate from the mRNA (Fig. 1.3.1.2).

1.3.2 The Initiation of Translation

It has been suggested that the mechanism of initiation of translation in eukaryotes is similar to that in prokaryotes, i.e. the ribosomal subunits bind 5' to the functional AUG initator codon at a "ribosome binding site" (Hagenbuchle *et al*, 1978; Lomedico & McAndrew, 1982; Sargan *et al*, 1982). However, this model currently has few advocates. The reasons for this are as follows. Firstly, this model does not explain the absolute requirement for a free 5'-end for initiation; eukaryotic translation will not initiate on circular mRNAs (Kozak, 1979; Konarska *et al*, 1981). Secondly, inspection of the 5'-proximal sequences of eukaryotic mRNAs reveals striking variability, not only in the position of the AUG codon, but also in the nucleotide sequences of the flanking regions (Kozak, 1978). This point has been well illustrated by the work of Sherman and coworkers (1980). They demonstrated that translation of the *CYC1* mRNA in *S.cerevisiae* could initiate with normal efficiency at any site within a region spanning 25 nucleotides around the normal AUG initiation codon. Their results suggested that there was no absolute requirement for a particular sequence 5' to the initiation codon (Sherman *et al*, 1980). These experiments were later extended, but their

conclusion remained unaltered (Baim et al, 1985a). While some eukaryotic mRNAs do show an impressive degree of complementarity with a purine rich segment near the 3'-terminus of 18S rRNA (Hagenbuchle et al, 1978), there is no consistency in the position of the pyrimidine rich sequence within mRNA molecules (Kozak, 1978). Thus, an acceptable model for initiation by eukaryotic ribosomes must be compatible with wide variations in both the location of the AUG codon relative to the 5'-cap and in the nucleotide sequence of the 5'-UTR.

It is now generally held that a "scanning mechanism", first proposed by Kozak in 1978, is a more likely model (see Fig. 1.3.2.1). In its simplest form, the model states that:

1. The 40S ribosomal subunit and associated factors bind the 5'-end of the mRNA (the presence of a 5'-cap promotes initiation from most eukaryotic mRNAs; Fig. 1.3.2.1 (A));

2. The 40S ribosomal subunit "scans" along the mRNA (Fig. 1.3.2.1 (B));

3. On reaching an AUG codon, the 40S ribosomal subunit stops "scanning", whereupon the 60S ribosomal subunit and associated factors bind to form the 80S initiation complex (Fig. 1.3.2.1 (C)).

The model as originally proposed implies that all 40S subunits should stop scanning at the first AUG initiator codon they encounter and, therefore, that the 5'-proximal AUG is used as the initiation codon. In most cases (over 90% of eukaryotic mRNAs) this is indeed the case (Kozak, 1982).

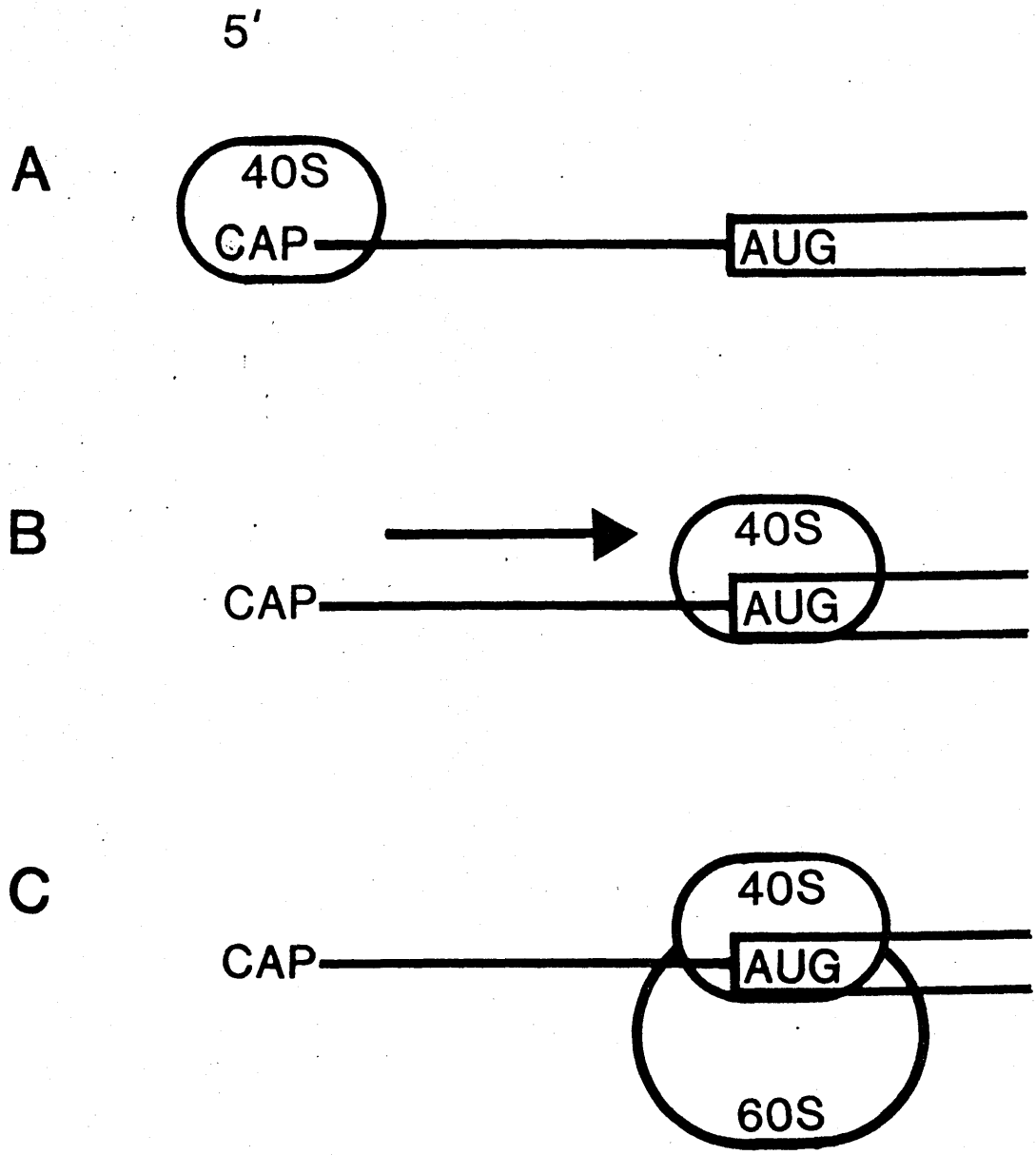


Figure 1.3.2.1 Scanning model for the initiation of eukaryotic translation

(see text for details)

Modifications to the model have been proposed to account for those mRNAs that do not initiate translation at the 5'-proximal AUG. It is suggested that when the 5'-proximal AUG is immediately adjacent to the 5' cap structure, then the scanning 40S subunit is unable to recognise it (Kozak, 1982). However, many upstream AUGs do not lie adjacent to the 5'-cap.

There is now considerable evidence to suggest that the sequence context around an AUG codon is important in defining its efficiency as a functional initiation codon (Kozak, 1981, 1982, 1983, 1984, 1986a; Liu *et al*, 1984; Morle *et al*, 1986; Szczesna-Skopura *et al*, 1986; Zitomer *et al*, 1984). Experiments using insertion mutagenesis and point mutagenesis (Kozak, 1984,1986a) have identified the following consensus sequence that is required for efficient translation initiation in mammalian cells:

$$5' \quad \overset{-5}{C}\ C\ (A/G)\ C\ C\ \overset{+1}{\underline{A}\ \underline{U}\ \underline{G}}\ \overset{+4}{G}\ G \quad 3'$$

The nucleotides at -3 and +4 appear to have the greatest influence on initiation efficiency. In cases where two or more AUG codons were found, or introduced, the AUG codon most frequently used to initiate translation was that with the optimal sequence context. A different consensus sequence has been derived for yeast from a comparison of the sequences of the 5'-UTRs of 96 mRNAs from *S. cerevisiae* (Hamilton *et al*, 1987). The optimum yeast consensus sequence around the AUG initiator codon appears to be:

$$\overset{-6}{} \qquad \overset{+1}{} \qquad \overset{+6}{}$$

$$5'\ A_{(n)}\ (A/U)\ A\ (A/C)\ A\ (A/C)\ A\ \underline{A}\ \underline{U}\ \underline{G}\ U\ C\ (U/C)$$

In highly expressed genes (e.g. glycolytic genes) an A-residue appears at positions -1 and -3 at frequencies of 89% and 100% respectively (Hamilton et al, 1987).

It is suggested that where an AUG initiation codon is present in a "strong" (i.e. optimal) sequence context, then this AUG will act as an efficient "stop-signal" for the scanning 40S subunits. However, if an AUG codon is present in a "weak" (i.e. suboptimal) sequence context, then a proportion of the scanning 40S subunits will fail to stop scanning at this AUG codon. Thus, AUG codons located upstream (i.e. 5'-) to a functional AUG initiation codon would be expected to depress initiation at the downstream AUG codon. However, the extent of this inhibition will depend on the sequence contexts of the upstream and downstream AUG codons. This inhibition can be at least partially suppressed by the presence of a translation termination codon in-frame with the upstream AUG codon(s) (Liu et al, 1984). Therefore, it appears that under certain circumstances ribosomes may be capable of reinitiating translation at another AUG codon after previously initiating, and terminating, at an upstream open reading frame (Kozak, 1984; Liu et al, 1984).

As well as stabilising mRNAs by protecting them from 5'-exonucleolytic attack (Furuichi et al, 1977), the 5'-cap

structure of mRNAs also plays an important role in influencing the efficiency of translational initiation of many mRNAs. It has been established that the 5'-cap structure increases the rate of translation initiation at the level of 40S-ribosome binding (for review see Banerjee, 1980). Several polypeptides (Cap Binding Proteins, or CBPs) have been identified which appear to interact specifically with the 5'-cap structure: 24CBP (CBP I or eIF-4E) and the CBP complex (CBP II or eIF-4F). These polypeptides have been shown to bind the 5'-cap structure and stimulate translation *in vitro* (Sonenberg *et al*, 1980; Tahara *et al*, 1981; Edery *et al*, 1984). Kozak (1980b) suggested that one function of the 5'-cap structure might be to mediate the denaturation of the 5'-proximal secondary structure of mRNAs in order to facilitate ribosome binding. The efficiency of denaturation of mRNA secondary structure has been shown to be influenced by CBPs (Lee *et al*, 1983; Altmann *et al*, 1985) and by the thermodynamic stability of the secondary structure (Lee *et al*, 1983; Pelletier & Sonenberg, 1985a; Lawson *et al*, 1986). It appears that the cap structure is recognised by CBP I, and that this recognition is not inhibited by secondary structure (Pelletier & Sonenberg, 1985b; Lawson *et al*, 1986). However, the ATP-dependent reaction catalysed by eIF-4F is inhibited by secondary structure and it has been suggested that it is this second reaction which is involved in destabilising mRNA secondary structure (Lawson *et al*, 1986). Monoclonal antibodies directed against CBPs inhibit the translation of folded,

capped eukaryotic mRNAs to a far greater extent than naturally uncapped mRNAs or native capped mRNAs which do not possess extensive secondary structure (Sonenberg et al, 1981). Also, Alfalfa Mosaic Virus (AMV) RNA-4, a naturally capped mRNA, appears almost independent of the cap structure for translation. This appears to be because the 5'-UTR of this mRNA is principally single-stranded (Gehrke et al, 1983).

A number of other workers have also shown that capped mRNAs with decreased secondary structure in their 5'-UTRs are less dependent on the CBP complex for translation (Lee et al, 1983; Pelletier & Sonenberg, 1985a,b; Kozak, 1986b; Lawson et al, 1986). This suggests that the secondary structure destabilising activity resides in (or depends upon) the CBP complex. The CBP complex has also been shown to exhibit mRNA discriminatory activity and, in certain circumstances, is possibly a limiting factor in the initiation of translation (Ray et al, 1983; Edery et al, 1984). The sum of these results suggests that mRNAs with extensive secondary structure in their initiation regions are discriminated against by the translational machinery of the cell and hence are inefficiently translated, while the translation of mRNAs with limited secondary structure at their 5'-ends is facilitated by the formation of the CBP complex. Extensive studies with prokaryotic mRNAs have established that, in these organisms also, mRNA structure plays an important role in translational regulation (for

review see Steitz, 1979).

Baim and co-workers (1985a) have reported that sequences 5'- to the AUG initiation codon apparently inhibit translation if they contain high proportions of G residues. This finding is consistent with the low proportion of G residues present in the leader regions of wild type yeast mRNAs (Hamilton et al, 1987). It is unclear why this should be the case. It is possible that G residues are simply inhibitory to scanning. Alternatively, their presence may influence the degree of secondary structure formation in the 5'-UTR.

The process of translation is summarised diagrammatically in Fig.1.3.1.2. The initiation complex (J4 in Fig. 1.3.1.2), contains the mRNA and the 80S ribosome with Met-$tRNA_f$ bound at the ribosomal P site. This complex is ready to accept an amino-acyl tRNA at the ribosomal A site and thereby begin the process of elongation.

1.3.3 Translational Elongation

The process of elongation (Fig. 1.3.1.2) can be divided into 4 main stages (reviewed by Moldave, 1985; Tuite, 1987) and is usually explained using the two-site model first proposed by Watson (1964):

(1) charging of aminoacyl tRNAs;

(2) binding of the amino-acyl tRNA to the ribosomal A site assisted by eEF-1;

(3) peptide bond formation, during which the peptide chain is transferred from the tRNA at the P site to the tRNA

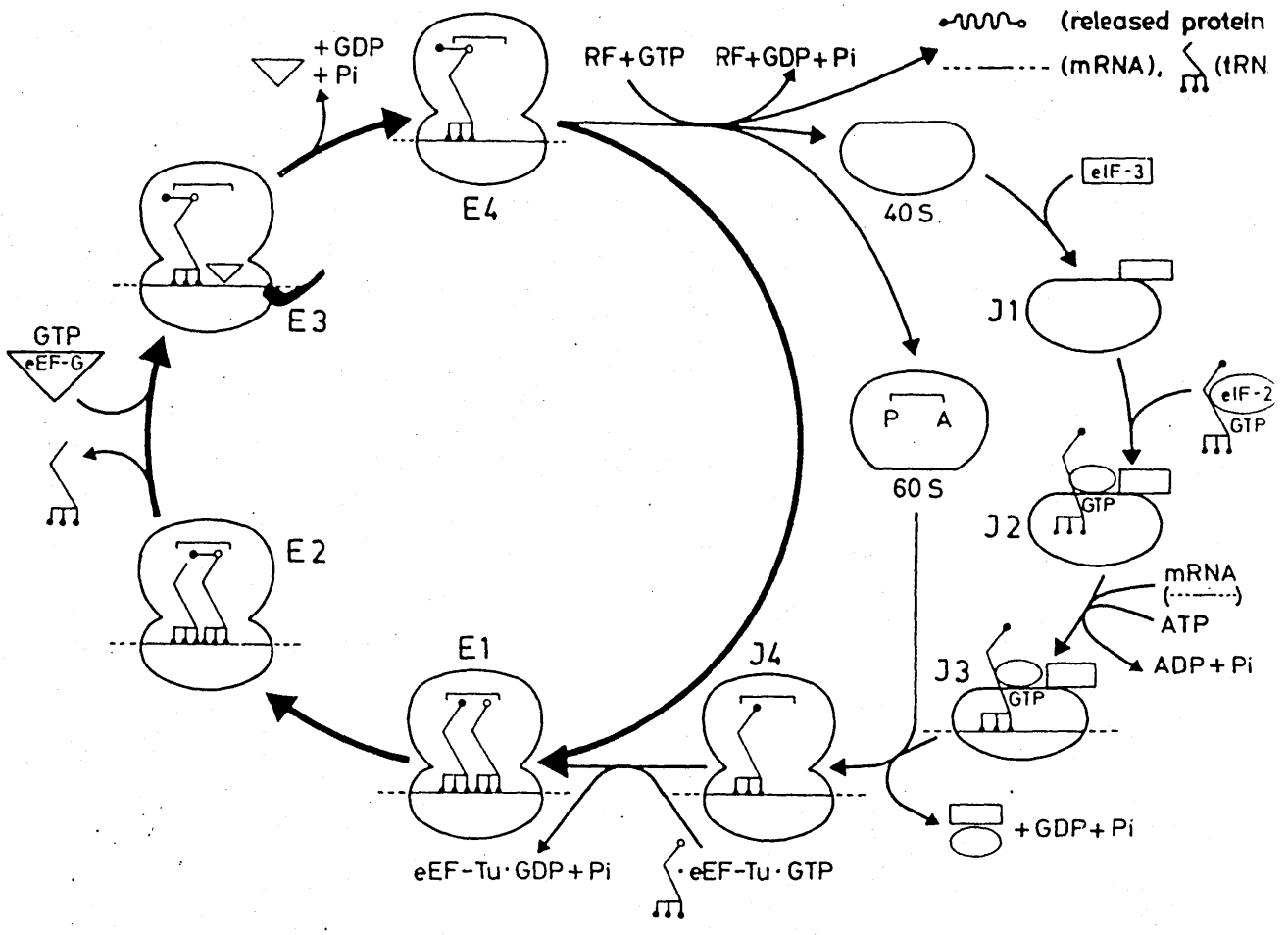


Figure 1.3.1.2 Scheme of eukaryotic protein synthesis

I1-I4: stages of initiation complex formation (cf. Fig. 1.3.1.1); E1-E4: stages of the repeating elongation cycle (thick arrows); P: binding site for peptidyl-tRNA; A: binding site for aa-tRNA; : met-tRNA$_f$ (initiator tRNA), elongator aa-tRNA, deacylated tRNA, recpectively.

at the A site;

(4) translocation of the peptidyl-tRNA from the ribosomal A site to the ribosomal P site and ejection of the uncharged tRNA from the P site.

The process of elongation has been reviewed recently (Nierhaus & Rheinberger, 1984; Moldave, 1985). The binding of an amino-acyl tRNA to the ribosomal A site is mediated by eEF-1 and GTP, which combine to form a ternary complex [eEF-1.GTP.amino acyl-tRNA]; the GTP is hydrolysed when this complex binds to the 80S ribosome. The second stage of the process is catalysed by the peptidyl tranferase activity which is thought to be part of the 60S ribosomal subunit. The reaction, which results in the formation of a new peptide bond, occurs between the nucleophilic amino group of the amino acid bound at the ribosomal A site and the esterified carboxyl of the peptidyl-tRNA terminal amino acid bound at the ribosomal P site. Translocation of the peptidyl-tRNA from the ribosomal A site to the ribosomal P site is catalysed by eEF-2, which first forms a binary complex with GTP, and then associates with the 80S ribosome at the large subunit. Hydrolysis of GTP is not required for the GTP-dependent binding of eEF-2 to ribosomes. The energy released by the hydrolysis of GTP may be used in the translocation reaction i.e. movement of the ribosome along the mRNA. Alternatively, the energy may be required for the release and recycling of eEF-2. Following translocation, eEF-2.GDP is released from the ribosome.

In *S. cerevisiae* a third elongation factor, eEF-3, is required in every cycle of the elongation process (Qin *et al*, 1987). Its exact function is still unknown, but inactivation of eEF-3 after initiation of translation has occurred results in an immediate block in translation. Immunoinactivation with a monoclonal antibody to eEF-3 showed that the factor is essential for the translation of natural mRNAs, and that eEF-3 is associated with polysomes but not with ribosomal subunits (Qin *et al* 1987). Further studies with partial reactions have shown that eEF-3 is not involved in amino acyl-tRNA binding to the ribosome, peptide bond formation, or translocation (Qin *et al*, 1987). Recent observations indicate that eEF-3 may be involved in the transphosphorylation of eEF-1.GDP to form eEF-1.GTP which is now ready for a further elongation reaction (Kamath & Chakraburtty, 1986).

The existence of more than two tRNA-binding sites on ribosomes has been proposed. Lake (1977) proposed an entry/recognition site before the A site, whilst Nierhaus and Rheinberger (1984) have proposed an exit site after the P site. It is possible that some of these sites may be functionally, but not necessarily physically, distinct from the two accepted ribosomal sites (for a review see Moldave, 1985).

There is a strong bias in the codon usage within efficiently expressed yeast genes (Ikemura, 1982; Bennetzen & Hall, 1982). Of the 61 possible coding triplets, 25 are preferred over the others (Table 1.3.3.1). For example, in

FIRST POSITION	SECOND POSITION				THIRD POSITION
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Term	Term	A
	Leu	Ser	Term	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Table 1.3.3.1 Codon bias in yeast.

The genetic code is tabulated in a standard format with the subset of preferred codons used in highly expressed yeast genes underlined (Bennetzen & Hall, 1982).

the S.cerevisiae gene encoding pyruvate kinase, 95% of the coding triplets belong to the subset of 25 preferred codons (Purvis et al,1987a). The degree of bias toward these 25 preferred codons in each gene appears to be related to the level of its mRNA in the cytoplasm: strongly expressed genes show more bias than genes exhibiting lower levels of expression (Bennetzen & Hall, 1982). Also, the subset of preferred codons correlates strongly with the relative abundances of the isoaccepting tRNAs (Ikemura, 1982; Bennetzen & Hall, 1982).

There would appear to be some general physiologic reason why those mRNAs encoding abundant proteins have highly biased codon usages, whilst those mRNAs encoding low abundance proteins have not. One possible reason is that the requirement for high protein levels exerts selective pressure for rapid and repetitive translation of the mRNAs concerned. The concentration of charged cognate tRNAs governs the step time required to add an amino acid opposite each codon (Bennetzen & Hall, 1982) and, therefore, rapid translation will be favoured by the use of codons corresponding to the abundant isoacceptor tRNAs. Consequently, mutation to codons for low abundance isoacceptors might, under certain circumstances, be deleterious. Hence, continued pressure for high rates of synthesis of a particular protein will act to select a set of preferred codons within the coding region of its gene. For those proteins not required at high levels, the rate of

translational elongation of their mRNAs is less likely to become rate-limiting, and therefore, their pattern of codon usage would be almost random. Imagine that a codon corresponding to a low abundance tRNA isoacceptor is used extensively in a high abundance mRNA. Translation of this mRNA would probably draw heavily on the small pool of this low abundance tRNA, causing a large proportion of these molecules to become uncharged. Consequently, the time taken to select this charged tRNA will increase, and the translation of all mRNAs containing this non-preferred codon would suffer a block in elongation. This would increase the risk of premature termination and/or translational error (Parker et al, 1978). Thus, the presence of a particular non-preferred codon in a highly expressed gene would have deleterious effects on a number of intracellular targets, and therefore, strong selective pressure towards the utilisation of abundant isoaccepting tRNAs would exist for abundant mRNAs. On the other hand, the use of low abundance tRNA isoacceptors by low abundance mRNAs would bring about little discharging of these isoaccepting tRNA pools. Therefore, for these mRNAs, there would be little selective pressure for single base changes to preferred codons

1.3.4 Termination of Translation

The formation of the last peptide bond followed by translocation (to move the peptidyl-tRNA and its mRNA codon to the ribosomal P site) brings a termination codon (UAA, UAG, or UGA) to the ribosomal A site. There are no known

normal tRNAs which correspond to these codons (Moldave, 1985). Yeast mutants exist which suppress translational termination using altered tRNAs capable of translating the termination codons (Gesteland et al, 1976; Sherman, 1982). A low level of natural suppression of termination has been recorded in yeast cell-free extracts (Tuite & McLaughlin, 1982). Interestingly, the UGA termination codon is only used in 20% of yeast mRNAs, UAA is used in 50% and UAG in 30% (Murayama et al, 1986). Furthermore, a second in-frame termination codon follows the first in many yeast mRNAs (Tuite, 1987).

In rabbit reticulocyte lysates a release factor (eRF) binds to the A site in the presence of GTP, and catalyses the termination reaction (reviewed in Moldave, 1985). The reaction involves the hydrolysis of GTP, and the hydrolysis of the peptidyl-tRNA ester bond to release the completed polypeptide chain, the deacylated tRNA, and usually, the ribosome from the mRNA. Hydrolysis of GTP is most probably required for dissociation of the eRF from the ribosome. One model suggests that when the P site is occupied by the peptidyl-tRNA and the A site by an eRF and GTP, this evokes the esterase activity at the peptidyltransferase centre of the large ribosomal subunit resulting in release of the free polypeptide (Moldave, 1985).

1.4 The Control of Translation

1.4.1 Introduction

There are a number of levels at which translation appears to be regulated. Control mechanisms have been demonstrated which involve sequestration of mRNA, rendering it reversibly non-translatable through the binding of extraneous protein factors. Certain structural components of an mRNA may also have a bearing on its translational efficiency. Furthermore, should translation factors be limiting, there exists the possibility that competition for these factors influences the relative efficiency of translation for different mRNAs. While some degree of control may be exerted at the levels of elongation (section 1.5) and termination (section 1.6), the major regulatory mechanisms appear to operate during initiation of translation. Indeed, it has been demonstrated that in yeast, initiation is normally the rate-limiting step in translation (Warner, 1982).

1.4.2 mRNA Binding Proteins and Messenger Ribonucleoprotein Complexes

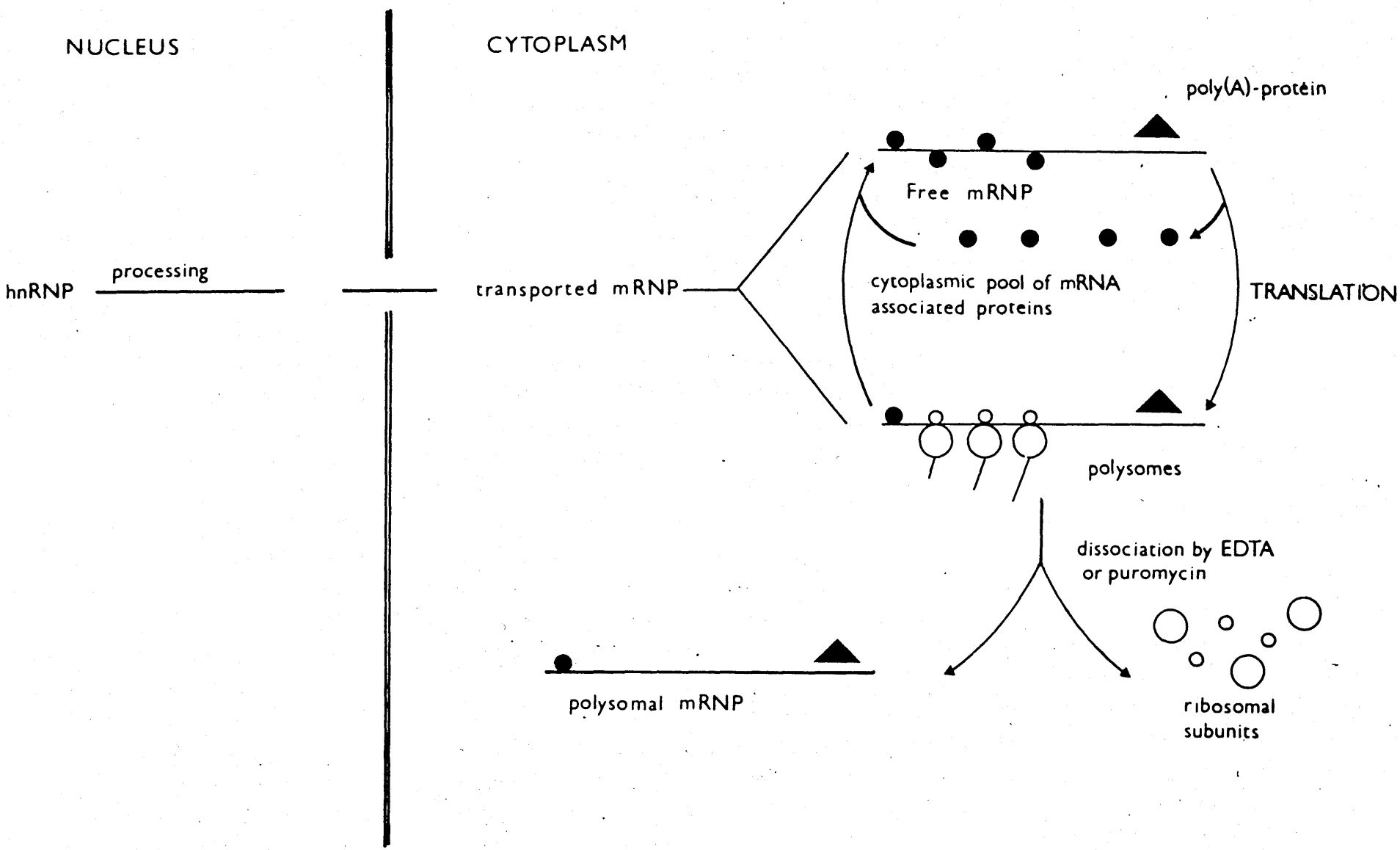
Free mRNPs are non-polysomal, cytoplasmic, protein-RNA complexes some of which are non-translatable <u>in vivo</u>. The mRNA contained within these non-translatable mRNPs is "masked" under certain developmental conditions (i.e. contained in a structure that is presumably refractive to translation) but can be selected for translation on an appropriate stimulus. mRNPs from sea urchin oocytes are not

translatable *in vivo*, but extraction with Na^+ ions was shown to render the mRNA translatable in a wheatgerm translation system (Jenkins *et al*, 1978). Therefore, some factor(s) contained within the mRNP functionally masks the mRNA. Similarly, phenol extraction of mRNP from surf-clam oocytes renders the mRNA competent for translation *in vitro* (Rosenthal *et al*, 1980). Therefore, some phenol-soluble component (probably protein) appears to block translation of the mRNA under physiologic conditions *in vivo* (Rosenthal *et al*, 1980). However, the natural unmasking of mRNAs *in vivo* need not be simply a case of stripping specific proteins from mRNPs; it may also involve the exchange of proteins attached to the mRNP with free cytoplasmic proteins (Young & Raff, 1979). This is not entirely surprising since it has been shown that polysomal-mRNP is a dynamic structure, with mRNA-associated proteins exchanging with a pool of free proteins in the cytoplasm (Greenberg, 1981). This is in contrast to the ribosomes themselves which seem to have a static association of proteins.

Quite how these discriminatory mechanisms work is unclear since not all mRNAs are found in free mRNPs. However, there appears to be some protein (or proteins) which binds in a specific manner to certain mRNAs, and thereby renders them non-translatable (Fig. 1.4.2.1; Jain *et al*, 1979). During the G2 growth phase of *Physarum polycephalum* for example, mature histone mRNA is stored in the cytoplasm as translationally inactive RNP complexes.

Figure 1.4.2.1 Model for translational control via mRNA sequestration in mRNP

(Jain *et al*, 1979)


NUCLEUS
CYTOPLASM
poly(A)-protein
Free mRNP
hnRNP
processing
transported mRNP
cytoplasmic pool of mRNA
associated proteins
TRANSLATION
polysomes
dissociation by EDTA
or puromycin
polysomal mRNP
ribosomal
subunits

Experiments performed *in vitro* have shown that the histone mRNA that accumulates in late G2 phase is translationally competent following extraction, but *in vivo* is somehow prevented from entering onto ribosomes until S phase begins (Laffler & Carrino, 1987). Interestingly, a set of RNA-binding proteins with an affinity specifically for histone mRNA has been revealed, some of which are unique to the mRNP fraction and are excluded from polysomes (Ruzdijic *et al*, 1985).

The expression in rat-liver of both heavy and light subunits of the iron storage protein, ferritin, is controlled at the level of translation (Zahringer *et al*, 1976;Aziz & Munro, 1986). In the absence of iron, ferritin mRNAs have been shown to be present as free mRNP, and to translocate to the polysome fraction within a few hours of the administration of iron (Aziz & Munro, 1986). Comparison of the sequences of the mRNAs which encode the heavy and light subunits showed a conserved sequence of 28 nucleotides in their 5'-UTRs (Aziz & Munro, 1987). Computer generated analyses suggest that the first 75 bases of the 5'-UTR of the ferritin mRNAs (which contain this highly conserved 28 base sequence) fold to form stem-loop structures (Aziz & Munro, 1987). Deletion of the first 67 nucleotides of the 5'-UTR of ferritin mRNAs causes loss of regulation by iron and an increase in the translation of these mRNAs (Aziz & Munro, 1987). Recently it has been shown that the stem-loop structure forms complexes with proteins in cytoplasmic extracts of rat tissues and cells. These complexes respond

to iron treatment in parallel with the time of translational activation of ferritin mRNAs (Leibold & Munro, 1988). Therefore, the binding of a specific cytoplasmic protein(s) to regulatory regions within the 5'-UTRs of the ferritin seems to modulate their translation in response to iron.

Developmental regulation of gene expression through this type of translational control mechanism has been reported in a number of other organisms including chick embryonic muscle cells (Jain *et al*, 1979), rabbit reticulocytes (Thomas *et al*, 1986), Surf clam *Spisula solidissima* (Rosenthal *et al*, 1980) and sea urchin oocytes (Jenkins *et al*, 1978; Young & Raff, 1979).

1.4.3 mRNA Translation Discriminatory Factors

The existence of mRNA-specific initiation factors which facilitate the translation of certain mRNAs has been proposed (Revel & Groner, 1978). On addition of partially purified eIF-3 to rabbit reticulocyte lysates, the degree of stimulation of translation was dependent on the type of tissue from which the factor was isolated (Heywood *et al*, 1974; Heywood & Kennedy, 1976). This was interpreted as indicating the existence of "discriminatory" eIFs (i.e. factors which are *specifically* required for translation of a particular mRNA sequence). However, these preparations may have differed in the amounts of contaminating factors which they contained and these contaminating factors could account for this "discrimination.

Other analogous examples of "tissue-specific"

translation have been reported. For example, although almost all mRNAs are translated to some extent in heterologous translation systems, the efficiency of translation may be very low (Ilan & Ilan, 1976). eIF-3 is composed of 7-10 different subunits and it has been suggested that these subunits might be interchangeable, or modifiable, and hence might be able to confer mRNA specificity on the initiation process (Trachsel *et al*, 1977). Although none of the recognised eIFs are dispensable, differential requirements for certain eIFs are observed for the optimal translation of specific mRNAs. The addition of eIF-4A to Krebs ascites cell-free systems stimulates the translation of encephalomyocarditis or mengo RNA to a greater extent than that of globin or oviduct mRNA (Wigle & Smith, 1973; Blair *et al*, 1977). In this same system, the addition of haem, under non-saturating concentrations of eIF-4A, stimulates the translation of globin and oviduct mRNAs to a greater extent than that of EMC or mengo RNAs (Wigle & Smith, 1973; Blair *et al*, 1977). In all cases, these experiments suffer from an inability to prove that discrimination is due to the factor described and not due to differential contamination with other factors.

The existence of mRNA discriminatory factors is no longer generally accepted. Most workers now believe that differences in translation efficiencies are due to differing abilities of mRNAs to compete for limiting translation factors. However, the subject is something of grey area

since it is sometimes difficult to determine whether an effect is due to differential affinities of a translation factor for different mRNA sequences or to the specific requirement of a factor for translation of a subset of mRNAs. This dilemma is well illustrated in the cases of the translation in the rat pancreas of two nonallelic forms of insulin (Cordell *et al*, 1982) and of the translation of the r-protein from *Dicytostelium* (Steel & Jacobsen, 1987).

In the rat pancreas, nonallelic genes encode two forms of insulin, *rat1* and *rat2* (Cordell *et al*, 1982). In a pancreatic tumour and in the normal pancreas, both genes appear to be transcribed to similar levels and equal amounts of each mature mRNA accumulate in the cytoplasm. However, *rat1* insulin accumulates in ten fold excess over *rat2* insulin in the tumour, whereas the two forms of insulin are present at similar levels in normal pancreatic tissue that has not been exposed to high glucose levels (Cordell *et al*, 1982). Therefore, some mechanism of selective translation appears to be operating in the tumour, and this may be related to the fact that in both the normal and the tumorous pancreatic tissue, *rat2*-mRNA does not appear to be capped at its 5'-end (Cordell *et al*, 1982). In normal pancreatic cells, insulin biosynthesis is controlled by glucose levels: rat islets exposed to high concentrations of glucose exhibit a 10:1 ratio of *rat1* to *rat2* insulin, whereas under conditions of low glucose, the two insulins are expressed at similar levels. The tumour cells are unresponsive to glucose and appear to be fixed in the glucose responsive

state. Sonenberg and co-workers (1980) purified and characterised a cap-specific binding protein which differentially stimulates the translation of fully-capped mRNAs as opposed to uncapped mRNAs. The activity of this CBP appears to be modulated by its association with other peptides (Tahara *et al*, 1981) and this may have a bearing on the induction of *rat1* insulin by glucose. It would appear that both capped and uncapped mRNAs can be translated, but that the efficiency of translation may be contingent, in some cases, on the activity of a CBP or possibly upon some other cap-independent mechanism. It is not possible to state categorically that this difference in insulin expression is due to the activity of a *discriminatory* translation factor, since it is conceivable that both capped and uncapped mRNAs require this CBP for translation. It seems likely that capped mRNAs have a greater affinity for the CBP described than do uncapped mRNAs. The author would favour the latter possibility.

The data from studies on the developmental expression of the ribosomal protein (r-protein) genes of *Dicytostelium discoideum* (Steel & Jacobsen, 1987) is also open to different interpretations. These genes show a drastic and specific decrease in expression during the first nine hours of development which is mediated by a block in the initiation of their translation. The relative abundance of r-protein mRNA remains essentially unchanged during the first nine hours of development and is comparable to levels

detected in growing cells (Steel & Jacobsen, 1987). The translational block is not the result of irreversible structural changes which inactivate r-protein mRNAs; these mRNAs remain translationally active when assayed in a wheatgerm cell-free extract. Also, on addition of the elongation inhibitor, cyloheximide, r-protein mRNAs are recruited onto polysomes *in vivo*. Furthermore, this change in translational efficiency is not due to cleavage of the poly(A)-tail (Steel & Jacobsen, 1987). It is possible that a protein factor (or factors) is specifically required for translational initiation of these mRNAs, and that the concentration of this factor(s) varies at particular developmental stages. Alternatively, it is possible that this exclusion of r-protein mRNAs is a consequence of a generalised decrease in the rate of translational initiation upon starvation or at the start of development, and is not due to specific changes in these mRNAs or their associated proteins. According to the model of Lodish (Lodish, 1974; Bergmann & Lodish, 1979; discussed in section 1.4.4) one would predict that if these mRNAs have inherently lower efficiencies of translational initiation, they will be disproportionately under-translated in conditions of limited translational initiation. The low degree of polysome loading of r-protein mRNAs in comparison to actin mRNA in vegetatively growing cells supports the idea that r-protein mRNAs might be relatively inefficient at initiating translation (Steel & Jacobsen, 1987). Thus, whilst this data initially appears to support a model of an mRNA

discriminatory factor, the data also supports a model of mRNA competition on the basis of different mRNA affinities for a translation factor(s).

1.4.4 mRNA Competition

There are at least seven different eIFs involved in the initiation of translation. Different eIFs appear to have different affinities for different mRNA sequences, and there appears to be competition amongst mRNA sequences for the components of translation initiation (Herson et al, 1979; Godefroy-Colburn & Thach, 1981; Walden et al, 1981; Rosen et al, 1982). Any nonspecific decrease in the rate of translation initiation at or before mRNA binding is thought to result in the preferential inhibition of translation of those mRNAs with lower rate constants for polypeptide chain initiation if:

(1) the mRNAs are competing with each other for limited initiation capacity, and

(2) the mRNAs bind components of the translational machinery at different efficiencies (Lodish, 1974).

Two basic mechanisms have been proposed for translational control mediated by competition at the level of initiation (Bergmann & Lodish, 1979):

(1) There may exist several parallel pathways for the initiation of translation in a given cell; thus there may be eIFs with differential mRNA specificities that are responsible for the initiation of translation on different classes of mRNA sequences.

(2) There may be only one pathway for the initiation of translation in a given cell, but different mRNA sequences may have differing affinities for the 40S ribosomal subunit, eIFs, or both. Thus, when the activity or concentration of these components is altered, this may lead to changes in the relative efficiencies of translation for these mRNAs.

The first mechanism, that of mRNA discriminatory factors, was discussed in the previous section (1.4.3) and, as stated, there is little reliable evidence to support it. However, there appears considerable evidence to support the possibility that individual mRNA species differ in their affinity for essential components of the translation process.

In the wheatgerm cell-free translation system, mRNAs normally translated at similar rates can differ markedly in their translation under competitive conditions. For example, Satellite Tobacco Necrosis Virus (STNV) mRNA functions without a 5'-cap (Herson et al, 1979). This mRNA has a relatively low rate of translation in vitro in comparison to other mRNAs, yet it appears to be strongly competitive when translated with other mRNAs (Herson et al, 1979). At low levels of STNV mRNA, this mRNA suppresses the translation of globin mRNA and, at higher levels of STNV mRNA, the translation of globin mRNA is completely suppressed. These differences appear to be related to the affinity of an mRNA sequence, other than the 5'-cap or the AUG translation initiation codon, for a component of the translation system.

That is, some component(s) of the translational system is rate-limiting, and the affinity of STNV mRNA for this component(s) appears to be greater than that of globin mRNA (Herson et al, 1979).

In a reconstituted cell-free system derived from rabbit reticulocytes containing excess globin mRNA, translation of beta-globin mRNA is approximately 50-fold more efficient than that of alpha-globin mRNA (Bergmann & Lodish, 1979). Addition of purified eIF-4B equalised the translation of these two mRNAs, suggesting that under normal reaction conditions, beta-globin mRNA has a significantly greater affinity for eIF-4B than has alpha-globin mRNA. In intact reticulocytes and in crude lysates, there is only a 1.4-fold difference in the amounts of alpha- and beta-globin synthesised (Bergmann & Lodish, 1979). Therefore, one would predict that eIF-4B must be present in near-saturating amounts in vivo. Should the concentration of eIF-4B decrease, then the translation of alpha-mRNA would be affected to a greater extent than the translation of beta-mRNA. Thus, a greater potential exists for the selective reduction of the translational efficiency of "poor" mRNAs by decreasing the concentration of limiting eIFs than by decreasing the concentration of activated ribosomal subunits (Bergmann & Lodish, 1979).

The translation of globin mRNA is inhibited by low concentrations of Mengovirus mRNA in rabbit reticulocyte lysates free from detectable traces of ds-RNA (Rosen et al, 1982). To a great extent this inhibition is relieved by the

addition of highly purified eIF-2, under conditions in which the overall rate of protein synthesis is not greatly stimulated. In the absence of Mengovirus mRNA, the translation of globin mRNA is not stimulated by the addition of eIF-2. Therefore, Mengovirus appears to compete efficiently with globin mRNA for eIF-2 (Rosen et al, 1982).

eIF-2 mediates the early steps in the initiation of translation. During initiation, eIF-2.GTP is converted to eIF-2.GDP, the latter being nonfunctional in initiation. To render eIF-2 functional, the GDP needs to be exchanged for GTP. Guanine nucleotide exchange factor (GEF) facilitates the displacement of GDP from eIF-2 and its replacement with GTP (reviewed in Proud, 1986). GEF fails to promote the GDP/GTP exchange when the alpha-subunit of eIF-2 is phosphorylated. Therefore, phosphorylation of eIF-2 inhibits GEF and stops the recycling activity. eIF-2 appears to be present in large excess over GEF. Therefore, an increase in the phosphorylation of eIF-2, for example from 10% to 30%, seems to be sufficient to rapidly inhibit initiation of translation. This mechanism appears to operate in rabbit reticulocytes (Proud, 1986), and HeLa cell GEF exhibits similar properties (Mariano et al, 1986), suggesting that the regulation of GEF activity by alpha-phosphorylation of eIF-2 might be a general mechanism for controlling translation at the level of initiation in mammalian cells.

In reticulocyte lysates, the viral core of vaccinia

virus inhibits the formation of [Met-tRNA$_f$.40S] initiation complexes. It appears that the viral core promotes limited alpha-phosphorylation of eIF-2, thereby inhibiting GEF activity and consequently causing a block at the level of translation initiation (Chakrabarti *et al*, 1987). Furthermore, the addition of partially purified eIF-2 and a factor from the rabbit reticulocyte cell supernatant reverses the inhibition of host translation induced by infection with vaccinia virus. Thus, phosphorylation of the alpha-subunit of eIF-2 appears to be a major mechanism of translational regulation. Interestingly, eIF-2 appears to be phosphorylated throughout the period of exponential growth in yeast (Romero & Dahlberg, 1986).

Host and viral mRNAs compete for the translational apparatus in reoviral-infected cells. Kinetic analyses suggest that the site of competition is an initiation factor which must bind to the mRNA before it can interact with the 40S ribosomal subunit (Golim *et al*, 1976). Competition experiments, using an *in vitro* translation system derived from Krebs ascites cells, between reovirus and globin mRNAs have revealed that these competitive effects could be relieved by the addition of eIF-4A or CBP II (which contains a subunit similar to eIF-4A; Ray *et al*, 1983). *In vitro*, both factors relieved competition among both capped and uncapped reovirus mRNAs according to similar hierarchies, suggesting that some feature other than the 5'-cap was being recognised by the factor (Ray *et al*, 1983).

To summarise, there exists considerable data to support

the idea that, in mammalian cells, different mRNA sequences compete with different efficiencies for specific initiation factors (most notably eIF-2, eIF-4A and eIF-4B). Therefore, the overall pattern of protein synthesis can be modulated by regulating the intracellular concentrations of these factors in their active form. A similar situation is thought to exist in yeast. However, the role of eIF-2 in the regulation of translation in this organism may be different from that in mammalian cells since yeast eIF-2 appears to be phosphorylated under condtions where translation is taking place (Romero & Dahlberg, 1986).

1.4.5 Upstream AUGs

Most eukaryotic mRNAs contain a single open reading frame (ORF), with a single functional translation initiation site that is usually the AUG closest to the 5'-end. The monocistronic character of most eukaryotic mRNAs, and occasional deviations therefrom, can be rationalised by a scanning mechanism in which both position (i.e. proximity to the 5'-end) and flanking sequences direct which AUG(s) initiate translation (section 1.3.2; Kozak, 1986a). If two AUG codons lie in different reading frames, and the first is in an unfavourable sequence context, some scanning 40S ribosomal subunits should reach the second AUG codon (Kozak, 1986a). The prediction that such mRNAs would synthesise two proteins in different reading frames has been verified for viral mRNAs, but not as yet for cellular mRNAs (De Haarr *et al*, 1985; Kozak, 1986a). This so-called "leaky scanning"

model (Kozak, 1986a) rationalises most of the known examples of dual translation initiation on animal viruses. However, this "leaky scanning" model cannot explain those situations where an AUG codon with a favourable sequence context occurs upstream of the functional initiation codon (Dixon & Hohn, 1984; Hughes et al, 1984; Kozak, 1984c; Liu et al, 1984). Such upstream AUG codons can severely depress the initiation of translation at the "authentic" start codon, the extent of the inhibition depending upon the sequences flanking the upstream AUG codon (Kozak, 1986c). This inhibition can be at least partially suppressed by the presence of a translation termination codon in-frame with the upstream AUG codon (e.g. Liu et al, 1984). The simplest explanation for such behaviour is that the ribosomes first translate the 5'-proximal ORF and then re-initiate translation at the next AUG codon downstream (Liu et al, 1984; Kozak, 1984). Agnoprotein, which is encoded by the leader region of the same mRNA that encodes SV40 late viral protein VP1, was the first functional "leader protein" detected (Jay et al, 1981; Margolskee & Nathaus, 1983; Ng et al, 1985). Most cellular mRNAs containing more than one ORF are probably not bifunctional in the sense of producing two biologically important proteins, since the leader-encoded products are generally small peptides. It is most likely that upstream minicistrons, where present in cellular mRNAs, are not present for their coding capacity, but for regulatory purposes.

This idea of a regulatory role is particularly appealing when a set of related genes all contain minicistrons as in the collagen family (Liau et al, 1984) or a family of neurotransmitter receptors (Noda et al, 1983; Kubo et al, 1986). However, the coding sequence of the human N-myc gene is near the 3'-end of its mRNA and is preceded by an ORF which could encode a basic protein of 86 amino acids (Stanton et al, 1986). Clearly, in this case the product of the upstream ORF may have a specific function. Also, the HMG coenzyme A reductase mRNA (which encodes an enzyme involved in cholesterol biosynthesis) might encode a functional leader protein since the largest transcript for this gene contains an upstream ORF of 106 codons (Reynolds et al, 1985). However, there is no absolute requirement for reinitiation in this case because the start codon for the upstream ORF has an unfavourable sequence context, and some HMG coenzyme A mRNAs are produced with shorter 5'-leaders containing no upstream ORFs.

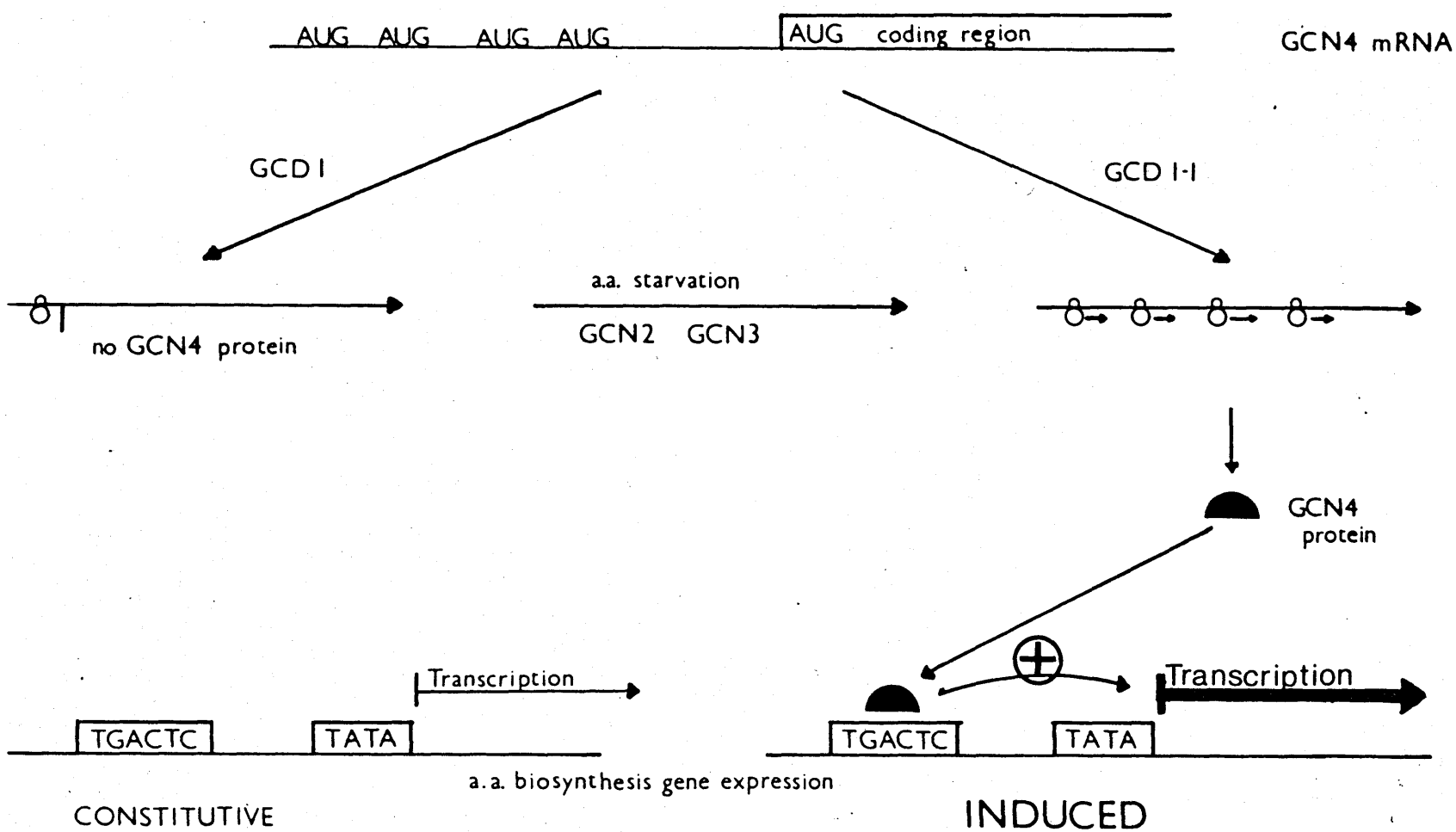
Recent results have suggested that the selection of internal AUGs by reinitiating ribosomes may be achieved via alternative mechanisms to "reinitiating ribosomes". Experiments in which a tricistronic mRNA was constructed and introduced stably into chinese hamster ovary cells, or transiently into COS monkey cells, showed a dramatic decrease in translation of the second ORF (ca. 150 fold) compared with the first ORF, whereas translation of the third ORF was only slightly reduced (ca. 3 fold; Kaufman et al, 1987). These results suggest the possible occurrence of

independent internal initiation, in contrast to reinitiation.

Perhaps the best characterised system of translational regulation involving upstream ORFs is the GCN4 gene of S.cerevisiae. The GCN4 gene encodes a positive transcriptional activator of unlinked amino acid biosynthetic genes (Hinnebusch, 1984). The level of GCN4 protein is regulated at the level of translation. The GCN4 transcript has an unusually long 5'-leader sequence of approximately 600 bases which contains four short upstream ORFs, and these are involved in the regulation of GCN4 mRNA translation in response to the availability of amino acids (Hinnebusch, 1984; Thireos et al, 1984). The 5'-leader functions in cis, along with the product of the GCD1 gene to repress the translation of GCN4 (Hope & Struhl, 1985; Tzamarias et al, 1986). However, under conditions of amino acid starvation, this translational repression is released, apparently by antagonism of the GCD1 product by the products of the GCN2 and GCN3 genes (Fig. 1.4.5.1; Hinnebusch, 1985). The situation becomes more complicated when the roles of the four upstream ORFs are examined using site-directed mutagenesis (Mueller & Hinnebusch, 1986). Each upstream AUG can decrease the translational efficiency of GCN4, but the two 3' proximal upstream AUGs are much more inhibitory than the 5' proximal AUGs. The first upstream AUG is required for efficient derepression of GCN4 expression under conditions of amino acid starvation, and this appears to be

Figure 1.4.5.1 Control of general amino acid biosynthesis: the role of *GCN4* translation

Genes under general amino acid control include *TRP1-5*; *LEU1,2,4,5*; *ARG2-8*; *HIS1-5* (Hinnebusch, 1986)


AUG AUG AUG AUG
AUG coding region
GCN4 mRNA
GCD 1
GCD 1-1
a.a. starvation
GCN2 GCN3
no GCN4 protein
GCN4
protein
Transcription
Transcription
TGACTC
TATA
TGACTC
TATA
a.a. biosynthesis gene expression
CONSTITUTIVE
INDUCED

mediated by antagonism of the inhibitory effects of the 3'-proximal upstream AUGs. The GCN2 product appears to be involved in overcoming the inhibitory effects of the 3'-proximal upstream AUGs, while the GCD1 product seems to modulate the antagonistic interaction between the first upstream AUG and the third and fourth upstream AUGs. Mutations in the upstream AUGs result in dramatic effects on GCN4 expression and so it is assumed that the upstream AUGs are recognised as efficient initiation codons. Also, each is followed by in-frame termination codons. In terms of the scanning model, the inhibitory effect of these upstream ORFs is thought to be due to inefficient reinitiation by yeast ribosomes at internal AUGs. Possible explanations of the control of GCN4 expression include:

1. under starvation conditions, translation of the 5'-proximal upstream ORFs suppresses initiation at the 3'-proximal upstream AUGs without affecting initiation at the "functional" GCN4 AUG, and

2. the 3'-proximal upstream AUGs are recognised efficiently under all conditions, but translation of the 5'-proximal upstream ORFs in some way makes initiation at the 3'-proximal upstream AUGs less inhibitory to subsequent reinitiation at the "functional" GCN4 start codon.

The second idea, that of translational coupling (i.e. the translational efficiency of the 3'-upstream ORFs is coupled to that of the 5'-upstream ORFs) has been shown to occur on certain bacterial polycistronic mRNAs (for review see Nomura et al, 1984). The role of the GCD1 product would

be to prevent these interactions under normal conditions, presumably by either suppressing initiation at the 5'-proximal upstream AUGs or by interfering with the translational coupling.

The yeast CPA1 gene encodes the glutaminase subunit of the arginine pathway carbamoyl phosphate synthetase. The expression of CPA1 is repressed by arginine at a post-transcriptional level (Messenguy et al, 1983). The 5'-leader contains a 25 codon upstream ORF, the product of which plays an essential negative role in the mechanism of repression (Werner et al, 1987). Deletion of the upstreamn ORF abolishes the repression of CPA1 expression in the presence of arginine, but does not lead to an overall increased level of expression, probably because the start codon for the upstream ORF has an unfavourable sequence context. Werner and co-workers (1987) have proposed a model for the arginine-specific repression of CPA1 translation (Fig. 1.4.5.2). They have suggested that in the presence of arginine, the product of the CPAR gene interacts with the CPA1 leader peptide on the ribosome. Formation of this complex would prevent other 40S ribosomal subunits from scanning further downstream to the start codon of the major ORF. In the absence of arginine, the CPAR gene product is thought to be unable to recognise the CPA1 leader peptide, the ribosome:leader peptide:CPAR product complex would not form, and this would allow 40S scanning to proceed to the next start codon, and hence translation of the carbamoyl-

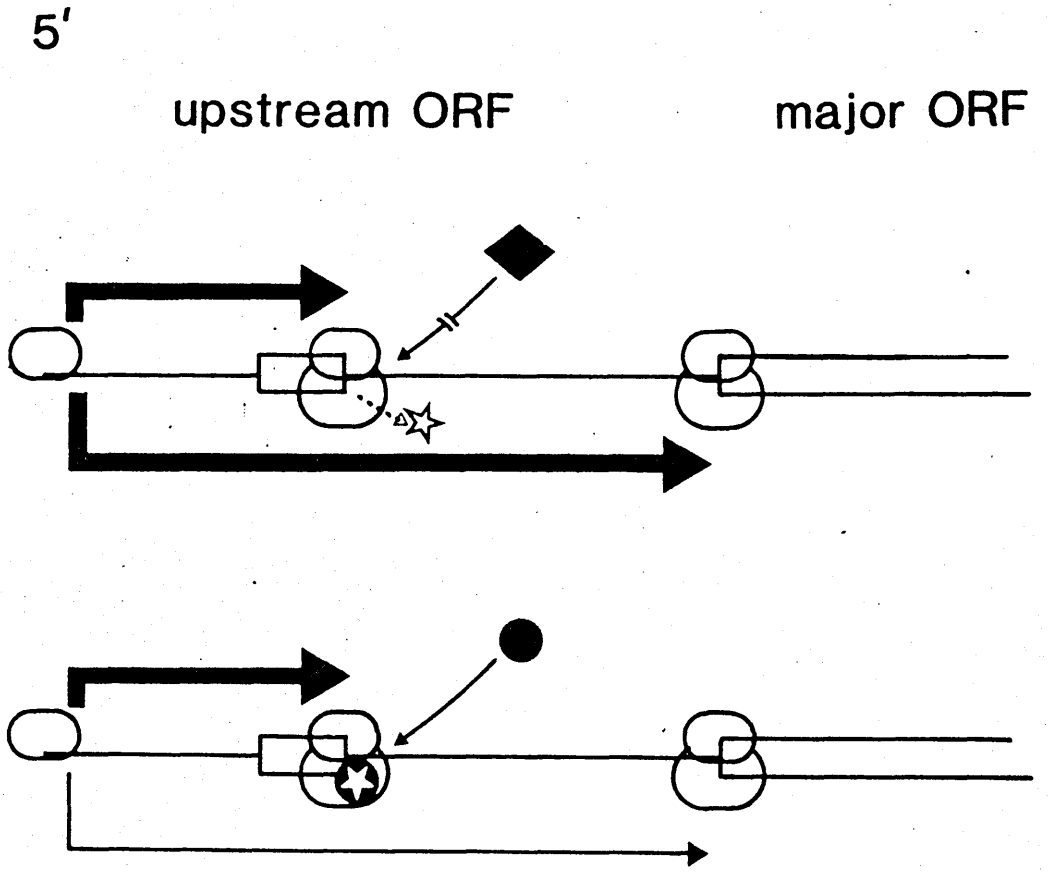


Figure 1.4.5.2 Model for translational regulation of CPA1

● CPAR product in presence of arginine; ☆ leader peptide; ◆ CPAR product in absence of arginine.

phosphate synthetase subunit would occur.

The 5'-leader sequence of the avian retrovirus (ASV) mRNA is approximately 380 nucleotides long, includes sequences required for viral replication, and contains 3 ORFs upstream of the "functional" AUG initiator codon for the *gag* and *env* genes. The analysis of various ASV mutants indicates that specific sequences in the 5'-leader (other than the previously identified consensus nucleotides which precede eukaryotic initiation codons; Kozak, 1983) can influence the efficiency of translation of this mRNA. Interestingly, the deletion of a small upstream ORF from the 5'-leader, which carries an "initiation codon" with an optimum sequence context, resulted in decreased expression from this mRNA (Katz *et al*, 1986).

Experiments have shown that polyamines negatively regulate the translation of mammalian ornithine decarboxylase mRNA (ODCase), thereby controlling their own synthesis (Kahana & Nathans, 1985). The mechanism for this has not yet been elucidated. Indeed, deletion of the leader causes a 40-fold increase in the translation of the ODCase mRNA *in vitro* (Kahana & Nathans, 1985). So far, there is no evidence linking the polyamine effect on ODCase synthesis in whole cells to the inhibitory effect of the 5'-leader on translation *in vitro*, but it is possible that the two observations are related. For example, in intact cells, the inhibitory effect of the 5'-leader may be prevented by the binding of a specific regulatory protein (perhaps ODCase itself), which is released in the presence of polyamines.

To summarise, the translation of some eukaryotic mRNAs is regulated via the presence of upstream ORFs. In contrast to mRNA competition (section 1.4.4) which provides a general mechanism for translational regulation, translational control that operates via upstream ORFs provides a mechanism which is specific for certain mRNAs.

1.4.6 mRNA Secondary Structure

The influence of mRNA secondary structure upon translation is manifested in at least three different ways: 1. Perhaps the most important is the influence of mRNA secondary structure upon the efficiency of translational initiation. Differential selection of mRNAs for translation can be exerted at the level of the entry of mRNA into initiation complexes with ribosomes. Intrinsic translational efficiencies appear to vary widely amongst mRNAs and appear significant in influencing the level of expression from particular mRNAs *in vivo* under competitive conditions (Walden *et al*, 1981). A major site of competition is thought to involve a step prior to mRNA-ribosome binding in which mRNAs must compete for a limiting component of the translational machinery (Ray *et al*, 1983). [However, competition can be viewed as involving all steps up to the completion of initiation, and depends upon which factor(s) is rate limiting.] 2. The presence of antisense RNA transcripts has been shown in a few specific cases to inhibit translation presumably through the formation of intermolecular hybrids. 3. The existence of stable

intramolecular secondary structures within the coding region of certain mRNAs have been shown to affect the rate of polypeptide chain elongation (Chaney & Morris, 1979). This section deals with the effects of long-range intramolecular, and inter-molecular secondary structure formation upon the initiation of translation. The effect of short range intramolecular mRNA secondary structure formation upon initiation has been discussed in section 1.3.2.

As mentioned previously (section 1.3.2), mRNAs with extensive secondary structure in their initiation regions appear to be discriminated against by the translational machinery and hence are inefficiently translated. The translation of zein mRNA from Zea mays in the wheatgerm cell-free translation system is inhibited, apparently by hybrid formation between the 5'- and 3'-UTRs of the mRNA. A large deletion in the 3'-UTR leads to a doubling in the translational efficiency of this mRNA (Spena et al, 1985). Long-range base-pairing interactions that bring 5'- and 3'-UTRs within close proximity have been predicted for a number of mature mammalian mRNAs including globins, insulins, growth hormone and interleukin-2 (Lockard et al, 1986; Konings et al, 1987). To date, no effects on translational efficiency have been observed for these mRNAs, except perhaps in the case of human alpha-globin mRNAs alpha1 and alpha2. When these mRNAs, which encode identical polypeptides, were translated in vitro, it was observed that alpha1-globin mRNA was translated with three times the

efficiency of alpha2-globin mRNA. It was suggested that the disparate translational efficiencies of the two alpha-globin mRNAs might be determined by the divergent sequences of their 3'-UTRs (Liebhaber & Kan, 1982). This in turn might influence the secondary structure around their translation initiation sites.

The normal RNA transcript of a gene could be rendered inactive for translation if it were to form a "duplex" with a complementary "anti-sense" RNA transcript (for review see Green et al, 1986). In prokaryotes, the replication of the plasmid ColE1 is thought to be inhibited by the formation of a duplex between a short anti-sense RNA and the 5'-end of the replication primer (Tomizawa & Itoh, 1981). The translation of Tn10 transposase is also regulated by the production of an anti-sense RNA transcript (Simons & Kleckner, 1983).

The ability of anti-sense RNA to inhibit gene expression in eukaryotes has also been demonstrated. Plasmids directing the synthesis of antisense thymidine kinase (TK) RNA substantially reduced TK expression in TK^- mouse cells carrying a plasmid containing a normally expressed TK gene (Izant & Weintraub, 1984). However, it is not known whether the inhibitory effects of eukaryotic anti-sense RNAs are mediated in the nucleus or the cytoplasm. There is weak evidence to suggest that they act within the nucleus (Brown, 1988). In contrast, anti-sense RNAs that are injected directly into the cytoplasm are thought to act as translational inhibitors (reviewed in Green et al, 1986).

For example, microinjection of anti-sense globin RNA into Xenopus cytoplasm selectively prevented globin translation (Melton, 1985). Synthesis of ribosomal protein L1 (RpL1) is selectively and efficiently inhibited by microinjection of anti-sense L1 RNA into Xenopus oocytes. RNase protection assays showed the formation of RNA:RNA duplexes in vivo and, unlike the globin and TK mRNAs, anti-sense RNAs complementary only to the 3'-terminal region of L1 mRNA repress translation as effectively as full-length anti-sense RNAs (Wormington, 1986). To my knowledge, there is only one reported example of a natural anti-sense RNA in eukaryotes and that is chicken muscle myosin translational-control RNA (tcRNA). tcRNA is an oligoribonucleotide composed of 20-30 bases which has been isolated from free mRNPs extracted from chicken muscle ((Bester et al, 1975; Heywood et al, 1975). This tcRNA complexes with myosin mRNA and it is suggested that two regions of the tcRNA are involved in rendering the myosin mRNA non-translatable (Heywood & Kennedy, 1976). An oligo(U)-sequence of the tcRNA is thought to base-pair with the poly(A)-tail of the mRNA, and another sequence in the tcRNA is thought to base-pair with part of the 5'-leader of the myosin mRNA (Heywood & Kennedy, 1976). Interaction of the myosin mRNA with the tcRNA is thought to circularise the mRNA and thereby inhibit ribosome binding (Heywood & Kennedy, 1976).

1.4.5 The Control of Elongation

The ability of tRNA isoacceptor pools to control the expression of genes has already been clearly demonstrated in the case of transcriptional attenuation in bacteria (Yanofsky, 1981). Under normal circumstances, differential rates of gene expression are not thought to be due to variable codon usage (Holm, 1986). However, it is predicted that non-preferred codons might limit gene expression at the level of translational elongation if their utilisation were to exceed normal levels (Holm, 1986; Varenne & Lazdunski, 1986). Anecdotal evidence is given by Jackson and Hunt (1982) that different ratios of isoaccepting tRNAs can alter the proportions of different proteins synthesised in a particular system. This type of control may well be due to variation in elongation rates mediated by differences in the codon usage of specific mRNAs. Should the rate of elongation be reduced by the effects of codon bias, initially this would have little effect on the overall pattern of protein synthesis. However, as the ribosome loading on the mRNAs increases, the pool of ribosomal subunits may become significantly reduced. This could lead to a reduction in the overall rate of initiation, and hence mRNAs with a higher affinity for the 40S initiation complexes would gain a translational advantage. In eukaryotes, specific pauses during translational elongation have been attributed to the interaction of the codon bias of certain mRNAs and the concentration of isoaccepting tRNAs (Lizardi *et al*, 1979; Morlon *et al*, 1983). Therefore, under

certain circumstances, regions of poor codon bias can decrease the rate of elongation along an mRNA.

When the silk fibroin mRNA from Bombyx mori was translated in a rabbit reticulocyte cell-free system, the addition of tRNA from silk glands was essential for the complete translation of the fibroin polypeptide (Lizardi et al, 1979). Furthermore, a large number of smaller sized polypeptides were observed. These were shown to be nascent fibroin chains of discrete size that accumulate due to discontinuities in translation. These discontinuities occurred at specific sites along the fibroin mRNA, and the relative duration of these "pauses" in translation could be experimentally modulated by changing the source of the supplementary tRNAs added to the in vitro system. These discontinuities or "pauses" in peptide chain elongation were also observed in vivo, at least under conditions of silk gland organ culture (Lizardi et al, 1979).

mRNA secondary structure formation may account for discontinuous translation in other systems (see section 1.4.6; Chaney & Morris, 1979) but several observations provide indirect evidence against a major role for mRNA secondary structure in the discontinuous translation of silk fibroin mRNA. Firstly, increased concentrations of potassium ions (which presumably stabilise mRNA secondary structures) lead to a decrease in the relative pause times in vitro. Secondly, different tRNA supplements altered the relative amounts of nascent chain accumulation at adjacent

elongation pause loci, suggesting that the primary influence is exerted by codon utilisation. Thirdly, the relative accumulation of nascent chains at adjacent loci was often different in the reticulocyte system as opposed to cultured gland cells. It seems unlikely that the secondary structure formed by the mRNA would be significantly different in either case. This evidence led to the proposal that these discontinuities in translational elongation reflect different times spent by the ribosome in recognition-binding reactions at each codon (Lizardi *etal*, 1979). While discontinuous translation occurs in intact silk gland cells, it is not known whether the rate-limiting step for fibroin translation *in vivo* is at the initiation or elongation phase of translation. Early in the fifth instar, the larval tRNA population is sub-optimal for fibroin mRNA translation, and therefore elongation may be limiting for this mRNA. Later in the fifth instar, fibroin mRNA is synthesised very rapidly whilst ribosome synthesis slows down, and the rate-limiting step is likely to be at the level of translational initiation.

Hoekema and co-workers (1987) have attempted to analyse the effects of codon bias on translation. Codon replacement experiments were carried out on the *PGK1* gene of *S.cerevisiae* to increase the number of non-preferred codons present in the *PGK1* coding region. They suggested that increasing the number of rare codons in the *PGK1* mRNA led to a reduction in its rate of translation (Hoekema *et al*, 1987). However, their experiments were based on assumptions

which may be invalid. Most of the decrease in protein synthesis could be directly attributed to a decrease in the steady-state level of the PGK1 mRNA. Hoekema and co-workers (1987) argue that since the rate of transcription is unlikely to be altered by changes in the coding sequence, then the observed decrease in PGK1 mRNA levels is due to disruption of normal PGK translation and hence indirectly to decreased PGK1 mRNA stability. However, it is much more likely that this decrease in PGK mRNA levels was due to the disruption of a downstream transcriptional activation sequence thought to be located within the coding region of PGK1 (Kingsman et al, 1985; Mellor et al, 1987). Thus, most of the decrease in protein levels was probably due to a decrease in PGK1 mRNA levels, and since no attempt was made to predict the mRNA secondary structure formation of the various mutants, the author believes that their data should be treated with extreme caution.

The distribution of non-preferred codons appears to be non-random in certain yeast genes (e.g. ARO1, TRP3; Purvis et al, 1987a). This has suggested a link between translation and protein folding which has not previously been considered. We have proposed that the way in which some proteins fold in vivo is affected by the presence of translational pauses, caused by local regions of poor codon bias (Purvis et al, 1987a). We suggested that some gene sequences may have evolved to control the rate of translational elongation such that the synthesis of defined

portions of their polypeptide chains is separated temporally. There is currently no direct evidence to support or disprove this hypothesis. However, experiments are currently being conducted in this laboratory to test this protein folding hypothesis.

The rate of translation elongation can also be influenced by the presence of secondary structure within the coding region of an mRNA. Baim and co-workers (1985b) have shown that a mutation allowing the formation of a stable hairpin-loop structure within the coding region of the CYC1 mRNA diminished the translation of this mRNA in S.cerevisiae to approximately 20% of wild type levels by causing inhibition of elongation. Studies on the accumulation of nascent peptide chains of the bacteriophage MS2 coat protein in MS2-infected E.coli have shown that the rate of translational elongation on this mRNA is not uniform (Chaney & Morris, 1979). The length of the nascent peptide chains which accumulate during coat protein synthesis has been correlated with the proposed secondary structure of the coat protein mRNA (Min Jou et al, 1972). The accumulation of nascent peptides occurs at, or near, regions of the mRNA where translating ribosomes would enter regions of RNA secondary structure. This led to the hypothesis that mRNA secondary structure can impede ribosome movement along the mRNA, thereby giving rise to the non-uniform size distribution of nascent peptides on polysomes (Chaney & Morris, 1979).

1.6 Regulation of Translational Termination

A bias is exhibited in the choice of termination codons employed in yeast mRNAs. The majority (56%) have UAA, 20% have UAG and 24% have UGA as their terminator (Tuite, 1987). As yet, there appears to be no evidence for any kind of consensus sequence around the terminator codon (Tuite, 1987). The majority of yeast terminators are followed shortly afterwards by a second in-frame termination codon, presumably to reduce the frequency of deleterious translational readthrough caused by the low-level of nonsense suppressors present in yeast (Tuite, 1987). As far as the author is aware, there is no evidence of translational control being exerted by the choice of termination codon.

1.7 The Phenomenon of Dosage Compensation

Dosage compensation is perhaps most familiar in terms of sex determination in Drosophila melanogaster where it has been observed that X-linked genes are expressed at similar levels in males (with one X-chromosome) and females (with two X-chromosomes) (for review see Baker & Belote, 1983). This mechanism acts at the level of transcription of individual genes. In the female there is a basal level of transcription from the X-chromosome equivalent to the transcriptional activity from the autosomes. In the male, male-specific lethal loci (msl) are expressed, and act to increase the transcription of X-linked genes. The exact

mechanism of this dosage compensation is not yet known, but it is believed that all X-loci compete for a limiting autosomally-encoded factor necessary for the transcription of these loci (Maroni & Lucchesi, 1980). This factor has not yet been identified.

In yeast, dosage compensation has also been observed to act upon some autosomally encoded genes (particularly ribosomal and histone genes) at the levels of RNA processing, mRNA stability, mRNA translation and protein stability, as well as at the transcriptional level (Warner et al, 1985; Lycan et al, 1987). This section will be limited to a discussion of dosage compensation acting at the translational level.

When a two-micron based plasmid containing the gene which encodes RP1 (ribosomal protein L3) was introduced into S.cerevisiae at a level of 5-10 copies per cell, transcription of the RP1 mRNA was increased 7.5 fold in comparison to the untransformed control. The transformed cells maintained a 3.5 fold increased level of mature RP1 mRNA, but the cells synthesised no more than 1.2 times the level of RP1 protein than did the control. The synthesis of at least 35 other ribosomal proteins in the transformed yeast cells was unaffected (Pearson et al, 1982). When present in excess, RP1 mRNA was seen to move from larger polysomes to smaller polysomes. Since the mean number of ribosomes per mRNA molecule was decreased, translational control of the synthesis of RP1 protein is most likely to occur by the transient inhibition of translation initiation,

although additional effects on translational elongation could not be ruled out (Warner et al, 1985). Therefore, a translational dosage compensation mechanism was operating upon the RP1 mRNA which caused RP1 mRNA to be translated with reduced efficiency when present in excess.

Analogous experiments have been performed on the S.cerevisiae CYH2 gene (Warner et al, 1985). When transcription of the CYH2 locus was increased 30 fold, there was a barely detectable increase in the levels of the CYH2-encoded protein. These results suggest that the initiation of translation of the CYH2 is somehow modulated, although additional effects on elongation cannot be ruled out. Again, no secondary effects on the synthesis of any other ribosomal proteins were observed. Thus it would appear that, as in the the example of RP1 expression above, expression of CYH2 is controlled at the translational level. The most likely mechanism is via some sort of feedback of the protein onto its mRNA but this has yet to be demonstrated.

It should be noted that this restriction of gene expression at the translational level is not thought to be a general phenomenon. In the case of the URA3 gene, the level of orotidine monophosphate carboxylase enzyme was proportional to the gene dosage up to a level of at least 44 copies per cell (Gerbaud & Guerineau, 1980). Also, when the yeast methionyl-tRNA synthetase gene was placed on an autonomously replicating plasmid, a 30 fold increase in the

level of enzyme activity was thought to be proportional to the increase in the gene dosage (Fasiolo *et al*, 1981).

1.8 Project Aims

The aims of this project were fourfold. Firstly, to set up a system to analyse translation in *S.cerevisiae*. Preference was given to an *in vivo* rather than an *in vitro* system for reasons discussed in Chapter 3. Secondly, To study the influence of codon bias upon the translation of the *PYK* mRNA of *S.cerevisiae*. Thirdly, to study the effects of secondary structure formation at the 5'-end of the *PYK* mRNA upon its translation. Finally, to investigate the phenomenon of translational dosage compensation acting upon the *PYK* mRNA (an observation which arose out of the investigations detailed above).

S.cerevisiae was chosen as the model system for this study for the following reasons:

1. The translational apparatus in yeast is believed to be similar to that of higher eukaryotes.
2. A wide range of recombinant DNA techniques (Struhl, 1983) and plasmids are available for introducing specifically modified genes into yeast (Parent *et al*, 1985).
3. There exists a strong background knowledge of *S.cerevisiae* genetics (Mortimer & Schild, 1985) which allows the potential for combined genetic, recombinant and biochemical approaches to studying yeast translation.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND ENZYMES

2.1.1 Radiochemicals

All radiochemicals were obtained from NEN, Wedgewood Way, Stevenage, Herts.

(^{32}P)5'α-dATP

(^{32}P)5'α-dCTP

(^{32}P)5'α-rUTP all at 600Ci/mmol

(^{32}P)5'γ-ATP at 7000Ci/mmol

2.1.2 Enzymes

T4 DNA ligase, DNA polymerase I (Kornberg), DNA polymerase I (Klenow fragment), and all restriction enzymes and restriction buffers were obtained from BRL, 3 Washington Road, Paisley, Scotland.

Ribonuclease A, deoxyribonuclease I, proteinase K, lysozyme and β-glucuronidase were obtained from Sigma, Fancy Road, Poole, Dorset.

SP6 RNA polymerase was obtained from NEN (see above)

Calf intestinal phosphatase was obtained from BCL, Bell Lane, Lewes, E. Sussex.

Zymolyase was obtained from Miles Laboratories Inc., Research Products Division, P.O.Box 37, Stoke Poges, Slough.

2.1.3 Chemicals and miscellaneous items

Acridine orange, agarose (electrophoretic grade), L-amino acids, ampicillin, dextran sulphate, diethylpyrocarbonate, dithiothreitol, ethidium bromide, glass beads (40 mesh), low melting point agarose, β-mercaptoethanol, MOPS (morphpropanesulphonic acid), polyethylene glycol (Mol. Wt.

3,350), Sephadex G50, sodium acetate, sodium HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), D-sorbitol and Triton X-100 were obtained from Sigma, Fancy Road, Poole, Dorset.

Boric acid, disodium hydrogen phosphate, D-glucose, phenol, potassium phosphate, sodium chloride, sodium dihydrogen orthophosphate, sodium phosphate, sucrose and tri-sodium citrate were obtained from Formachem, 80 Kirk St., Strathaven, Scotland.

Calcium chloride (Analar), formaldehyde (37%^{W}/v), lithium chloride (Analar), sodium deoxycholate and trichloroacetic acid were obtained from BDH Chemicals Ltd., Poole, England.

Caesium chloride and formamide (A.R.,99.5%) were obtained from Rose Chemicals Ltd., 83 Darenth Road, London.

Ethylenediaminetetraacetic acid (EDTA) and sucrose were obtained from Koch-Light, A & J Beveridge Ltd., 5 Bonnington Road Lane, Edinburgh

Deoxynucleotide triphosphates (dNTPs) and RNA Guard were obtained from Pharmacia Ltd., Midsummer Boulevard, Central Milton Keynes, Bucks.

Biogel (100-200 mesh) and Dowex 50W-X8 beads were obtained from Bio-Rad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Herts.

Magnesium chloride (Analar) was obtained from May and Baker, Dagenham, England.

Tris base was obtained from BCL, Bell Lane, Lewes, E.Sussex.

Brij 58 was obtained from Aldrich, The Old Brickyard. New Road, Gillingham, Dorset

Phage Lambda DNA and the Hybridot Manifolds were obtained from BRL, 3 Washington Road, Paisley, Scotland.

Nitrocellulose (Scleicher & Schuell, 0.45μm pore size) was obtained from Andermann & Co., London Road, Kingston-on-Thames, Surrey.

The Flowcell was obtained from Cecil Instruments Ltd., Milton Industrial Estate, Milton, Cambridge.

Polyallomer tubes (38 ml) were obtained from Beckman, Turnpike Road, High Wycombe, Bucks.

X-ray film was obtained from Kodak Ltd., Kodak House, Station Road, Hemel Hempstead.

2.1.4 Growth Media

Bactopeptone, tryptone, yeast extract and yeast nitrogen base (without amino acids) were obtained from Oxoid Ltd., Wade Road, Basingstoke, Hants.

Bacto-Agar was obtained from Difco, U.K.Div., P.O.Box 14B, Central Ave., E.Moseley, Surrey.

2.2 ORGANISMS AND GROWTH CONDITIONS

2.2.1 Saccharomyces cerevisiae : the strain used throughout this study was DBY746, α , His-3, Ura3-52, Leu2-3, Leu2-1 trp1-289

a) Complete medium (YPG)

Glucose 2%

Bactopeptone 2%

Yeast extract 1%

b) Minimal/selective medium (GYNB)

Glucose 2%

Yeast nitrogen base 0.65%

(without amino acids)

(amino acids were added to 50ug/ml where required)

c) Solid media

2% Bacto-Agar was added to GYNB and YPG before autoclaving.

Cultures of DBY746 and transformed DBY746 were grown for 3 days on GYNB plates containing the appropriate amino acids. The plates were sealed with Parafilm and stored at 4°C for up to 3 months before subculturing.

Liquid cultures were grown at 30°C with vigorous shaking (250 rpm). To obtain cultures in mid-logarithmic growth:

(i) 2.5ml of YPG or 25ml of GYNB (with amino acid supplement) were inoculated from cultures stored on plates and grown for 48 hours with vigorous shaking to obtain a stationary culture;

(ii) a small volume (usually 25-100ul) was then added to the appropriate medium for the experiment (prewarmed to

30°C) and grown for 12-16 hours at 30°C with shaking;

(iii) for polysome preparations, the stationary culture was added to 400ml of prewarmed YPG to give an absorbance at 600nm of 0.015-0.050. This was then grown at 30°C with shaking until an absorbance at 600nm of approximately 0.6 was reached.

2.2.2 Escherichia coli : the strain used for all experiments was C1400: ED8767, supE, supF, hsd5, met-, recA, L512 (Cami & Kouilsky, 1978).

a) L-Broth

Tryptone	1%
Yeast extract	0.5%
Glucose	0.1%
NaCl	0.5%

b) Solid media

2% Bacto-Agar was added to L-Broth prior to autoclaving. In order to maintain selective pressure for the presence of plasmids, Ampicillin was added at 50-100ug/ml or (rarely) Tetracycline at 5-10µg/ml

E.coli cultures were grown at 37°C with shaking for 3-4 hours in 2.5ml of L-Broth with the antibiotic supplement; 1.5ml of this culture was then added to 300ul of glycerol and, after mixing, the strain stored at -70°C for long periods. In order to use such stored strains, they were first streaked out onto L-Agar plates containing the appropriate antibiotic and incubated overnight at 37°C. These plates could be stored for short periods at 4°C.

To obtain liquid cultures:

(i) 5ml of L-Broth containing the appropriate antibiotic were inoculated in the morning with a single colony from a storage plate and incubated at 37°C with shaking for 6-8 hours;

(ii) the culture described above was added to 400-800ml of prewarmed L-Broth containing the antibiotic and incubated at 37°C with shaking overnight.

2.3 PREPARATION OF POLYSOMES FROM S.CEREVISIAE

All glassware used for work involving RNA was heat-baked at 200°C overnight to render RNases inactive. Plasticware was soaked in 0.1% DPC overnight, while glass beads were washed in concentrated nitric acid, rinsed and then heat baked at 200°C overnight. All solutions termed 'RNase free' (except those containing amine groups) were treated with 0.1% DPC overnight followed by autoclaving at 15lb.in^{-2} for 15 min. Compounds containing amine groups (e.g. Tris, EDTA) were taken from previously unopened bottles and added to RNase free water. Gloves were always worn when working with RNA.

2.3.1 Solutions

All solutions were autoclaved at 15lb.in^{-2} for 15 min.

Solution A 1M Sorbitol

25mM EDTA, pH 8

Solution B 1.2M Sorbitol

10mM EDTA

100mM NaCitrate, pH 5.8

20 x SSC 3M NaCl

0.3M Na_3Citrate

10X Polysome lysis buffer

1M NaCl

30mM $MgCl_2$

100mM Tris, pH 7.4

Phenol

saturated by vortexing with 0.1M Tris (not pH'd) and containing 1-2 crystals of 8-hydroxyquiniline.

2.3.2 Procedure

The method used is an adaptation of a procedure described by Hutchison & Hartwell (1967). 400ml of prewarmed YPG were inoculated from a 48 hr stationary culture to an absorbance at 600nm of 0.02-0.05 (YPG blank), and grown overnight at 30°C with vigorous shaking until an absorbance at 600nm of approximately 0.6 was reached. 80ml of this culture were then added to 160ml of ethanol and stored at -20°C for RNA extraction at a later date. The remaining cells were harvested by centrifugation at 1100 x g for 5 min at room temperature. The pellet was resuspended in solution A, containing 50mM DTT (added fresh), and incubated at 30°C for 20 min with gentle shaking. The cells were then harvested by centrifugation at 760 x g for 5 min at room temperature. The pellet was resuspended in 50ml of

3%(^{V}/v) B-glucuronidase in solution B and incubated at 30°C for approximately 15 min with gentle shaking. Sphaeroplast formation was monitored by light microscopy, and was judged adequate when over 90% lysis was seen on addition of 35%(^{W}/v) N-lauryl sarcosine. The sphaeroplasts were then harvested by centrifugation at 760 x g for 3.5 min at room temperature. The pellet was resuspended in 50ml of osmotically stabilised YPG (YPG containing 1.2M Sorbitol) and incubated at 30°C with gentle shaking for 90 min (this is termed the Recovery Phase).

Recovered sphaeroplasts were harvested by centrifugation at 760 x g for 3.5 min at room temperature. The pellet was resuspended in 500µl of RNase free lysis buffer on ice, then sodium deoxycholate and Brij 58 were added sequentially to 0.5% each. The lysate was vortexed for 5 sec to ensure complete mixing, and then centrifuged for 60 sec in an Eppendorf microfuge (12,000 x g) at 4°C. 100µl of the supernatant was added to 400µl of RNase free lysis buffer and then 500µl of this diluted lysate carefully layered onto a 37ml 10-50% ^{W}/w sucrose gradient; 500ul of the undiluted lysate was carefully layered onto another sucrose gradient. The gradients were centrifuged using a Beckman SW28 rotor for 2.75 hours at 25,500rpm and 4°C. The gradients were then drawn, from the bottom, through a spectrophotometer flowcell using a spectrophotometer (LKB Ultraspec 4050) to continuously monitor the absorbance at 260nm, the trace being recorded on a BBC chart recorder

(Goertz Metrawatt; model SE120). Fractions of approximately 700µl from the gradient containing the undiluted lysate were dripped directly into eppendorf tubes containing 420µl of 20 x SSC and 280µl of 37%(W/v) formaldehyde. The fractions were vortexed to ensure adequate mixing, heated to 60°C for 15 min and then stored at -70°C until required for dot-blotting (section 2.8.4.1). The diluted sample was used to monitor the A_{260} of the polysomes, and material from this gradient was not stored. The undiluted material was too concentrated to obtain a satisfactory A_{260} profile, but was ideal for mRNA analysis.

2.4 ISOLATION OF RNA FROM S.CEREVISIAE

2.4.1 Solutions

a) Lysis buffer

0.1M	LiCl
0.1M	Tris, pH 7.5
0.01M	DTT (added fresh on day of experiment)

b) TNES buffer

0.2%	SDS
50mM	EDTA
50mM	Tris, pH 7.5
100mM	NaCl

2.4.2 Isolation of RNA from fresh cells

RNA was isolated according to the procedures of Lindquist (1981). 100ml of a YPG culture in mid-logarithmic

growth phase (A_{600}nm = 0.5-0.8, YPG blank) were harvested by centrifugation at 960 x g for 5 min at 4°C. The pellet was resuspended in 5ml lysis buffer and the resulting solution transferred to RNase free tubes containing :

7g	Glass beads (450-500u mesh)
500µl	10% SDS
2.5ml	Phenol
2.5ml	Chloroform

and vortexed continuously for 5 min, followed by centrifugation at 12000 x g for 5 min at 4°C. The aqueous phase was removed and added to 5ml of Phenol/Chloroform (1:1), vortexed for 1 min and then centrifuged at 12000 x g for 5 min at 4°C. This was repeated and then the aqueous phase was added to 2 volumes of diethyl ether and vortexed for 30 sec. A second ether extraction of the aqueous phase was then performed, followed by precipitation of the aqueous phase by the addition of sodium acetate to a final concentration of 0.1M and 2 volumes of ethanol. RNA prepared in this way was stored under ethanol until required. The integrity of the RNA was checked by subjecting a small amount to non-denaturing (TBE) gel electrophoresis, the gel being stained with ethidium bromide and the RNA visualised under UV light. Yields were estimated to be routinely above 25µg.

2.4.3 Storage of cells for RNA preparation

If necessary cells may be stored at -20°C with the addition of 2 volumes of ethanol to the culture. RNA was

then extracted when convenient (usually within 1 week).

Cells stored under ethanol were harvested by centrifugation at 9800 x g at 4°C for 5 min. The resulting pellet was then used in the procedure described above (2.4.2).

2.4.4 Mini RNA preps

In order to determine the abundance of the PYK mRNA in large numbers of yeast transformants, a method was devised for the isolation of RNA from small volumes of culture. YPG cultures (usually 5ml) were grown overnight and stored with the addition of 2 volumes of ethanol at -20°C.

When required, 3ml of the stored culture in ethanol was harvested by centrifugation at 12000 x g in 2 eppendorf tubes for 5 min at 4°C. The cell pellets were resuspended in 200µl of lysis buffer. To this was added 50µl of 10% SDS, approximately 0.9g glass beads (i.e. until the beads were visible just above the meniscus of the liquid), then 250µl of phenol, pH 7.0 and 250µl of chloroform. The whole mix was then vortexed continuously for 5 min. This was followed by two further extractions of the aqueous phase with phenol/chloroform (250ul of each) and two extractions with 500µl of diethylether. The RNA was then precipitated from the aqueous phase by the addition of sodium acetate to 0.1M and 2 volumes of ethanol and stored at -20°C. Yields were routinely estimated to be between 5-10µg.

2.4.5 Extraction of RNA from polysomal fractions

Fractions from polysome gradients (section 2.3) were pooled and precipitated by the addition of sodium acetate to a final concentration of 0.1M and then 2 volumes of ethanol and left overnight at -20°C. The samples were centrifuged at 12000 x g in an eppendorf microfuge for 5 min at 4°C, the ethanol removed and the pellets dried by vacuum dessication. The dried pellets were then resuspended in 400ul of TNES buffer containing 100µg/ml of proteinase K. (This solution was predigested at 37°C for 30 min to destroy any contaminating nucleases). The samples were incubated at 37°C for 30 mins and then extracted 3 times with 800µl of phenol/chloroform (1:1), vortexing for 2 min to mix the phases, and centrifuging for 5 min at 12000 x g in an eppendorf microfuge to separate the phases. The aqueous phase was then extracted twice with 800ul of diethylether, and finally precipitated by the addition of sodium acetate to a final concentration of 0.1M and then 2 volumes of ethanol and stored at -20°C.

2.5 ISOLATION OF DNA FROM E.COLI AND S.CEREVISIAE

2.5.1 Solutions

Unless otherwise stated, solutions were autoclaved at 15lb.in^{-2} for 15 min.

Solution A (lysis mix)

50mM	Glucose
25mM	Tris,HCl, pH 8.0
10mM	EDTA

Solution B

0.2M NaOH

1% SDS

(made up fresh on day of experiment)

Solution C (high salt soln.)

3M K (orNa) acetate (anhydrous)

Made with the minimum quantity of water to dissolve the salt, then glacial acetic acid added to the required volume; pH 4.8.

For 500mls of solution, quantities were:

123g Na acetate

ca. 30mls dH_2O

ca. 350mls glacial acetic acid

not autoclaved

Solution D (TE Buffer)

0.1M EDTA

10mM Tris.HCl, pH 7.6

(usually stored as 10 x TE)

Solution E (Gradient Buffer)

50mM NaCl

30mM Tris.HCl, pH 8.0

5mM EDTA

Solution F (Stet Buffer)

0.8%	Sucrose
0.5%	Triton X-100
50mM	Tris.HCl, pH 8.0
1mM	EDTA

not autoclaved

Solution G (Yeast Buffer 1)

1M	Sorbitol
20mM	EDTA
20mM	KPO_4, pH 7.5

Solution H (Yeast Buffer 2)

1M	Sorbitol
0.2%	Triton X-100
150mM	NaCl
20mM	Na HEPES, pH 7.5
5mM	KCl
1mM	EDTA

Solution I (Yeast Buffer 3)

40mM	Tris,HCl pH 7.5
5mM	Na acetate
1mM	EDTA

Ethidium bromide plates

1%	Agarose
0.5µg/ml	EtBr

2.5.2 Large scale preparation of plasmid DNA from *E.coli*

One litre cultures were grown as described in section 2.2.2. Cells were harvested by centrifugation at 9800 x g

for 10 min. The pellets were resuspended in 30ml of solution A to which was added 80mg of lysozyme in 10ml of solution A (made up fresh) and left for 10 min on ice. 80ml of solution B was added, mixed and the samples left for a further 5 min on ice. 60ml of solution C was then added and, after mixing thoroughly by inversion, the mixes were kept for a further 60 min on ice with occasional mixing by inversion. This was followed by centrifugation at 12400 x g for 10 min at 4°C, the supernatants were filtered through a tea-strainer and retained. 2 volumes of ethanol were then added and, after mixing, they were left for at least 1 hour at -20°C. (Improved yields were obtained by leaving overnight at -20°C) The DNA precipitates were harvested by centrifugation at 9800 x g for 5 min, resuspended in 50ml solution D and then 2 volumes of ethanol were added and the samples incubated for at least 2 hours at -20°C. The DNAs were then harvested by centrifugation at 9800 x g for 5 min, resuspended in 27ml of solution E, to which was added 29g of CsCl and 0.5ml of ethidium bromide solution (10mg/ml). The density was adjusted to 1.56-1.58 g ml^{-1}. These solutions were then subjected to ultracentrifugation in a Beckman Vti50 rotor for 16 hours at 45,000rpm and 20°C. The plasmid bands produced were removed using a 1ml syringe inserted into the side of each tube. The plasmid DNAs were purified by chromatography through a Biogel column (100-200 mesh, 20cm length column) containing 2ml of Dowex ion-exchange resin at the bottom to remove the ethidium bromide. Void volume fractions containing the plasmid DNAs were identified

by spotting 1ul of each onto ethidium bromide plates and viewing under UV light. Fractions containing plasmid DNA were precipitated by the addition of 2 volumes of ethanol and stored at -20°C.

2.5.3 Rapid (small-scale) preparation of DNA from E.coli

(Camy & Kourilsky, 1978).

Cultures of 1.5ml were grown as described in section 2.2.2, transferred to 1.5ml eppendorf tubes, and the cells harvested by centrifugation at 12000 x g in an Eppendorf microfuge for 15 sec. The cell pellets were resuspended in 350µl of solution F, to which was added 25µl of a fresh lysozyme solution (10mg/ml in solution F). The tubes were immediately placed in a boiling water bath for 40 sec and then centrifuged at 12000 x g in an Eppendorf microfuge for 15 min at 4°C. The resulting pellets were removed using a toothpick, and the DNA precipitated from the supernatants by the addition of 40µl of 3M Na acetate and 410µl of cold isopropanol. Samples were left for 5 min at ambient temperature. The DNAs were then pelleted by centrifugation at 12000 x g in an Eppendorf microfuge for 5 min at 4°C and dried by vacuum dessication. The pellets were resuspended in 100µl of dH_2O and 5-10µl of these solutions were used for each restriction digest.

2.5.4 Isolation of DNA from S.cerevisiae

2.5.4.1 Isolation of DNA from intact cells

Cells from a mid-logarithmic YPG culture, grown at 30°C, with gentle shaking (A_{600}=0.6-0.8, YPG blank), were harvested by centrifugation at 3800 x g for 5 min. The cell pellet was resuspended in 5ml of solution G and then re-centrifuged at 3800 x g for a further 5 min. The pellet was resuspended in 200µl of solution H and 50µl of a fresh DTT solution (2mM in solution H) was then added. Zymolyase dissolved in solution H was added to a final concentration of 10 mg/ml and the mixture incubated at 37°C. Lysis, which was monitored by light microscopy, was usually complete in 15-30 min; care was taken not to lyse longer than necessary. When digestion was complete, the volume was made up to 5ml with solution I, adding SDS to a final concentration of 1%, EDTA to 100mM, and NaCl to 100mM. Proteinase K (predigested at 37°C for 30 minutes) was added to a final concentration of 250µg/ml and digestion allowed to proceed at 37°C for 90 min. On completion of the digestion, equal volumes of phenol (Tris-saturated, pH 7) and chloroform were added, the tubes vortexed for 1 min, and then centrifuged at 12100 x g for 10 min. The aqueous phase was then extracted a further two times with phenol/chloroform followed by two ether extractions. Finally, the DNA was precipitated by the addition of sodium acetate to a final concentration of 0.1M and two volumes of ethanol. DNA was stored at -20°C under ethanol.

2.5.4.2 Isolation of DNA from sphaeroplasts

Sphaeroplasts after recovery (section 2.3.2) were harvested by centrifugation at 760 x g for 5 min. The pellets were resuspended in 2ml of solution I to which was added :

300µl	10% SDS
600µl	0.25M EDTA
75µl	4M NaCl
60µl	25mg/ml proteinase K (predigested at 37°C for 30 min)

The tubes were vortexed briefly and then incubated a 37°C for 45 min. The procedure was then the same as detailed above (2.5.4.1) beginning with the phenol/chloroform extractions.

2.6 MANIPULATION OF DNA

2.6.1 Restriction and DNA Modification

Restriction digests of DNA were carried out at a DNA concentration of 50µg/ml. Restriction buffers used were those recommended and supplied by BRL (REACT^{TM} buffers), and restriction enzymes were usually used in large excess - typically 5-10 units of enzyme per 1ug DNA. The digests were carried out at 37°C (except Sma1 which was used at 30°C) for 3-4 hours, and small samples were subjected to TBE gel electrophoresis to check that digestion was complete. When it was necessary to restrict large quantities of DNA, enzyme concentrations were decreased and the reactions left

at 37°C overnight.

In order to decrease self-ligation of vectors (see below) vector DNA was treated by the addition of 22 units of Calf Intestinal Alkaline Phosphatase per 5μg DNA and incubated at 37°C for 30 min. The enzyme was then heat inactivated at 70°C for 10 min. Phosphatasing reactions were usually carried out in the restriction buffer used for the original digest.

2.6.2 Ligation of DNA

Ligations were carried out in a total volume of 20μl containing :

66mM Tris,HCl pH 7.6

10mM DTT

6.6mM $MgCl_2$

1mM ATP.

The ratio of insert DNA to vector DNA was fixed at around 5:1 for optimum ligation. 10 units of T4 DNA ligase were used for "sticky-ended" ligations and 20 units for "blunt-ended" ligations. Ligation reactions were incubated at 18°C overnight and diluted 5 times before transformation of E.coli.

2.6.3 Transformation of E.coli

E.coli cells were made competent for DNA uptake by the calcium chloride method (Mandel & Higa, 1970). Large batches of competent cells were made up and stored as 200μl aliquots containing 17.6% glycerol at -70°C. (Dr IJ Purvis

was responsible for the availability of most of the competent cells used). When required, aliquots of competent cells were thawed on ice and to 50µl of competent cells was added 10µl of a diluted ligation mix. This was mixed and left for 15 min at 4°C, followed by 'heat-shock' at 37°C for 5 min. 1ml of L-broth (containing 0.1% glucose) was added and the transformation mix incubated at 37°C for a further 45 min. The cells were then plated out onto L/B plates containing the appropriate antibiotic for selection of cells that take up the plasmid and incubated at 37°C overnight. The plasmids in a proportion of the resulting colonies were purified (section 2.5.3) and then subjected to restriction analysis (section 2.6.1).

2.6.4 Construction of Probes Used in This Study

2.6.4.1 Plasmid Probes

The actin mRNA was analysed using SPACT9. A 1.5kb BamH1/HindIII fragment of the actin gene from plasmid pYA301 (a subclone of pYA208; Gallowitz & Sures, 1980) was subcloned into the BamH1/HindIII sites in the polylinker of SP64 to form SPACT9 (Fig. 2.6.4.1). pYA301 was a generous gift from Jean Beggs. This plasmid was linearised with EcoR1 and Xho1 to prepare the template for riboprobe synthesis (section 2.9.3)

The β-galactosidase mRNA was analysed using a nick-translated (section 2.9.2) fragment of the β-galactosidase coding region. A 3kb BamH1 fragment from pMC1871 (Casabadan et al, 1983) containing the whole β-galactosidase coding

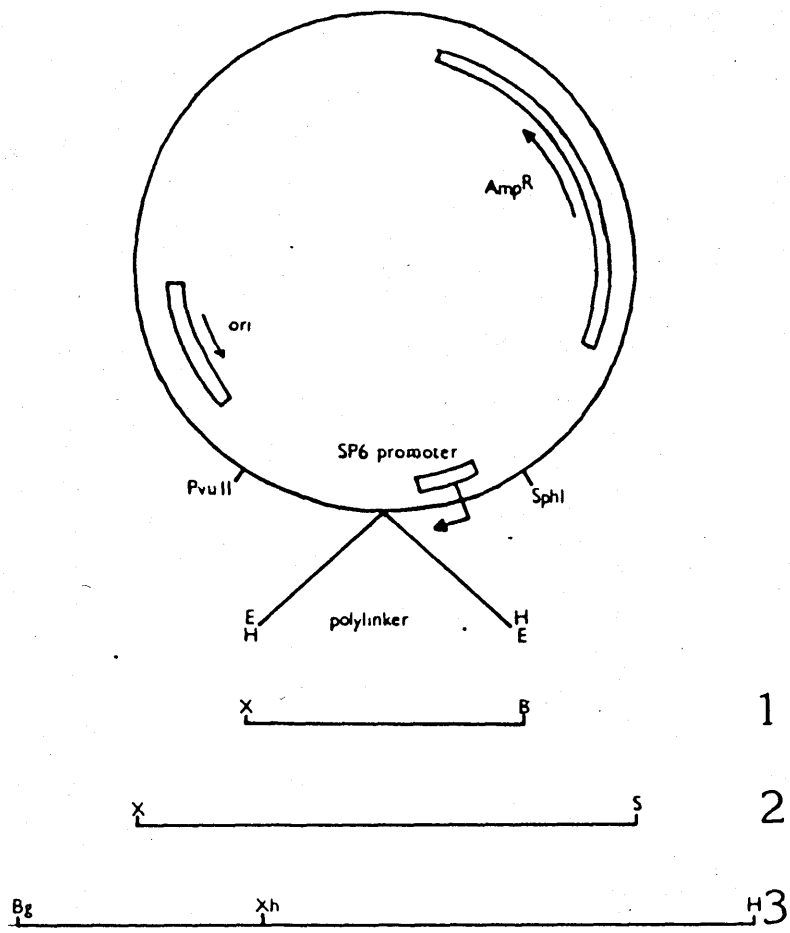


Figure 2.6.4.1 Details of plasmids used for riboprobe synthesis in this study.

1. SPK2 (in SP65); 2. SP65R (in SP65); 3. SPACT9 (in SP64). ori: bacterial origin of replication; Amp^R: ampicillin resistance gene (β-lactamase). Restriction enzyme sites - B: BamH1; Bg: BglII; E: EcoR1; H: HindIII; S: Sst1; X: Xba1; Xh: Xho1

region was isolated using the "Gene-clean" procedure (Vogelstein & Gillespie, 1979).

The PYK mRNA was analysed using SPK2. A 550bp Xba1/BglII fragment of the PYK gene (Burke et al, 1983) was subcloned into the Xba1/BamH1 sites in the polylinker of SP65 to form SPK2 (Fig. 2.6.4.1). This plasmid was linearised with EcoR1 and PvuII to prepare the template for riboprobe synthesis (section 2.9.3).

18S rRNA was analysed using SP65R. A 1kb Sst1/Xba1 fragment of the yeast 18S rRNA gene from pY1rG12 (Petes et al, 1978) was subcloned into the Sst1/Xba1 sites in the polylinker of SP65 to form SP65R (Fig. 2.6.4.1). This plasmid was linearised with HindIII to prepare the template for riboprobe synthesis (section 2.9.3).

2.6.4.2 Oligonucleotide Probes

The sequences of the oligonucleotide probes designed for use in this study are given in Table 2.6.4.1. Oligonucleotides were synthesised by Dr. V.B.Math of the Biochemistry department.

Number	Sequence	Specificity
130	5'-CCTCTTGGGGTTTCGACGTAACCGACAACACC-3'	RP1
133	5'-GGAGCAGTGATGACAACCTTCTTGGCACCAGCG-3'	G3PD
038	5'-TAATATCTTCATTCAATCATGATTC-3'	all PYK
220	5'-ATCTTTCTAATCTAGACATTGTGATG-3'	WT PYK
223	5'-CCAATTCAGCTGGGTGTCTTGGCAA-3'	1C PYK
224	5'-GCTAGCTCTGCGGGATGGCGGGGTA-3'	2D PYK

Table 2.6.4.1

Sequences and Specificities of all Oligonucleotides Employed in This Study

The G3PD mRNA was analysed using a 33 base oligonucleotide which is complimentary to a region common to all 3 genes of the G3PD gene family (Holland *et al*, 1983).

The RP1 mRNA was analysed using a 32 base oligonucleotide which is complementary to a short region of the *tcm1* gene (Schultz & Friesen, 1983).

Four oligonucleotides were used to analyse the PYK mRNA:

1. 038, a 25 base oligonucleotide which is complementary to to a region immediately 3' to the *PYK* coding region (Purvis *et al*, 1987b). This oligonucleotide will hybridise to all the *PYK* mRNAs produced during this study.
2. 220, a 26 base oligonucleotide which is complementary to a region spanning the ATG translation start site of the *PYK* gene. This oligonucleotide will only hybridise to the wild-type *PYK* mRNAs produced during this study.
3. 223, a 25 base oligonucleotide which is complementary

to a region within the insertion of plasmid pLD1(35)1C (see Chapter 5 for more details). This oligonucleotide will only hybridise to PYK mRNAs produced from plasmid pLD1(35)1C.

4. 224, a 25 base oligonucleotide which is complementary to a region within the insertion of plasmid pLD1(35)2D (see chapter 5 for more details). This oligonucleotide will only hybridise to PYK mRNAs produced from plasmid pLD1(35)2D.

2.6.5 Transformation of S.cerevisiae

2.6.5.1 Solutions

All solutions were autoclaved at 15lb.in^{-2} for 15 min.

Solution A	1M	Sorbitol
	25mM	EDTA, pH 8
Solution B	1.2M	Sorbitol
	10mM	EDTA
	100mM	Na citrate, pH 5.8
Solution C	1.2M	Sorbitol
	10mM	$CaCl_2$
Solution D	10mM	$CaCl_2$
	10mM	Tris.HCl, pH 7.5
Solution E	2 volumes	1.8M Sorbitol
	1 volume	3 x YPG (2.2.1)

Bottom agar 2% Glucose

0.65% YNB (without amino acids)

2% Bacto-agar

1.2M Sorbitol

(amino acids added to 50µg/ml autoclaving)

Top agar 2% Glucose

0.65% YNB (^{W}/o amino acids)

3% Bacto-agar

1.2M Sorbitol

(amino acids added to 50µg/ml after melting, agar kept at 48 ^{o}C until required)

2.6.5.2 Procedure

100ml of a culture in mid- to late- logarithmic growth (A_{600} = 0.7-0.8) was used for each transformation.

Cells were harvested by centrifugation at 960 x g for 5 min at room temperature. The cells were resuspended in 50ml of solution A containing 50mM DTT (made up fresh on day of experiment and then filter sterilised) and incubated with gentle shaking for 20 min at 30^{o}C. The cells were re-harvested by centrifugation at 960 x g for 5 min at room temperature. The cell pellet was resuspended in 50ml of solution B containing 2-3% (^{V}/v) β-glucuronidase (made up fresh on day of experiment and then filter sterilised) and incubated with gentle shaking for 15-30 min at 30^{o}C. (Sphaeroplast formation was monitored by light microscopy and was judged adequate when over 90% of cells lysed on the addition of 35%(^{W}/v)N-lauryl sarcosine.) The sphaeroplasts

were harvested by centrifugation at 960 x g for 3.5 min at room temperature and were washed twice in 50ml of 1.2M sorbitol, centrifuging each time at 960 x g for 3.5 min at room temperature. The washed sphaeroplasts were resuspended very gently in 100µl of solution C to give a thick suspension. DNA (5-15µg, in 10-15µl) was added to 50µl of sphaeroplasts in sterile eppendorf tubes, and these were left for 15 min at room temperature. 500µl of 20% PEG in solution D (made up fresh and filter sterilised) was then added and left for approximately 45 sec before centrifuging at 12000 x g in an Eppendorf microfuge for 1 sec. The PEG was carefully removed, the sphaeroplasts resuspended in 100µl of solution E and incubated for 30 min at 30°C. The sphaeroplasts were diluted to 1 ml by the addition of 1.2M sorbitol, and 200µl of this suspension was plated onto bottom agar plates with 10 ml of top agar (equilibrated at 48°C). The plates were incubated at 30°C; colonies usually appeared 4-7 days after transformation. Putative transformants were then streaked onto selective plates (GYNB agar containing the appropriate amino acids) to check that they exhibited the correct auxotrophic markers.

2.7 ELECTROPHORESIS OF NUCLEIC ACIDS

2.7.1 Solutions

(unless otherwise stated, all solutions were autoclaved at 15 lb in^{-2} for 15 min)

Solution A (TBE)

89	mM	Tris.HCl, pH 8.3
89	mM	Boric acid
2.5	mM	EDTA

Solution B (TE)

0.1	mM	EDTA
10	mM	Tris.HCl, pH 7.6

Solution C (10 x MOPS)

0.2	M	Morphpropanesulfonic acid
0.05	M	Sodium acetate
0.01	M	EDTA

adjusted to pH 7.0 with NaOH. Stored in the dark, without autoclaving.

Agarose gel loading buffer

0.4%	Bromophenol blue
50%	Glycerol

Formaldehyde gel loading buffer (RNase free)

0.4%	Bromophenol blue
50%	Glycerol
1mM	EDTA
0.4%	Xylene cyanol

MFF (RNase free)	µl/ml
50% Formamide	500
6% Formaldehyde	162
1 x MOPS	100
dH_2O	238

2.7.2 Agarose Gels

Electrophoresis of nucleic acids was generally carried out in 0.8-1.0% agarose in TBE. Gels were run at voltages not less than 2.5 V cm^{-1} and not more than 10 V cm^{-1}, until the bromophenol blue dye front approached the end of the gel. Gels containing DNA or RNA were stained in 0.5µg/ml ethidium bromide after electrophoresis, or by adding 0.5µg/ml ethidium bromide to the agarose solution immediately prior to casting the gel. Visualisation of the DNA or RNA was by exposure of the stained gel to ultraviolet light using a Chromato VUE transilluminator, model TM36 (Ultraviolet Products Inc.). Important gels were photographed using a polaroid camera with red filter (Wratten 3A).

2.7.3 Low melting-point (LMP) agarose gels

Isolation of individual restriction fragments for cloning was performed using 0.8% LMP agarose gels, run in TBE at no higher than 50V. The fragment of interest was cut out of the gel after electrophoresis; 5 volumes of TE added to the gel slice, followed by heating for 30 min at 70°C. The resulting solution was then used in ligation reactions (2.6.2)

2.7.4 Formaldehyde gels

1.5% agarose gels containing formaldehyde were prepared and run according to the methods of Maniatis and co-workers (1982). For each 100ml of agarose solution required, 1.5g of RNase free agarose was dissolved in 73ml of RNase free

water by heating in a microwave oven. The solution was allowed to cool to about 60°C, whereupon 10ml of solution C (10 x MOPS) and 16.2ml of 37% (W/v) formaldehyde were added and the gel cast immediately. RNA solutions were denatured by the addition of 8 volumes of MFF, followed by heating for 15 min at 60°C. One volume of loading buffer was added before the gel was loaded. Gels were run at about 10 V cm^{-1} in 1 x MOPS (which was recirculated) until the bromophenol blue dye front approached the end of the gel.

2.8 Filter Hybridisations

2.8.1 Solutions

Solution A [SSC (standard saline citrate)]

0.15M NaCl

0.015M Na_3citrate

Solution B (20 x SSCP)	g l^{-1}
NaCl	193.2
Na_3citrate	88.4
Na_2HPO_4	38.0
$NaH_2PO_4.H_2O$	19.2

Solution C (100 x Denhardts solution)	g l^{-1}
Bovine Serum Albumin	20
Ficoll	20
Polyvinylpyrollidine	20

(NB dissolve in order shown)

2.8.2 Northern Blotting

A formaldehyde gel was placed face down on 3 sheets of Whatmann 3mm paper soaked, and in contact with, 20 x SSC. A piece of nitrocellulose (presoaked in dH_2O, followed by 20 x SSC) was placed on the gel, followed by 3 pieces of Whatmann 3mm paper cut to the size of the gel. On to this were placed 3-4 layers of absorbent nappy pads, cut to size, followed by a 1.5kg weight. The gel was blotted overnight with 20 x SSC. After blotting. the nitrocellulose filter was baked for 3 hours at 80°C (Thomas, 1980).

2.8.3 Southern Blotting

DNA gels were blotted using procedures adapted from those of Southern (1975). TBE gels were soaked in 0.2M NaOH, 1.5M NaCl for 30 min, and then in 3.0M NaCl, 0.5M Tris.HCl, pH 7.5 for 60 min. The gels were then treated as for Northern blotting (section 2.8.2).

2.8.4 Dot Blotting

2.8.4.1 RNA

RNA was dot blotted using procedures adapted from those described by White & Bancroft (1982). Ethanol precipitates of RNA were harvested by centrifugation at 12000 x g at 4°C, vacuum dessicated, and resuspended in 200µl of RNase free water. To this was added 200µl of 6:4, 20 x SSC:37% (w/v) formaldehyde. The samples were vortexed and heated for 15 min at 60°C. RNA samples were stored in this form at -70°C. The treatment of RNA from polysome gradients has been

described elsewhere (section 2.3.2)

When required, the RNA samples were thawed, vortexed and diluted if necessary by the addition of 15 x SSC. A sample size of 100µl per dot was usually used. A nitrocellulose filter (presoaked in dH_2O, then 20 x SSC and allowed to dry) was placed in the 96-well BRL Hybridot manifold, a vacuum applied, and the samples added to the wells. After the samples had been drawn through, 100µl of 15 x SSC was added to each well to wash the sample through. The manifold was dismantled with the vacuum still applied, the filter lifted off and baked for 3 hours at 80°C.

2.8.4.2 DNA

The DNA samples dissolved in water were denatured by the addition of NaOH to 0.1M followed by incubation for 15 min at 37°C. Then, HCl and Tris (pH 7.5) were each added to final concentrations of 0.1M. The samples were diluted where necessary with 15 x SSC and dotted immediately onto nitrocellulose, as described in the previous section (2.8.4.1).

2.8.5 Hybridisations

Prehybridisation fluid was added to nitrocellulose filters sealed into plastic bags and these were prehybridised for at least 2 hours (usually 6 hours) at the appropriate temperature. The hybridisation conditions used are described in Table 2.8.5.1

Component (ml)	Nick-translated probes	Riboprobes	Oligont. probes
20xSSC	2.7	2.1	2.5
Formamide	4.5	5.85	(a)
50% Dextran sulphate (W/v)	0.5	0.5	1.0
100x Denhardt's	0.45	0.42	0.5
10%SDS	0.45	0.42	0.5
0.4M $NaPO_4$	0.45	0.42	0.5
0.25M EDTA	0.36	0.33	0.4
dH_2O	0.6	=	(a)
Total volume	10ml	10ml	10ml
Temperature of incubation	42°C	42°C	37°C

Table 2.8.5.1: Hybridisation Conditions for Different Types of Probe

(a) The concentrations of formamide in the hybridisation mixes of the various oligonucleotide probes used are given in Table 2.8.5.2

Oligonucleotide	% formamide	%G+C	Length
038	20	24	25
220	35	31	26
223	30	52	25
224	40	68	25
G3PD	20	58	33
RP1	20	56	32

Table 2.8.5.2: The Concentrations of Formamide used in Hybridisations with Different Oligonucleotide Probes (the conditions were optimised experimentally)

The length of hybridisation was usually 16 hours for nick-translated and ribo-probes, and 24 hours for oligonucleotide probes.

After hybridisation with nick-translated and ribo-probes, the filters were washed three times for 5 min each in 2 x SSCP (containing 0.1% SDS) at room temperature, and three times for 15 min each in 0.4 x SSCP (containing 0.1% SDS) at 55°C. If the background hybridisation, estimated using a GM monitor, was still thought to be too high, the filters were washed a further two times in 0.2 x SSCP (containing 0.1% SDS) at 55°C.

Following hybridisation with oligonucleotide probes, the filters were washed three times for 15 min each in 6 x SSC at room temperature and two times for 15 min each in 6 x SSC at 45°C

The washed filters were sealed in plastic bags and exposed to Kodak X-Omat film, usually for 16 hours in an

autoradiography cassette using an intensifying screen. Film was developed using a Kodak X-Omat automatic processor.

After hybridisation and autoradiography, all dot blot filters were cut up into individual dots, placed into scintillation vials, 1ml of Ecoscint added and then the radioactivity counted in a Beckman LS1801 scintillation counter.

2.9 PREPARATION OF RADIOACTIVE PROBES

2.9.1 Solutions

All solutions were autoclaved for 15 min at 15lb.in^{-2}

Solution A (Klenow buffer)

0.066% Gelatin
10mM $MgCl_2$
10mM Tris.HCl, pH 7.4

Solution B (5 x SP buffer)

200mM Tris.HCl, pH 7.5
30mM $MgCl_2$
25mM NaCl
10mM Spermidine
50mM DTT (added after autoclaving)

Solution C (10 x Kinase buffer)

100mM KCl
70mM Tris.HCl, pH 7.6
10mM $MgCl_2$
5mM DTT (added after autoclaving)

Solution D (Column buffer)

100mM NaCl

10mM Tris.HCl, pH 7.5

1mM EDTA

2.9.2 Nick-translated probes

The following reaction mix was used, the components being added in the order recorded;

klenow buffer	45.0µl
dCTP,dGTP,dTTP (10mM)	1.0µl of each
β-mercaptoethanol (diluted $^1/_{40}$ in dH_2O)	1.0µl
DNase I (0.5µg/ml)	0.7µl
dH_2O	4.8µl
[^{32}P]-dATP	2.5 or 5.0µl
E.coli polymerase I	1.0µl
plasmid DNA (0.5-2µg)	2.0µl
	60µl

The reaction mixture was incubated for one hour at 14°C, after which the reaction was stopped by the addition of 4µl 0.25M EDTA, pH8.0.

2.9.3 Riboprobes

2.9.3.1 Preparation of DNA

The DNA template (25-50µg) was truncated by restriction at an appropriate site, or sites. Two restriction sites were usually chosen to reduce readthrough on non-truncated template. Readthrough caused problems with background

hybridisation. A small sample was subjected to electrophoresis to check that restriction was complete, and the remainder precipitated by the addition of two volumes of ethanol. The DNA precipitate was then harvested by centrifugation in an Eppendorf microfuge at 12000 x g for 5 min at 4°C. After removing the ethanol, the pellet was dried in a vacuum dessicator. The pellet was resuspended in RNase free water and was extracted three times with one volume of RNase free phenol and one volume of RNase free chloroform. This was followed by two extractions with two volumes of RNase free ether, followed by precipitation of the DNA by the addition of sodium acetate to a final concentration of 0.1M and two volumes of RNase free ethanol. Before use, the RNase free DNA was harvested by centrifugation at 12000 x g in an Eppendorf microfuge, dried by vacuum dessication and resuspended in RNase free water to give an estimated concentration of 0.5µg/ul. The actual concentration was estimated by gel electrophoresis, comparing a small portion of the sample with known amounts of other DNAs.

2.9.3.2 Synthesis

Synthesis of RNA probes was carried out using adaptations of procedures described by Melton and co-workers (1984) and Krieg & Melton (1984).

The following reaction mix was used, all components were RNase free and were added in the order recorded. For preparation of more than one probe, the mix was increased in

proportion to the number of probes required, and 20ul added to each DNA sample.

5 x SP buffer	5.00µl
ATP,CTP,GTP (10mM)	1.25µl of each
RNAGuard (27units/µl)	1.25µl
BSA (1mg/ml)	1.25µl
H_2O	2.25µl (+ 2.5µl)
*cold-UTP	1.00µl
[^{32}P]-UTP	5.00µl (2.5µl)
SP polymerase (9units/µl)	0.5µl
Total volume	20µl

* cold-UTP [10mM] was diluted by 1 in 20 to 1 in 40 with RNase free water depending upon the required specific activity of the probe being synthesised.

1µl of this reaction mix was removed and added to 9µl of H_2O for TCA precipitation to enable calculation of unincorporated label. The remainder of the mix was added to the DNA (0.5-2µg) and, after mixing, incubation was for one hour at 37°C. After incubation, 1µl of the mix was removed and added to 9µl of H_2O to enable calculation of incorporated label by TCA precipitation. The remainder was stored at -20°C.

2.9.3.3 TCA precipitation

2 x 4µl of the diluted reaction mixes were spotted onto Whatmann 3mm filters, presoaked in 10mM ATP. The filters were placed into a perforated beaker suspended in a beaker of 5% TCA stirred using a magnetic stirrer and bar. The

temperature was maintained at 4°C. The filters were washed 4 times in 5% TCA for 15 min at 4°C, and once in ethanol for 15 min at 4°C. The filters were then dried and placed in scintillation vials containing 1ml of water and subjected to Cherenkov scintillation counting. The efficiency of Cherenkov counting was approximately 40%. Incorporation of UTP into riboprobes was usually of the order of 50-75%.

2.9.4 End-labelling of oligonucleotide probes

Synthetic oligonucleotides were ethanol precipitated, vacuum dessicated and resuspended in dH_2O. Their concentrations were determined using spectrophotometry (an A_{260} of 1 is equivalent to 40µg/ml of single-stranded DNA). The following reaction mix was used:

10 x kinase buffer	4µl
oligonucleotide	300ng
gamma-^{32}P-ATP	5µl
T4 polynucleotide kinase	1µl
dH_2O	to 40µl

The reaction was incubated at 37°C for 1 hour.

2.9.4 Sephadex-G50 column chromatography

Nick-translated and oligonucleotide probes were separated from unincorporated radioisotopes by gel filtration on Sephadex-G50 columns (20cm in length, 0.5cm in diameter). 5µl of Dextran Blue (50mg/ml) and 5µl of Phenol Red (50mg/ml) were added to the reactions. The samples were then loaded onto Sephadex-G50 columns previously

equilibrated with column buffer (Solution D). Fractions of approximately 500µl were collected. Those containing the Dextran Blue contained the nick-translated probes and were pooled. For oligonucleotide probes, fractions eluted with and just before the Dextran Blue were pooled. 1µl of the pooled fractions was added to 1ml of dH_2O and subjected to Cherenkov scintillation counting. Specific activities of the probes were usually around 10^6-10^7 cpms/µg. In no case were more than 10^6 cpms per ml hybridisation fluid used. Both oligonucleotide and nick-translated probes were boiled prior to their use, for 5 min and 10 min respectively.

2.10 Estimation of β-Galactosidase Activity in Yeast

(Purvis et al, 1987c)

Cells were harvested by centrifugation at 5000 x g for 5 min and resuspended in 1ml of assay buffer (100mM Na_2PO_4, 1mM $MgSO_4$, 100mM B-Mercaptoetanol, pH 7.0) per 0.1g wet-weight cells. After resuspension, 2g of glass beads (0.45mm diameter) per 0.1g cells was added and the mixture vortexed for 1 min. 1ml of the lysate was centrifuged at 12000 x g for 10 min and dilutions of the supernatant used for enzyme assays. 0.8ml of the supernatant (or diluted supernatant) was added to 0.2ml of ONPG (4mg/ml), vortexed briefly, and left at 30°C for a measured time until the colour developed. The reaction was stopped by the addition of 0.5ml of 1M sodium carbonate, and the absorbance read at 420nm. Relative enzyme activity is expressed as functional β-galactosidase tetramers per plasmid-bearing cell.

CHAPTER 3

RESULTS AND DISCUSSION:

THE DEVELOPMENT OF A METHOD

3.1 Results

A major aim of this project was to assess the effects of specific manipulations within the yeast PYK gene upon the translation of its mRNA *in vivo*. Therefore, the first priority of this project was to set up a system to directly analyse translation in *S.cerevisiae*. Translation is a complex process, involving a large number of cellular factors. It is not unreasonable to assume that there may be problems in using an *in vitro* system to analyse translation of modified PYK mRNAs, particularly if a heterologous system is used (e.g. rabbit reticulocyte lysate or wheatgerm extract). It is considered unlikely that an *in vitro* system will be able to closely mimic *in vivo* concentrations of translation factors, ions and NTPs. Furthermore, concentrations of tRNAs are unlikely to be similar *in vitro* and *in vivo*, and the *relative* abundances of tRNAs will be different in heterologous systems. Also, *in vitro* analyses require the use of "naked" RNA i.e. proteins which bind *in vivo* may not be bound in these studies (e.g. the poly(A) binding protein (Adams *et al*, 1986)). Other RNA binding proteins which bind *in vivo* may not do so *in vitro*, and there may also exist the added complication of mRNA-specific proteins. Although a yeast *in vitro* translation system was available (Tuite & Plesset, 1986), there was some concern as to how closely this could be expected to mimic conditions in an intact cell; many of the criticisms of heterologous systems described above also apply to homologous systems. Thus it was decided to attempt direct,

in vivo measurements of translation, where the mRNA under consideration would be translated in its normal cellular environment, and all translation factors would be present in locally correct concentrations.

One of the methods commonly used to analyse translation *in vivo* involves determination of the distribution of mRNAs on polysomes, usually by fractionation of crude post-mitochondrial supernatants upon sucrose density gradient centrifugation. The first step in such procedures involves cell lysis. The two main approaches to achieving cell breakage in *S.cerevisiae* are firstly, to shear the cells by vortexing them with glass beads and, secondly, to lyse yeast sphaeroplasts by the addition of detergent(s). The major advantage of the glass bead method is, that almost until the point of cell breakage, the cells are in a physiologically "normal" state. However, this method has several disadvantages. Firstly, polysomes may be sheared during vortexing with the glass beads. Secondly, the polysomes are exposed to endogenous RNases for a significant period (approximately 6 minutes) during the cell breakage. Finally, cell breakage is often inefficient and variable. On the other hand, the second procedure which involves lysis of sphaeroplasts has several advantages. Firstly, this method would seem to cause only minimal amounts of shearing of polysomes. Secondly, polysomes are only exposed to released endogenous RNases for only a short period (approximately 90 seconds) before centrifugation since it is

possible, technically, to lyse the sphaeroplasts and load the post-mitochondrial supernatants onto sucrose gradients extremely rapidly. Finally, lysis of sphaeroplasts is efficient and reproducible; routinely, over 90% lysis is obtained. The major disadvantage of this method is that physiologically, conditions within sphaeroplasts are not identical to those in intact cells. However, it has been shown that, in medium stabilised with sorbitol, the rates of synthesis of RNA and protein are approximately the same in intact cells and sphaeroplasts (Hutchison & Hartwell, 1967). Therefore, procedures involving detergent-mediated lysis of sphaeroplasts were used since it was thought this might give results which could be related to whole cells with the most confidence. In order to minimise any possible artefactual errors, only those chemicals necessary for sphaeroplast lysis were employed. RNase- and translation-inhibitors (e.g. cycloheximide) that have been used in previous studies to enhance polysome profiles (Hartwell *et al*, 1970; Kraig & Haber, 1980), were not employed in this study. Reliance being placed on speed of preparation of polysomes.

Having established the method of choice for cell lysis, the next stage was to develop a suitable procedure for sedimentation of yeast polysomes on sucrose density gradients. A number of different methods have been used previously (Hutchison & Hartwell, 1967; Hartwell *et al*, 1980; Kraig & Haber, 1980) and the performance of some of these were tested in this study. After using sucrose gradients of 5-25%(^{W}/v) and 10-50%(^{W}/v), it was decided that

sucrose gradients of 10-50%($^{W}/_{W}$) gave the most satisfactory separation of polysomes. After centrifugation, the gradients were fractionated from the bottom using a capillary tube suspended just above the bottom of the centrifuge tube, in an attempt to avoid dislodging material which had pelleted. (This was not always successful). Gradients were drawn, using an LKB peristaltic pump, through a flow-cell placed in a spectrophotometer monitoring continuously at a wavelength of 260nm. The spectrophotometer was attached to a chart recorder in order to obtain a profile of the absorbance at 260nm for each gradient. Unfortunately, due to the limited absorbance range of the spectrophotometer (0-2.5 units), in order to obtain a useful absorbance profile it was necessary to run a dilution of each transformant assayed; an undiluted sample contained too much material (see Fig 3.1.1). Therefore, as described in chapter 2.3.2, two gradients were run for each sample; a diluted sample to monitor A_{260}, and a concentrated sample to use in determining mRNA distributions.

It was conceivable that the absorbance profile observed was due to the separation of random aggregates of RNA, proteins, and ribosomal subunits, and was not due to differential sedimentation of polysomes. Experiments were devised to exclude this possibility.

A post-mitochondrial supernatant was prepared from a 200ml culture of DBY746. One-fifth (100µl) of the post-mitochondrial supernatant was added to 500µl of lysis buffer

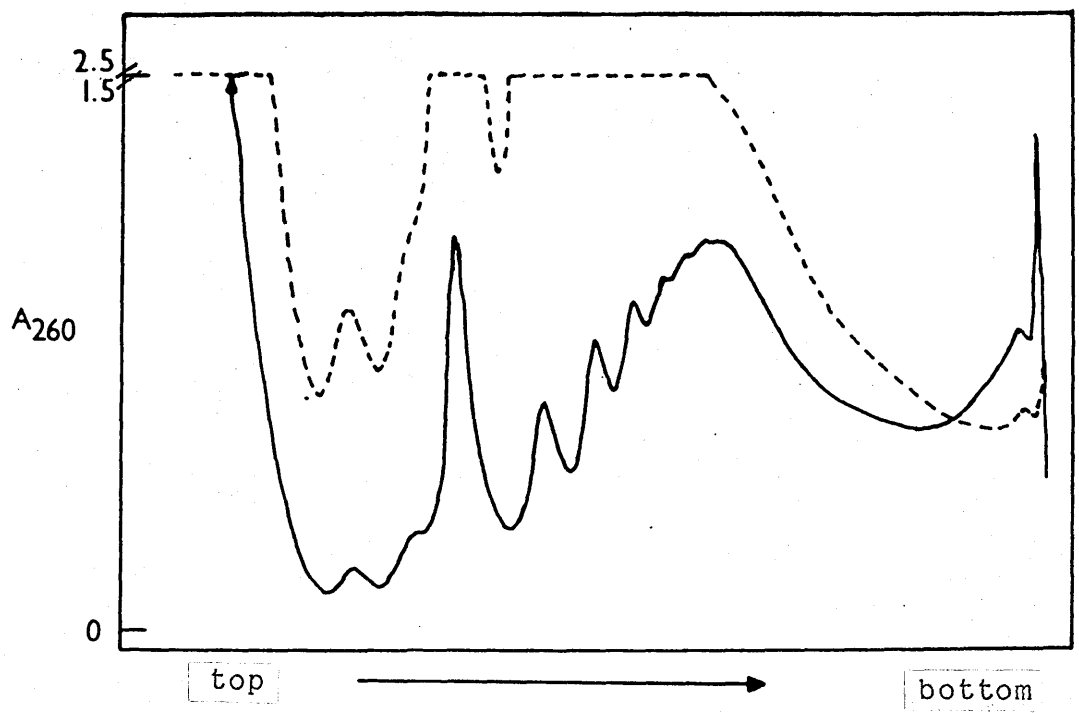


Figure 3.1.1 Absorbance profiles at 260nm of diluted (continuous line) and undiluted (dotted line) samples from DBY746 loaded onto sucrose gradients

The arrow below the diagram represents the direction of sedimentation

containing 60μg of RNase A and was incubated at 37°C for 3 min. A further one-fifth was treated in a similar fashion, but without the addition of RNase A. The samples (500μl) were then subjected to sucrose density gradient centrifugation. The results of the experiment are shown in Fig 3.1.2. RNase is thought to cleave mRNA between ribosomes. Therefore, aggregates should be unaffected by the addition of RNase while polysomes should be degraded to monosomes. It can be clearly seen that, in the presence of RNase A, there is almost complete loss of the rapidly-sedimenting "polysomal" material; the vast majority of the material being concentrated in the "monosomal" area of the gradient, as has been previously observed (Brown & Hardman, 1980).

Chelation of Mg^{2+} ions by EDTA is known to cause dissociation of polysomes into ribosomal subunits and mRNP particles (Hamilton & Ruth, 1969). Thus, EDTA was added (to a final concentration of 25mM) to one-half of a post-mitochondrial supernatant (prepared from DBY746 as described above) immediately prior to sucrose density gradient centrifugation. An equivalent volume of lysis buffer was added to the other half of the supernatant prior to centrifugation. The resulting absorbance profiles of the two gradients are shown in Fig 3.1.3. Addition of EDTA caused the rapidly-sedimenting "polysomal" material to sediment almost exclusively in the positions occupied by ribosomal subunits.

In an analogous experiment, the addition of

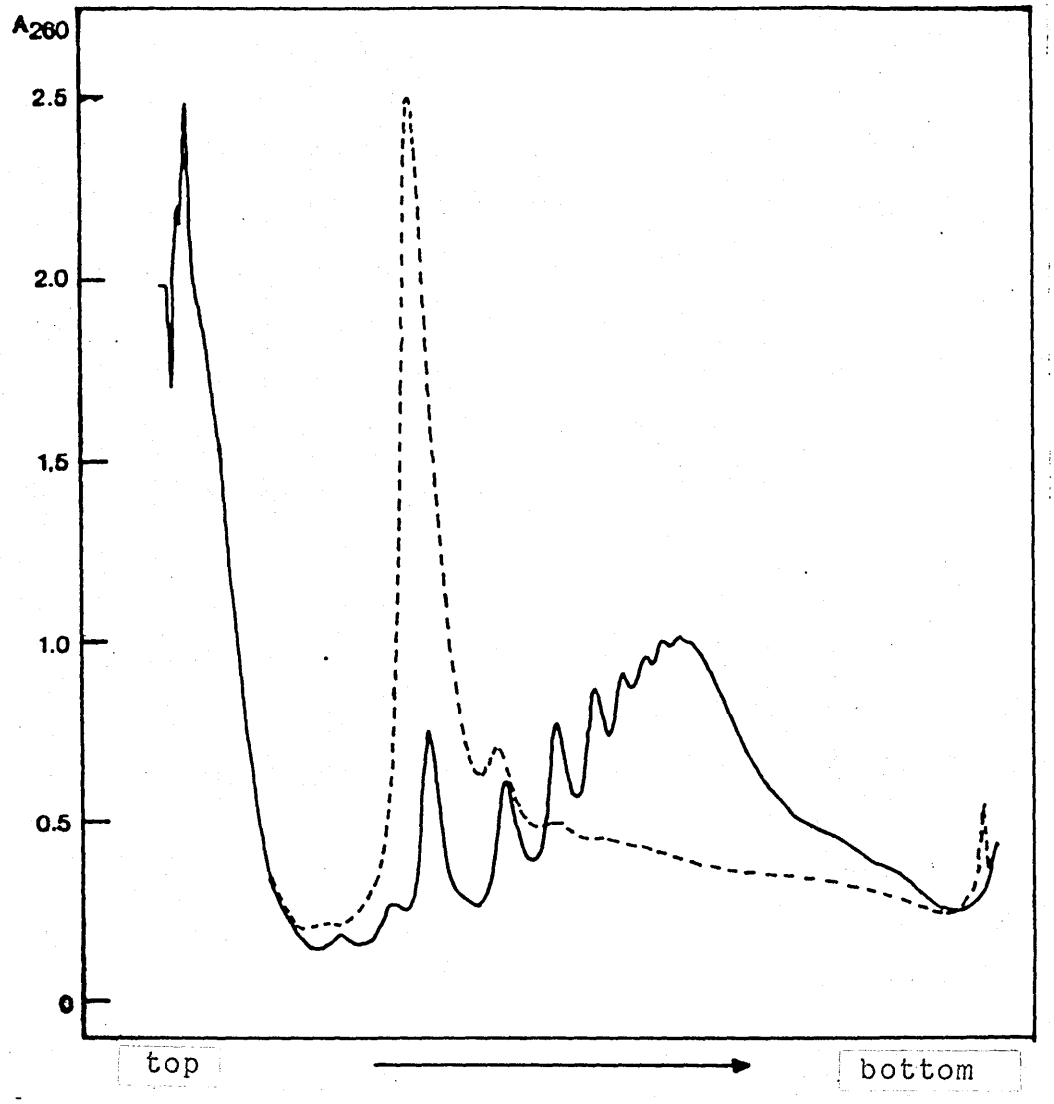


Figure 3.1.2 The effect of RNase A on yeast polysomes

A post-mitochondrial supernatant was prepared from DBY746. One-fifth was subjected to sucrose density gradient centrifugation with no prior treatment to obtain the control profile (continuous line), while 60µg RNAse A was added to a further one-fifth before centrifugation (dotted line). The arrow below the diagram represents the direction of sedimentation.

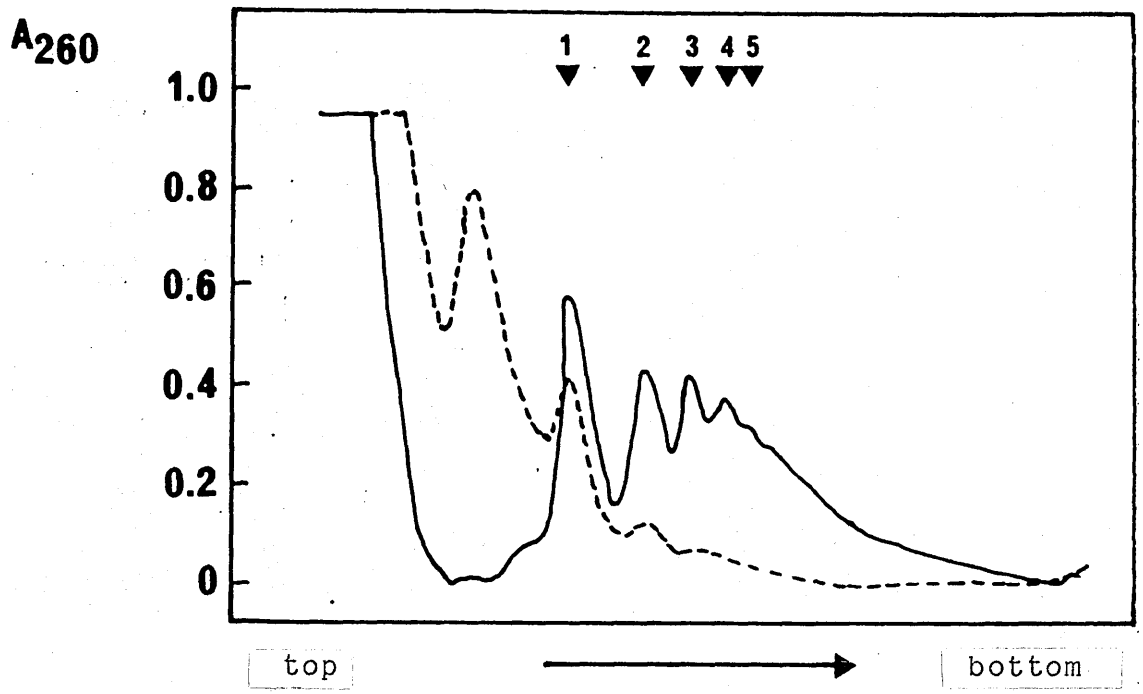


Figure 3.1.3 The effect of EDTA on yeast polysomes

A post-mitochondrial supernatant from DBY746 was split into two equal fractions. The first was subjected to sucrose density gradient centrifugation with no prior treatment to obtain the control profile (continuous line), while EDTA was added to the second fraction (final concentration 25mM) before centrifugation (dotted line). The arrow below the diagram represents the direction of sedimentation, and the arrows at the top show the positions of polysomes containing 1, 2, 3, 4 and 5 ribosomes respectively.

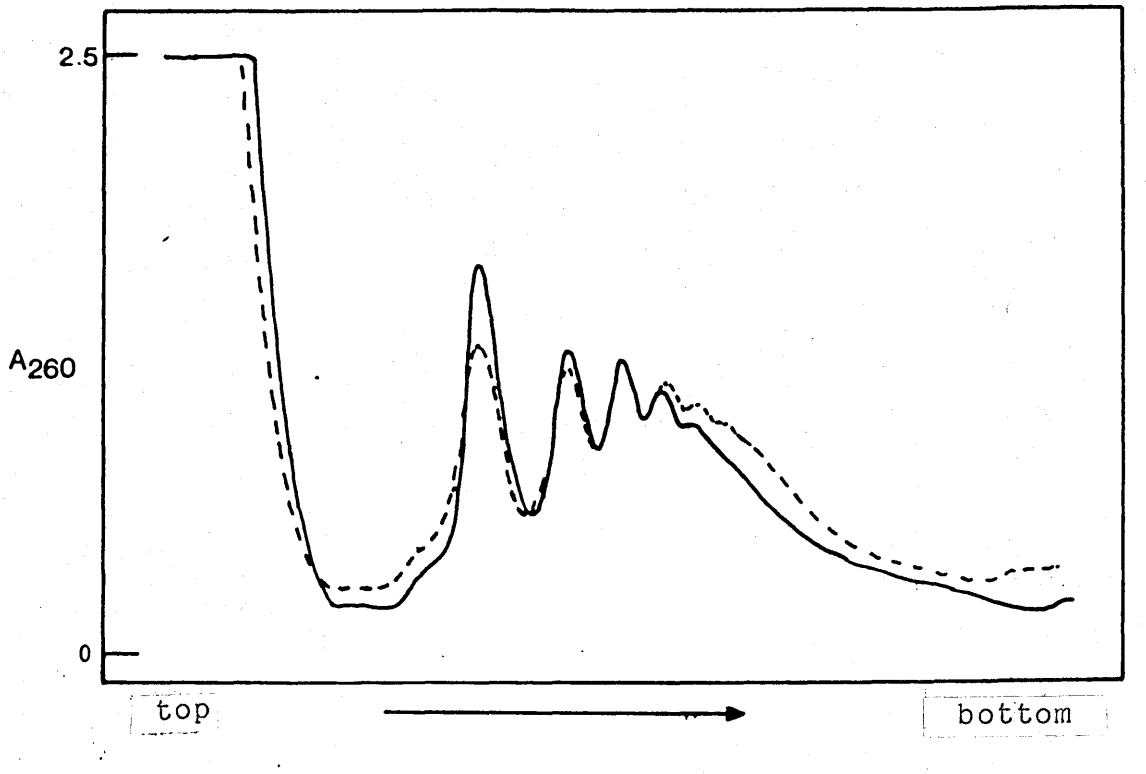


Figure 3.1.4 The effect of cycloheximide on yeast polysomes

A post-mitochondrial supernatant from DBY746 was split into two equal fractions. The first was subjected to sucrose density gradient centrifugation with no prior treatment to obtain the control profile (continuous line), while cycloheximide was added to the second fraction (final concentration 5mM) before centrifugation (dotted line). The arrow below the diagram represents the direction of sedimentation.

cycloheximide (an inhibitor of translational elongation) to a final concentration of 5mM, for the 10 min immediately prior to lysis, led to a reduction in the size of the "monosome" peak and a general increase in the rate of sedimentation of the "polysomal" material (see Fig 3.1.4).

Therefore, bythe three criteria described above, the absorbance profiles at 260nm represent the separation of yeast polysomes.

The last stage of the method involves the quantitation of specific mRNAs across yeast polysome gradients. Before embarking on mRNA quantitation experiments, it was important to demonstrate that the mRNA contained within polysome gradients was intact.

The integrity of RNA from across polysome gradients was tested by gel electrophoresis and Northern blotting. A polysome gradient prepared from DBY746 was split into eight equal fractions, these fractions were precipitated with ethanol and the RNA extracted from them as described in section 2.4.5. The RNA was suspended in H_2O and one-half of each sample loaded onto a non-denaturing agarose gel and subjected to electrophoresis. After staining with EtBr, the gel was photographed under UV light (Fig. 3.1.5). The observation that the 25S and 18S rRNAs were intact and that there was little smearing of these bands was generally found to indicate that the RNA preparation as a whole was intact. Agarose gel electrophoresis was used to check the yields of all RNA and mini-RNA preparations. It would appear that the

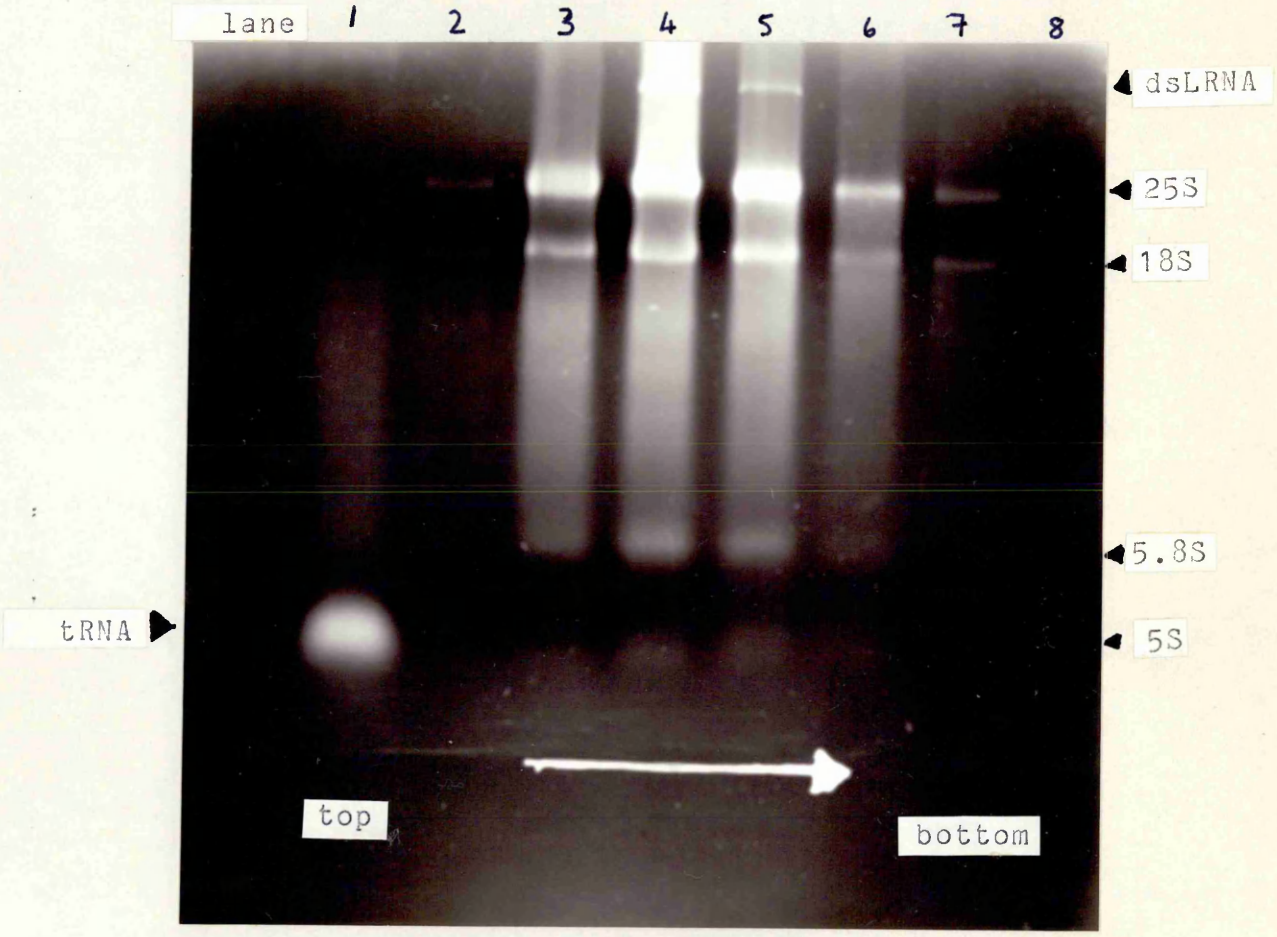


Figure 3.1.5 Integrity of RNA from polysome gradients

A polysome gradient prepared from DBY746 was split into eight equal fractions, the RNA was extracted and subjected to TBE gel electrophoresis. The arrow below the diagram represents the direction of sedimentation.

RNA is intact across the polysome gradient. The fractions at the top of the gradient probably contain low molecular weight RNA (e.g. tRNAs) which is smearing due to the speed at which the gel was run. RNA isolated from a second polysome gradient was subjected to Northern blotting and challenged with a probe for the PYK mRNA (nick-translated SPK2). The autoradiograph shows that the PYK mRNA is intact across the polysome gradient (Fig. 3.1.6). Differential degradation of mRNA in different regions of the gradient appears not to have taken place.

The distribution of specific mRNAs across polysome gradients has been tested using the following probes. Actin mRNA was analysed using riboprobes synthesised from SPACT9 (chapter 2.6.4). The β-galactosidase mRNA was analysed using a nick-translated fragment of the β-galactosidase coding region (chapter 2.6.4). The glyceraldehyde-3-phosphate dehydrogenase mRNA was analysed using the labelled oligonucleotide 133 (chapter 2.6.4). The RP1 or L3 mRNA was analysed using the labelled oligonucleotide 130 (chapter 2.6.4). The pyruvate kinase mRNA was analysed either by using riboprobes synthesised from SPK2 or by labelled oligonucleotides (chapter 2.6.4). 18S rRNA was analysed using nick-translated SP65R (chapter 2.6.4).

Preliminary experiments were performed with each probe to ensure all hybridisation reactions were quantitative. Probes were calculated to be present in at least 5-fold excess, and RNA dilution series were used in initial experiments to check that hybridisation was indeed

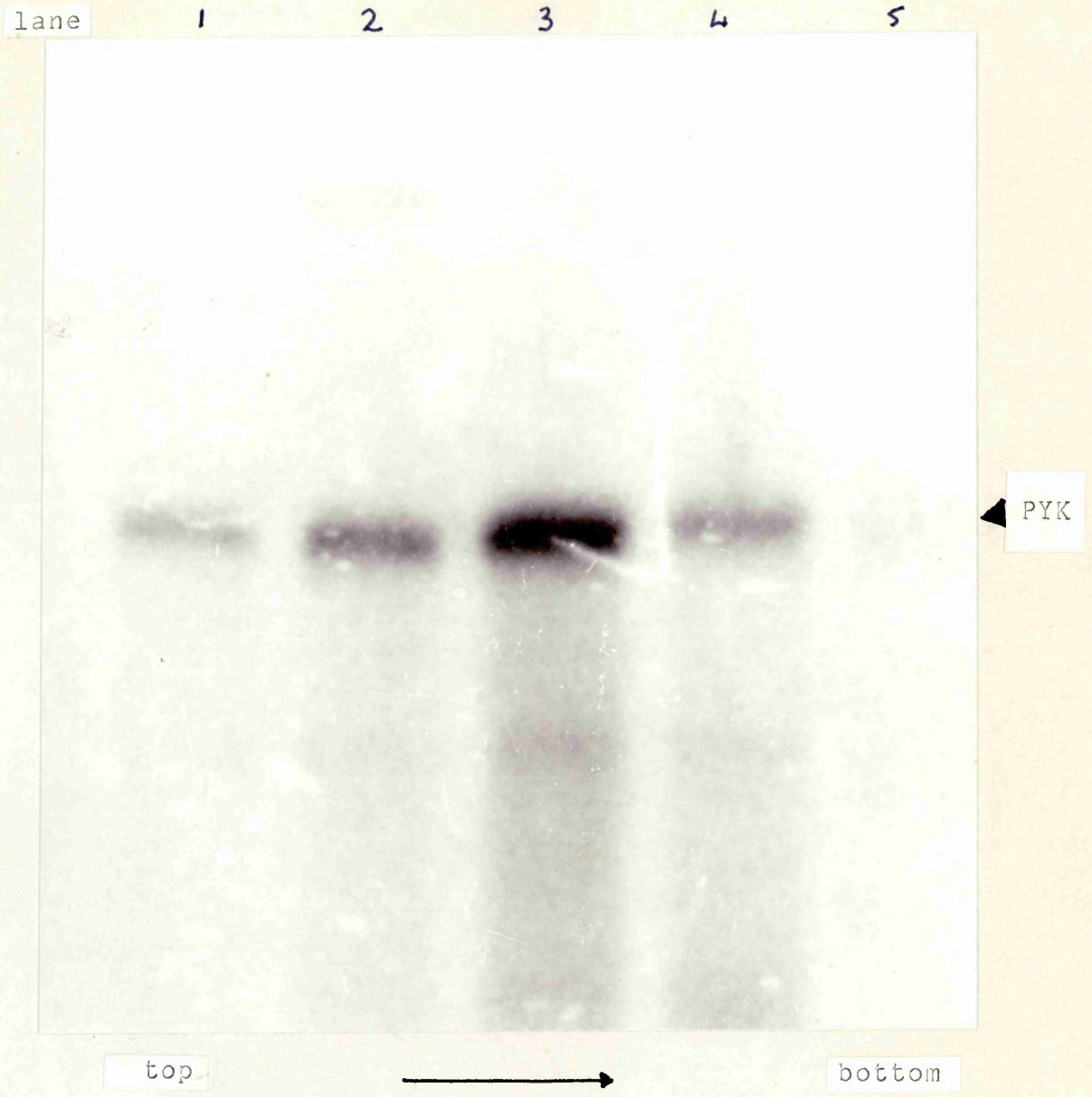


Figure 3.1.6 Integrity of the PYK mRNA across a polysome gradient

RNA was isolated from a polysome gradient of DBY746, subjected to Northern blotting and challenged with a probe specific for the PYK mRNA. The arrow indicates the direction of sedimentation.

quantitative. Some results are shown in Fig. 3.1.7.

After hybridisation and autoradiography, the nitrocellulose filters from different gradients were cut up into individual dots and subjected to scintillation counting. The results for each fraction were then converted into a percentage of the total radioactivity bound to the filter, and the data plotted graphically. Fig 3.1.7 illustrates the results of one such experiment. In some cases, the debris sedimenting at the bottom of the tube was seen to be disturbed during fractionation of the gradients. Any hybridisation resulting from fractions containing this debris was subsequently adjusted. Also, after examination of the autoradiograph, fractions whose activity was obviously spurious were discarded.

An examination of the absorbance profiles at 260nm revealed a relationship between distance from the top of the gradient and the number of ribosomes on an mRNA. When the logarithm of the ribosome loading of the mRNA is plotted against the sedimentation of the complex (in mm), the data approximate to a straight line (Fig. 3.1.8). By crude extrapolation of this graph, it was possible to estimate approximately where an mRNA carrying ten ribosomes would sediment.

The positions of sedimentation of polysomal complexes were estimated from the A_{260} profiles using diluted post-mitochondrial supernatants and these were marked on the graphs showing the distribution of the total radioactivity

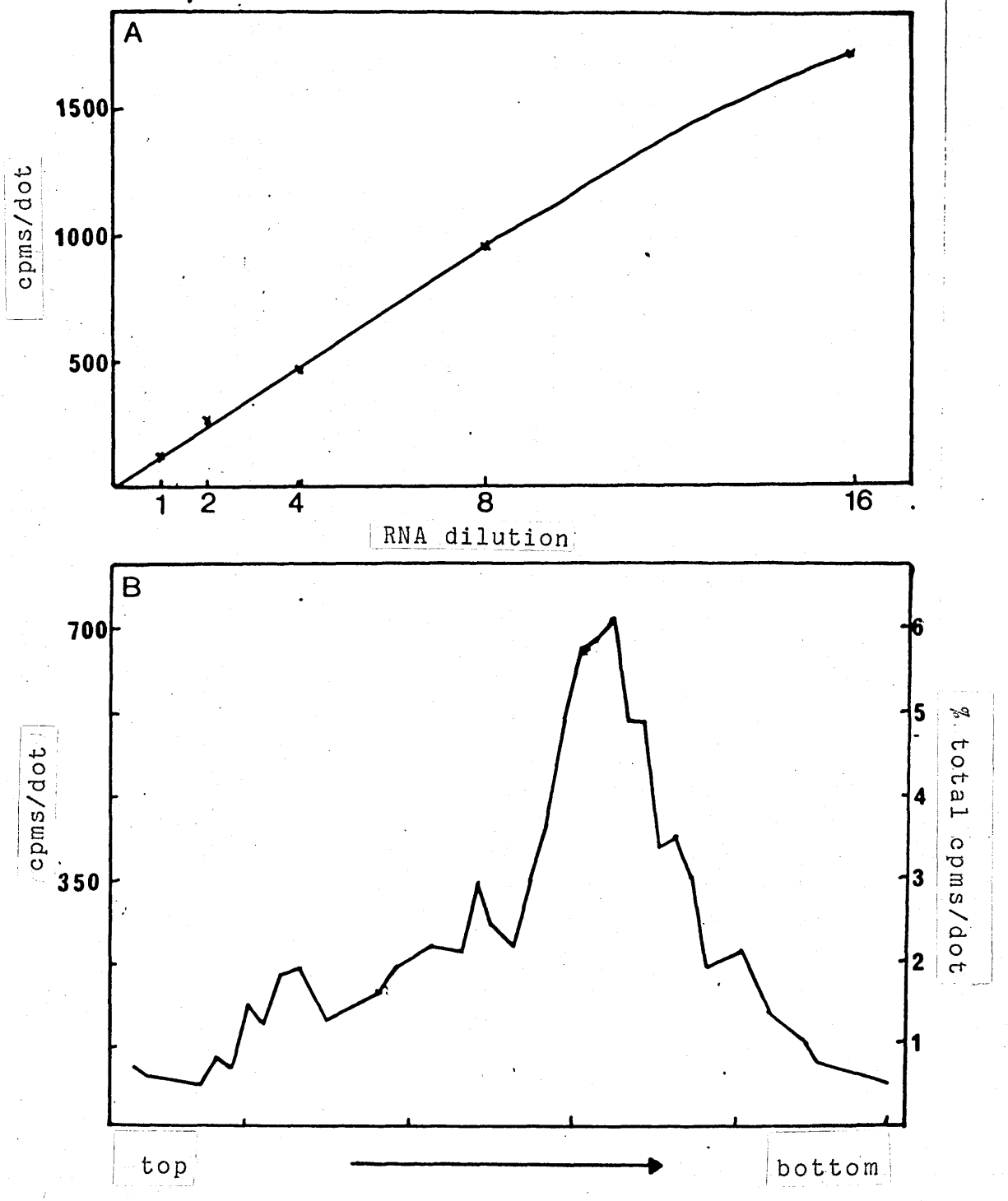


Figure 3.1.7 Demonstration of quantitative hybridisation

A: set of RNA dilution standards; B: the distribution of the PYK mRNA is given both in terms of cpms/dot (left hand axis) and %total cpms/dot (right hand axis)

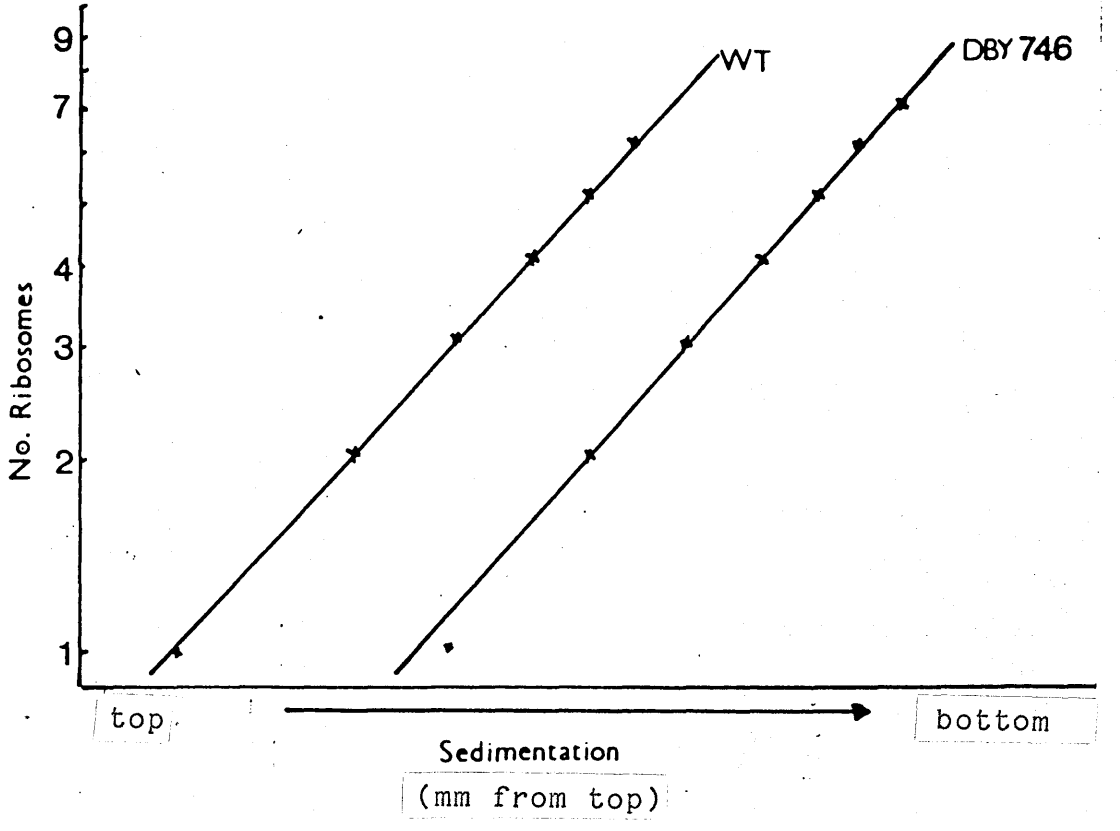


Figure 3.1.8 Relationship between the ribosome loading of an mRNA and its sedimentation on sucrose density gradients

for each probe used. To facilitate the comparison of the distributions of different mRNAs, these graphs were marked into different regions of sedimentation and the amount of the total radioactivity present in each region was estimated by cutting out and weighing each region of the graph.

As far as was possible, the distributions of mRNAs on polysome profiles were compared within gradients, and not between gradients, in order to minimise errors.

3.2 The Method in Use

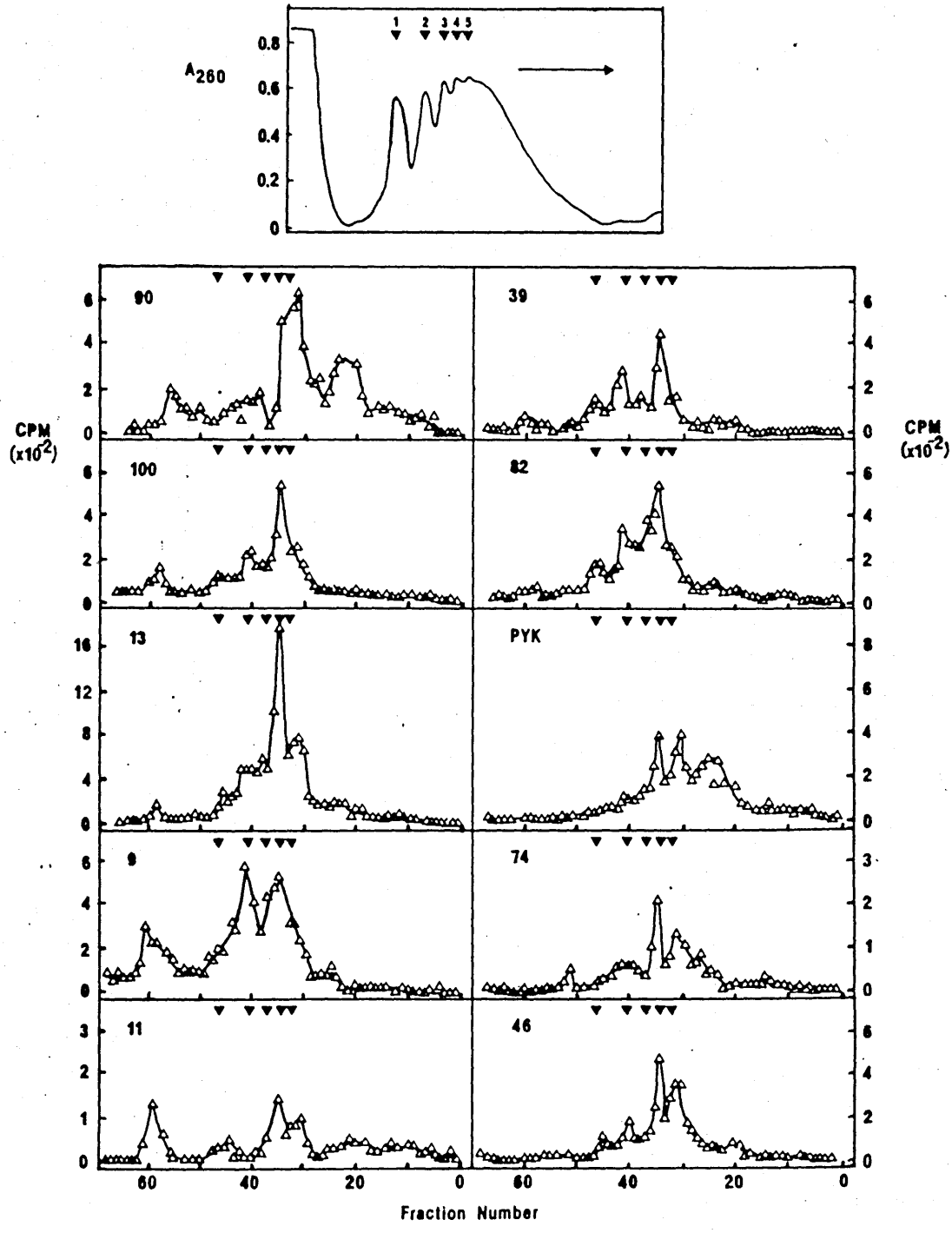
This method of polysome analysis has been used in this laboratory in a number of projects. T.C.Santiago has shown the existence of two populations of differing mRNA stability in yeast; one population is considerably more stable than the other when mRNAs of similar length are compared (Santiago *et al*, 1986). In a collaborative experiment, polysome preparations were made from *S.cerevisiae* DBY746. The distribution across polysome gradients of 10 specific yeast mRNAs of known half-life (6.6->100 min) was analysed to test if there was a relationship between mRNA translation and degradation. Although the mRNAs varied in their distribution across the same polysome gradients, no obvious correlation was observed between the stability of an mRNA and its ribosome loading *in vivo* (Fig. 3.2.1; Santiago *et al*, 1987). A similar result was obtained in a second experiment. This observation has strong implications concerning the mechanisms of mRNA degradation in yeast (Brown *et al*, 1988). It strongly suggests that the ribosome

Figure 3.2.1

Comparison of the distribution of ten different mRNAs of different half-life across the same polysome gradient. The polysome gradient shown at the top of the figure was divided into 68 equal fractions, which were used to prepare 10 identical dot blots for analysis of the mRNAs shown. The small arrows at the top of each profile show the position of polysomes containing 1 to 5 ribosomes, respectively. The length and half-life of each mRNA are given below:

mRNA	length (bases)	half-life (minutes)
90	1,100	6.6 +/- 0.67
100	500	10.4 +/- 1.1
13	740	12.1 +/- 1.0
9	440	16.5 +/- 1.5
11	700	18.0 +/- 2.2
39	380	18.3 +/- 1.5
82	370	22.3 +/- 3.1
PYK	1,600	59.9 +/- 7.8
74	1,050	83.4 +/- 9.2
46	550	>100

(from Santiago *et al*, 1987)

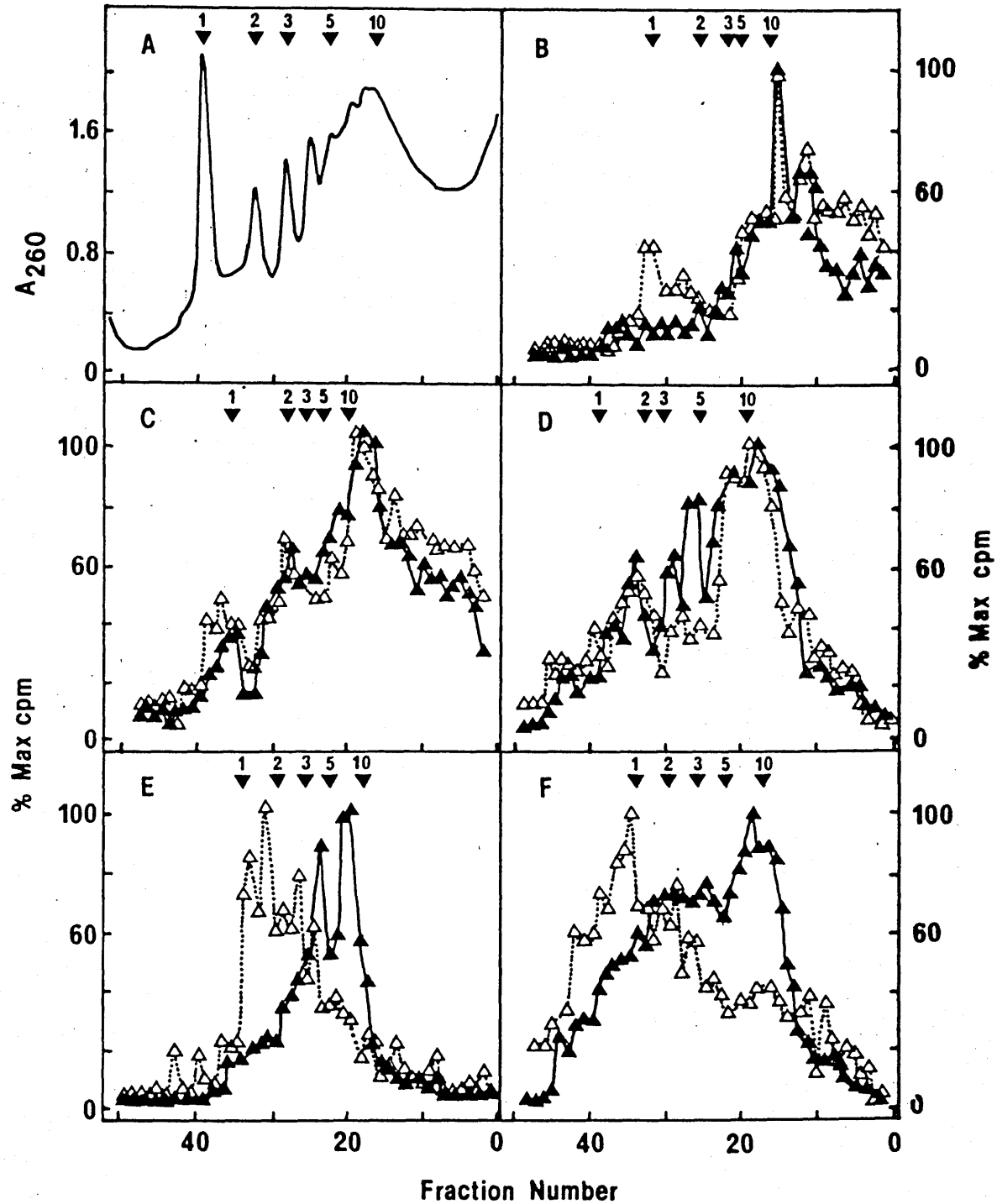

A260
1 2 3 4 5
0.8
0.6
0.4
0.2
0
90
39
100
82
13
PYK
9
74
11
46
CPM (x10^-2)
CPM (x10^-2)
60
40
20
0
Fraction Number

loading upon a natural mRNA does not in itself determine the stability of that mRNA in yeast (Santiago *et al*, 1987).

Polysome gradients were also prepared, in conjunction with Dr.I.J.Purvis, in experiments to investigate the effects of alterations within the 3'-UTR of the *PYK* mRNA upon its stability and translation *in vivo* in *S.cerevisiae* (Purvis *et al*, 1987b). The replacement of approximately 50bp of the 3'-UTR of the *PYK* gene, with a single unit (in either orientation), or a dimer, of a modified pUC13 polylinker, was found to have little effect on the distribution of the *PYK* mRNA across polysome gradients relative to the wild-type *PYK* mRNA internal control (Fig. 3.2.2). Interestingly, the deletion of this 50bp from the 3'-UTR, or the insertion of a strong inverted repeat sequence at the site of the deletion, both affect the translation of the *PYK* mRNA (Fig. 3.2.2). The basis for this result is unknown, but one common feature of these modifications is that each would bring sequences at the 3'-end of the 3'-UTR close to the termination codon (Purvis *et al*, 1987b). This might inhibit translation of the mRNA in some as yet unknown manner. Effects of the 3'-UTR upon mRNA translation have been observed in other systems (Zaret & Sherman, 1984; Kruys *et al*, 1987).

Figure 3.2.2

Comparison of the distribution of wild-type and modified PYK mRNAs across polysome gradients. Polysome gradients (e.g.A) from strains bearing different plasmid-borne PYK constructs were divided into 48 equal fractions and used to prepare dot blots. Different PYK mRNAs were quantified using specific oligonucleotides; ▲ chromosomal PYK mRNA; △ plasmid-borne 3'-UTR-modified PYK mRNA. The different gradients shown are polysome gradients prepared from DBY746 transformed with single-copy plasmids carrying each modified PYK gene: B = one polylinker inserted; C = one polylinker (opposite orientation to B); D = two polylinkers in tandem repeat; E = three polylinkers, two in inverted repeat (predicted to form a very stable stem-loop structure); F = 50bp deletion. (from Purvis et al, 1987b)


A
B
C
D
E
F
1
2
3
5
10
A_{260}
1.6
0.8
0
100
60
% Max cpm
40
20
Fraction Number

CHAPTER 4

RESULTS AND DISCUSSION:

IS THE *PYK* MESSENGER RNA OF *S.CEREVISIAE* SUBJECT TO DOSAGE COMPENSATION AT THE LEVEL OF TRANSLATION?

4.1 Introduction

This investigation arose out of the results from an initial experiment to look at the effects of manipulations of the PYK gene on the translation of its mRNA (these manipulations are described in detail in Chapter 5). The results of this initial experiment suggested that the PYK gene of S.cerevisiae was subject to dosage compensation at the translational level. Therefore, experiments were devised in an attempt to investigate this phenomenon further.

4.2 Transformation and Results

DBY746 was transformed separately with the five plasmids supplied by Searle: pLD1(35) WT, 1C, 2D, 3K and 4H. (These plasmids are described in Chapter 5. Briefly, pLD1(35)WT consists of the wild-type PYK gene cloned onto the multicopy vector pJDB207. pLD1(35)1C, 2D, 3K and 4H only differ from pLD1(35)WT in that they contain 42bp insertions into the unique Xba1 site at the 5'-end of the PYK coding region.) One transformant with each plasmid was selected and, along with the host strain, subjected to polysome analysis. The cultures were grown to approximately the same optical density at 600nm (A_{600}=0.6), whereupon the cells were harvested and sphaeroplasted. After a standard recovery period (Chapter 2.3), a proportion of the sphaeroplasts were stored under ethanol (for RNA extractions at a later date) whilst the remainder were lysed, and a post-mitochondrial supernatant was centrifuged on sucrose

density gradients. The gradients were fractionated and the RNA in each was denatured by treatment with formaldehyde. A measured proportion of each fraction was dot-blotted onto nitrocellulose, and these filters were challenged with radioactive probes specific for the PYK mRNA (nick-translated SPK2), actin mRNA (nick-translated SPACT9) and the RP1 mRNA (end-labelled oligonucleotide 130). The amount of radioactivity bound to individual dots was determined by scintillation counting, and the data is presented graphically in Figs. 4.2.1, 4.2.2 and 4.2.3. The gradients have been divided into regions bearing 0-2, 3-6, 7-10 and >10 ribosomes, and the proportion of each specific mRNA in these regions determined to facilitate comparisons. This data is presented in Tables 4.2.2, 4.2.3 and 4.2.4. Table 4.2.1 illustrates the distribution of the material from each of the gradients which absorbs at 260nm (mainly RNA). It can be seen that these gradients are quite similar.

An examination of the data for the control mRNAs, actin (Fig. 4.2.2 and Table 4.2.3) and RP1 (Fig. 4.2.3, and Table 4.2.4) leads to the conclusion that, although differences between the gradients are evident, the distributions of the two control mRNAs are fairly similar. On the whole, the polysome gradient from the transformant containing the WT plasmid does appear to exhibit a slight bias toward lower polysomes than those carrying plasmids pLD1(35)1C, 2D, 3K and 4H. In contrast, that of DBY746 appears slightly biased toward larger polysomes. These differences may be due to slight differences in the physiological states of the cells

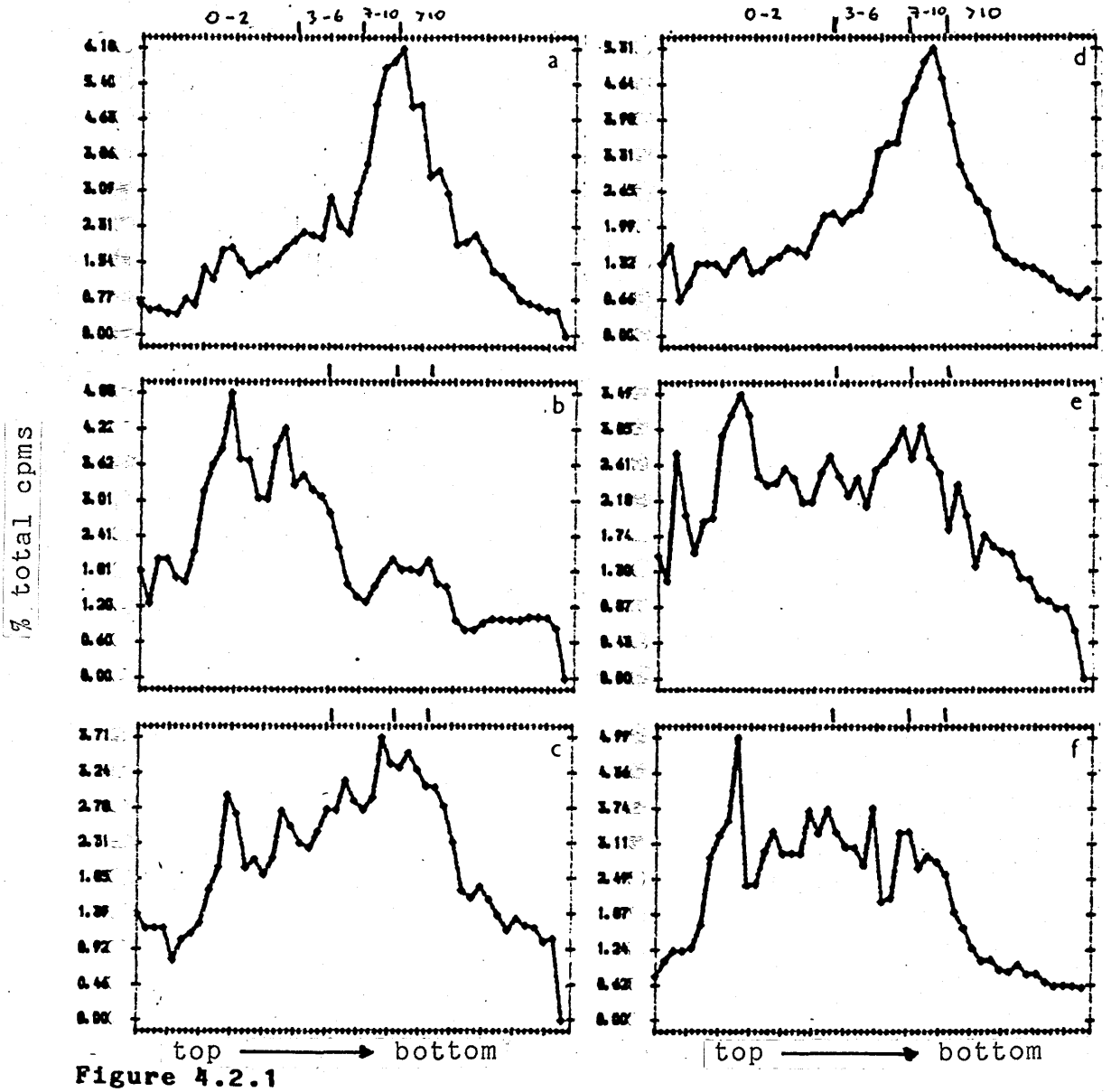


Figure 4.2.1

Distribution of the PYK mRNA across polysome gradients prepared from host (DBY746) and transformed (WT, 1C, 2D, 3K & 4H) strains. (see also Table 4.2.2)

	Strain	TCF		Strain	TCF
a	DBY746	11740	d	2D	10255
b	WT	13739*	e	3K	24598
c	1C	16031	f	4H	12018

* WT gradient diluted 3-fold before dotting

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 10 ribosomes respectively.

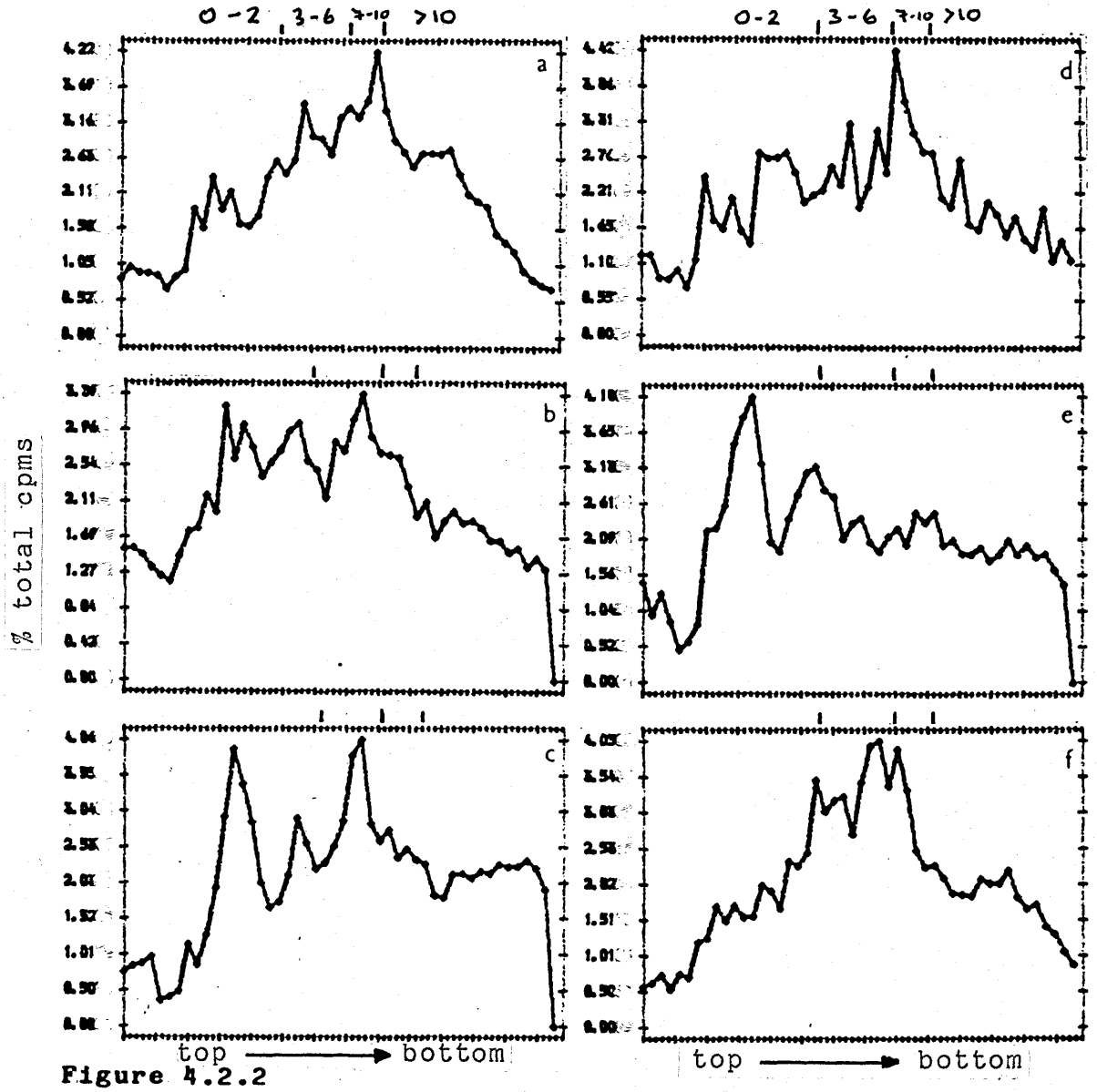


Figure 4.2.2

Distribution of the actin mRNA across polysome gradients prepared from host (DBY746) and transformed (WT, 1C, 2D, 3K & 4H) strains. (see also Table 4.2.3)

	Strain	TCF		Strain	TCF
a	DBY746	9920	d	2D	14794
b	WT	3147*	e	3K	20489
c	1C	18777	f	4H	11884

* WT gradient diluted 3-fold before dotting

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 10 ribosomes respectively.

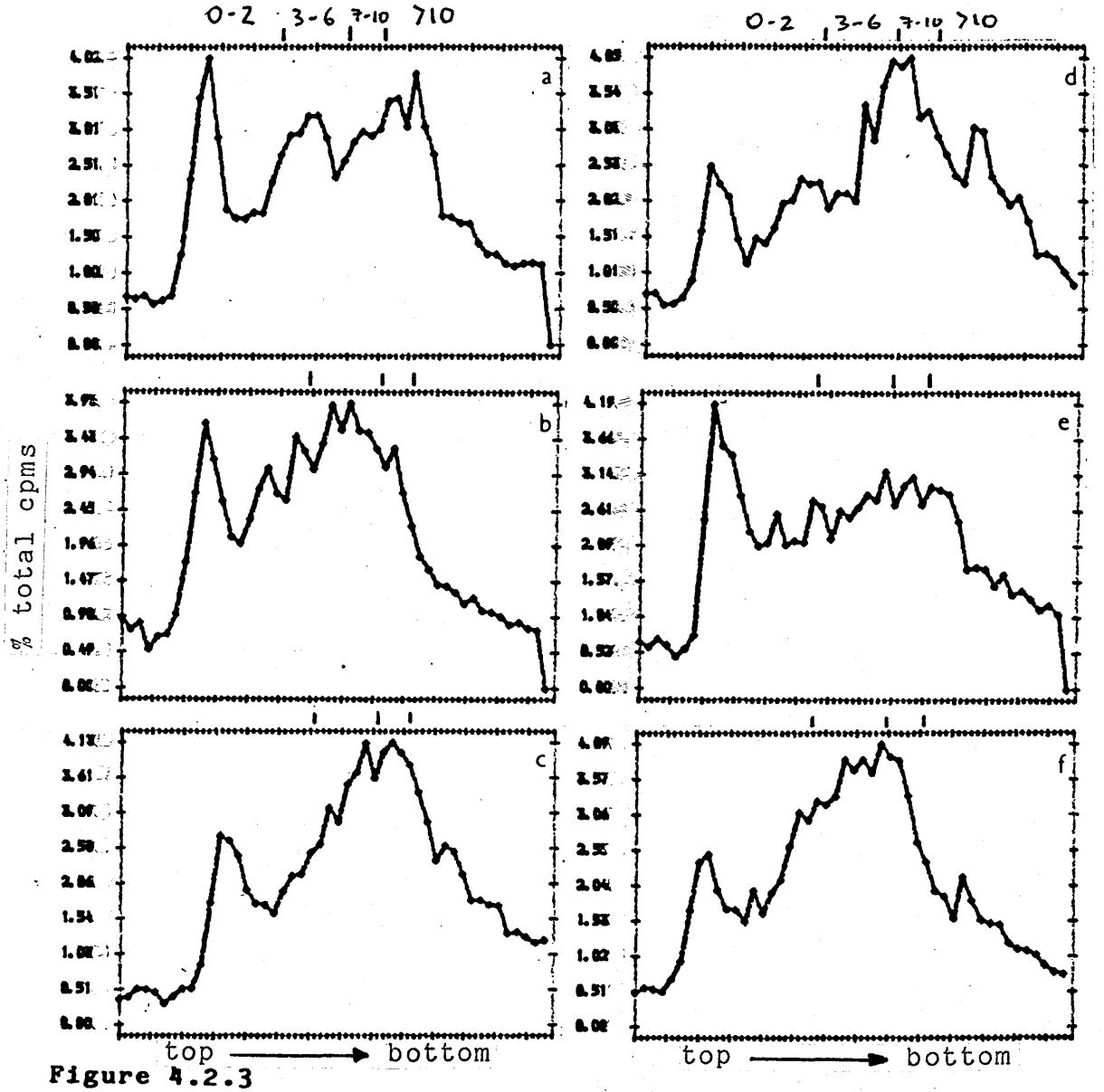


Figure 4.2.3

Distribution of the RP1 mRNA across polysome gradients prepared from host (DBY746) and transformed (WT, 1C, 2D, 3K & 4H) strains. (see also Table 4.2.4)

	Strain	TCF		Strain	TCF
a	DBY746	7729	d	2D	7555
b	WT	6649*	e	3K	8159
c	1C	10892	f	4H	7683

* WT gradient diluted 2-fold before dotting

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 10 ribosomes respectively.

Strain	Polysome fraction (% total absorbance profile)				PYK mRNA (% total)
	0-2	3-6	7-10	>10	
746	25	30	25	21	0.56 ± 0.2
WT	25	35	22	18	5.93 ± 2.9
1C	21	38	22	19	3.14 ± 1.3
2D	21	29	29	21	1.81 ± 0.8
3K	22	32	21	25	4.31 ± 1.1
4H	21	32	28	19	1.96 ± 0.3

Table 4.2.1

Distribution of the absorbance profiles at 260nm into specific regions of polysome gradients prepared from host (DBY746) and transformed (WT, 1C, 2D, 3K & 4H) strains.

Strain	Polysome fraction (% total cpms)				PYK mRNA (% total)
	0-2	3-6	7-10	>10	
746	15	17	16	52	0.56 ± 0.2
WT	73	15	5	8	5.93 ± 2.9
1C	36	35	16	13	3.14 ± 1.3
2D	22	29	24	25	1.81 ± 0.8
3K	47	21	12	20	4.31 ± 1.1
4H	53	25	11	11	1.96 ± 0.3

Table 4.2.2

Distribution of the PYK mRNA across specific regions of polysome gradients prepared from host (DBY746) and transformed (WT, 1C, 2D, 3K & 4H) strains.

Strain	Polysome fraction (% total cpms)			
	0-2	3-6	7-10	>10
746	24	24	13	40
WT	45	27	12	17
1C	45	23	11	21
2D	36	24	15	24
3K	36	24	15	25
4H	26	32	16	26

Table 4.2.3

Distribution of the actin mRNA across specific regions of polysome gradients prepared from host (DBY746) and transformed (WT, 1C, 2D, 3K & 4H) strains.

Strain	Polysome fraction (% total cpms)			
	0-2	3-6	7-10	>10
746	30	24	11	35
WT	43	31	13	13
1C	34	26	20	21
2D	28	26	17	29
3K	41	24	12	23
4H	34	34	15	18

Table 4.2.4

Distribution of the RP1 mRNA across specific regions of polysome gradients prepared from host (DBY746) and transformed (WT, 1C, 2D, 3K & 4H) strains.

for each transformant, despite the fact that they were all harvested at similar A_{600} readings. Alternatively, these differences may simply be due to differences in the pouring of the sucrose gradients.

The data on the distribution of the PYK mRNAs (Fig. 4.2.1, and Table 4.2.2) would appear to indicate that, compared to the slight differences observed between gradients for the control mRNAs, increasing the level of the PYK mRNA leads to a decrease in the ribosome loading of this mRNA. This effect on PYK mRNA translation is most evident when the gradients for DBY746 and pLD WT are compared; these strains exhibit an approximately 10-fold difference in PYK mRNA levels (DBY746 = 0.56%, WT = 5.93%) and there is a dramatic reduction in the ribosome loading on the PYK mRNA in the WT transformant. This effect of decreasing ribosome loading with increasing PYK mRNA level appears to hold for transformants pLD WT, 1C, 2D and 3K. However, transformant 4H exhibits a lower ribosome loading than might be predicted on the basis of this relationship. It seems likely that the insertion in the PYK 4H gene causes a decrease in the ribosome loading of this mRNA (this observation was confirmed in later experiments - see section 5.3).

It was concluded from this experiment that some form of dosage compensation mechanism was operating which seems to inhibit gross overexpression of the PYK gene, and that it was acting at the level of translation. That is, an increase in the level of PYK mRNA was being at least

partially counterbalanced by a decrease in the ribosome loading of this mRNA. Whilst this initial observation pointed toward the existence of an interesting phenomenon, interpretation was complicated by the fact that the transformants analysed contained dissimilar PYK genes.

Further experiments were devised to investigate this dosage compensation phenomenon further, whilst excluding this complication. It was decided to concentrate on yeast transformants which carry the wild-type PYK plasmid (pLD1(35)WT), and yet exhibit different levels of PYK mRNA. RNA was prepared from twelve transformants using the mini-RNA preparation described in section 2.4.4, and the abundance of the PYK mRNA in these transformants determined by dot-blotting (Table 4.2.5). Using this data, three transformants (WM4, WM5 and WN2) which contained widely differing PYK mRNA levels, were selected for comparison with the host strain. These strains were grown up and polysome preparations carried out as described in section 2.3. The results are presented in Fig. 4.2.4 and Fig. 4.2.5 and summarised in Tables 4.2.6-9. An examination of the three control mRNAs (actin, G3PDH and RP1), reveals that the polysome gradients from DBY746, WM4 and WM5 were very similar. Unfortunately, gradient WN2 was considerably different from the others with much more material sedimenting in the monosomes and relatively low molecular weight polysomes. It is possible that this was due to some RNase contamination in this preparation. Whilst this difference does make comparisons between gradients more

Strain	PYK mRNA (% total mRNA)	Strain	PYK mRNA (% total mRNA)
WTA	2.0	WM3	1.4
WTC	0.6	WM4	8.9
WTE	0.6	WM5	4.0
WTF	1.7	WN1	1.7
WTG	3.5	WN2	2.0
WTI	0.8	WN3	1.0

Table 4.2.5

Preliminary measurements of PYK mRNA abundance from 12 pLD(1)35 WT transformants.

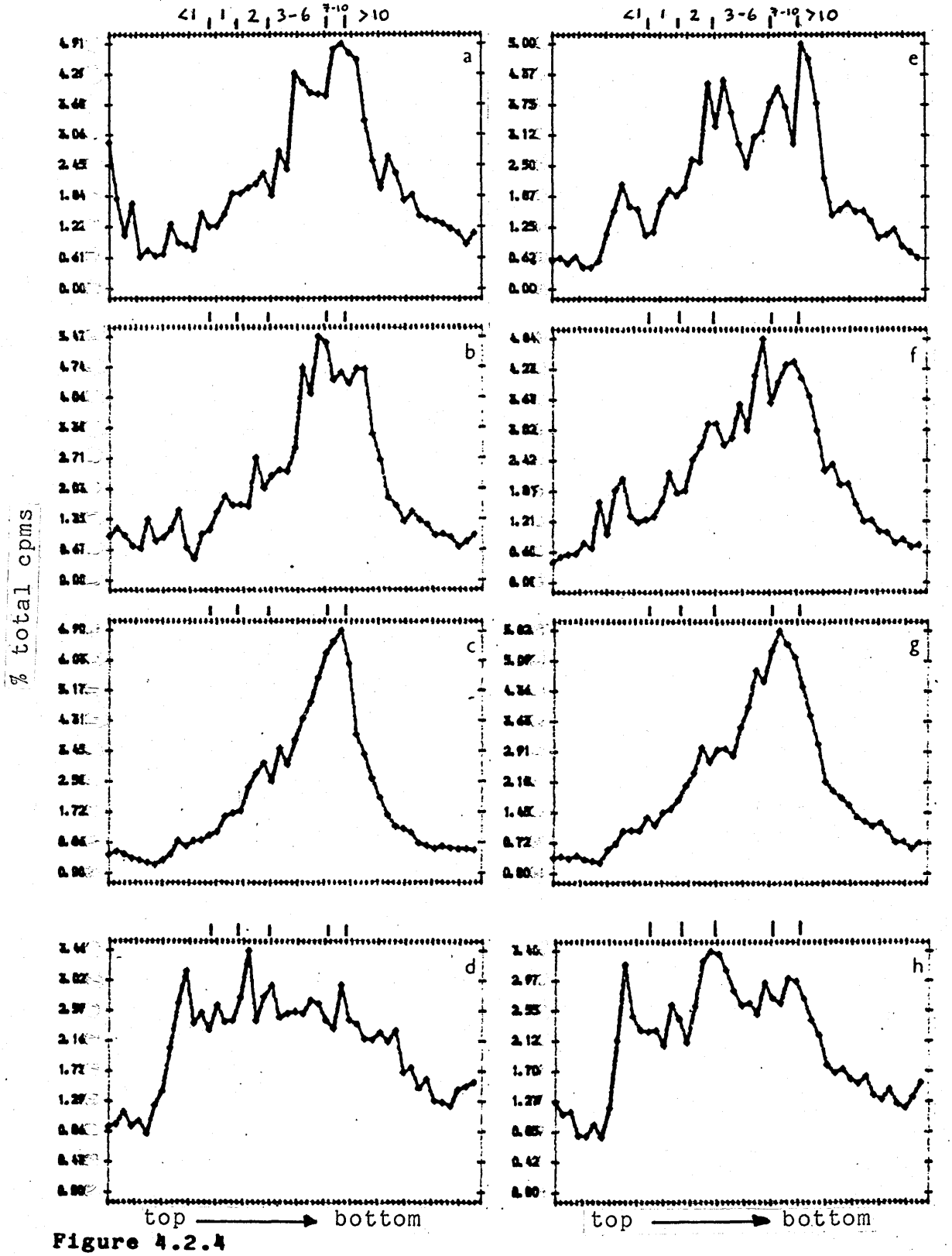


Figure 4.2.4

Distribution of the PYK, actin, G3PD and RP1 mRNAs across polysome gradients prepared from transformed strains WM4 and WM5.

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 10 ribosomes respectively.

	Strain	mRNA	TCF		Strain	mRNA	TCF
a	WM4	PYK	74460	e	WM5	PYK	154135
b	WM4	ACTIN	84243	f	WM5	ACTIN	136025
c	WM4	G3PD	36201	g	WM5	G3PD	34615
d	WM4	RP1	3989	h	WM5	RP1	3886

Figure 4.2.4 (cont'd)

	Strain	mRNA	TCF		Strain	mRNA	TCF
A	WN2	PYK	60839	E	DBY746	PYK	69751
B	WN2	ACTIN	59749	F	DBY746	ACTIN	77882
C	WN2	G3PD	14236	G	DBY746	G3PD	9247
D	WN2	RP1	3071	H	DBY746	RP1	4172

Figure 4.2.5 (cont'd)

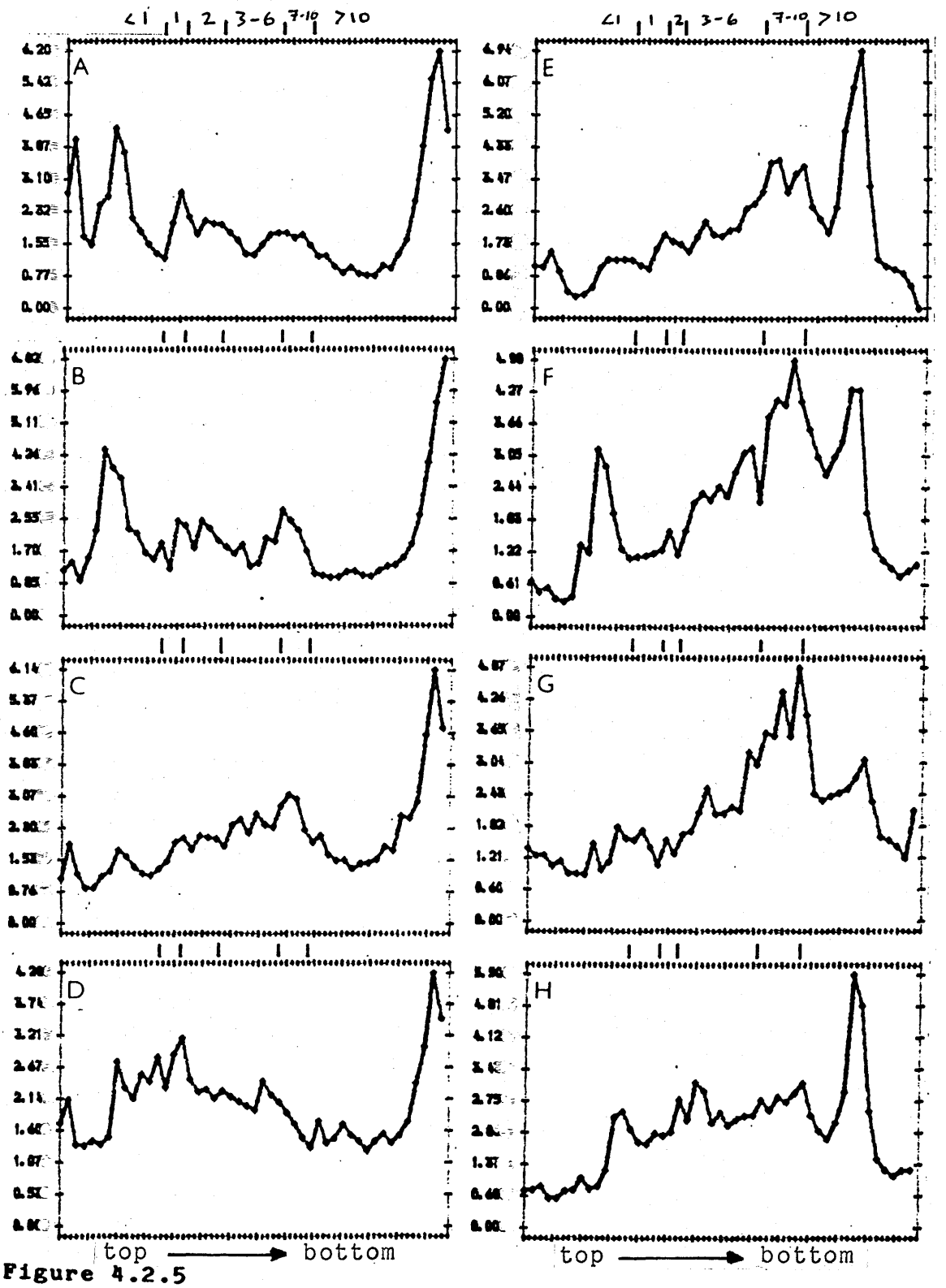


Figure 4.2.5

Distribution of the PYK, actin, G3PD and RP1 mRNAs across polysome gradients prepared from host (DBY746) and transformed (WN2) strains. (see also Figure 4.2.4 and Tables 4.2.6-9)

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 10 ribosomes respectively.

Strain	Polysome fraction (% total cpms)					PYK mRNA (% total)
	<1	1-2	3-6	7-10	>10	
DBY746	12	12	18	29	30	0.7 ± 0.3
WM4	10	13	24	25	28	1.0 ± 0.4
WM5	10	17	25	21	28	1.1 ± 0.3
WN2	43	25	18	10	5	2.0 ± 0.9

Table 4.2.6

Distribution of the PYK mRNA across specific regions of polysome gradients prepared from host (DBY746) and transformed (WM4, WM5 & WN2) strains.

Strain	Polysome fraction (% total cpms)				
	<1	1-2	3-6	7-10	>10
DBY746	17	12	12	30	30
WM4	9	13	25	26	27
WM5	11	16	24	25	24
WN2	44	23	16	14	3

Table 4.2.7

Distribution of the actin mRNA across specific regions of polysome gradients prepared from host (DBY746) and transformed (WM4, WM5 & WN2) strains.

Strain	Polysome fraction (% total cpms)				
	<1	1-2	3-6	7-10	>10
DBY746	9	8	29	33	22
WM4	6	15	27	33	19
WM5	6	16	25	31	24
WN2	17	21	31	21	10

Table 4.2.8

Distribution of the G3PD mRNA across specific regions of polysome gradients prepared from host (DBY746) and transformed (WM4, WM5 & WN2) strains.

Strain	Polysome fraction (% total cpms)				
	<1	1-2	3-6	7-10	>10
DBY746	10	30	16	23	20
WM4	20	25	25	17	14
WM5	16	26	20	17	21
WN2	28	28	28	10	6

Table 4.2.9

Distribution of the RP1 mRNA across specific regions of polysome gradients prepared from host (DBY746) and transformed (WM4, WM5 & WN2) strains.

difficult, it does nevertheless seem clear that there are no significant differences in the distribution of the PYK mRNA across the different gradients. This is contrary to what had been anticipated given the apparently strong evidence for dosage compensation in the experiment shown in Fig.4.2.1-3. The reason for this apparent discrepancy became obvious when the levels of PYK mRNA in these strains were re-measured using RNA extracted from the same culture that was used for the polysome preparations. The results of this analysis are presented in Table 4.2.10. The data show that a large decrease had occurred in the PYK mRNA levels during the analysis of transformants WM4 and WM5. That is, from the time that the PYK mRNA levels of these transformants was first measured, to the time of the polysome preparations, the PYK mRNA levels had decreased considerably. Although the actual growth rates of these transformants were not measured in this study, the presence of colonies of different sizes upon transformation might perhaps have been interpreted as the results of plasmid-induced stress. Certainly when the transformants were first grown in culture they appeared to grow appreciably slower than the untransformed host DBY746. During the time of these experiments, the transformants were maintained by sub-culturing (approximately every 2 months) onto fresh selective agar followed by storage at 4°C.

This decrease or "wind-down" in PYK mRNA levels is most probably a consequence of decreased plasmid copy number. It

Strain	PYK mRNA (% total mRNA)	
	Initial value	Value at time of polysome analysis
WM4	8.9	1.0 ± 0.4
WM5	4.0	1.1 ± 0.3
WN2	2.0	2.0 ± 0.9
DBY746	0.6	0.7 ± 0.3

Table 4.2.10

Abundance of PYK mRNA measured in host (DBY746) and transformant (WM4, WM5 & WN2) strains before storage, and after storage prior to polysome analysis.

should be emphasised that more than 90% of the cells in the cultures used for the polysome preparations were LEU^+. (The plasmid borne auxotrophic marker was LEU2). The basis for this decrease in PYK mRNA levels ("wind-down") is not known. It seems likely that the presence of the PYK gene itself on the multicopy plasmid was the cause of this "wind-down" effect. The plasmid vector used in these studies was a derivative of 2-micron and, according to Murray and co-workers (1987), clones of cells with elevated levels of 2-micron (and presumably 2-micron based) plasmids tend to maintain a high copy number. Also, since 2-micron itself appears unable to counter upward fluctuations in copy number (Murray et al, 1987), it was suggested that the average copy number of the population might be expected to increase due to infrequent unequal segregation of 2-micron, followed by its amplification in the cell containing fewer plasmids (Murray et al, 1987). Our data appear to indicate that this 2-micron based plasmid does not follow this tendency when it carries the PYK gene. It therefore seems likely that there is strong selection pressure against overexpression of the PYK gene. Thus, those cells carrying fewer gene copies may have a selective advantage. This may not be surprising, since PYK is known to be one of the two sites of regulation of glycolytic flux. Disruption of PYK expression might well lead to metabolic imbalances detrimental to the growth of the cell. Glycolytic flux could be disrupted in two ways:

1. Increased levels of the PYK enzyme would lead to increased glycolytic flux. This might be detrimental to the

cell, possibly because it will lead to a decrease in the levels of important glycolytic intermediates.

2. Increased levels of PYK mRNA would lead to an increase in dosage compensation and this might possibly decrease the translation of PFK mRNA (PFK is the other regulatory step in glycolysis). A reduction in the translation of PFK mRNA would decrease glycolytic flux and thereby decrease generation of ATP. (This could have been tested by analysing the translation of PFK mRNA; but a probe was not available). It is possible that PYK overexpression reduces the viability of the yeast cells during storage. Experiments are currently in progress in this laboratory to further characterise this observed "wind-down" in PYK mRNA levels.

When the data on PYK mRNA levels for this experiment is considered (Table 4.2.10), it is no longer surprising that the PYK mRNA distributions across the different gradients are so similar (Fig. 4.2.4). Clearly, the similar polysomal distributions were due to the unfortunate fact that the PYK mRNA levels had decreased in WM4 and WM5 during the period of storage prior to polysome analysis.

Paul Moore (a new PhD student in the laboratory) has recently confirmed the observation of dosage compensation. He transformed DBY746 with pLD1(35) WT and selected a relatively fast-growing and a relatively slow-growing transformant (transformants which formed a large or small colony, respectively), anticipating that the difference in

growth rate was due to disparate levels of PYK mRNA. No preliminary determination of PYK mRNA levels in these transformants was performed, as in the previous experiment the PYK mRNA levels had decreased during the time taken to perform this analysis. These two transformants were immediately subjected to polysome analysis, the results of which are given in Fig. 4.2.6 and Table 4.2.11. The data shows that of the two gradients, the ribosome loadings for the control mRNAs, G3PD and RP1, are slightly higher for WT1 than for WT2. When this difference between the gradients is taken into account, it appears that the ribosome loading on the PYK mRNA of WT1 is lower than that for WT2. Both transformants show poorer ribosome loadings for the PYK mRNA than that for the host strain. Therefore, translational dosage compensation of PYK mRNA translation has now been confirmed in two independent experiments.

An experiment was performed to analyse this phenomenon further. This experiment was designed to delineate the cis-acting features within the PYK mRNA which were important in the dosage compensation mechanism. Since the majority of translational control systems so far examined appear to operate at the level of translation initiation (see section 4.3 for more details) it was decided to concentrate on the 5'-UTR of the PYK mRNA.

The plasmid YCpPKG2 (Fig. 4.2.7; Purvis et al, 1987c) contains the 5'- and 3'-UTRs of the PYK gene from S.cerevisiae fused to practically the entire coding region of the E.coli β-galactosidase gene. This plasmid contains

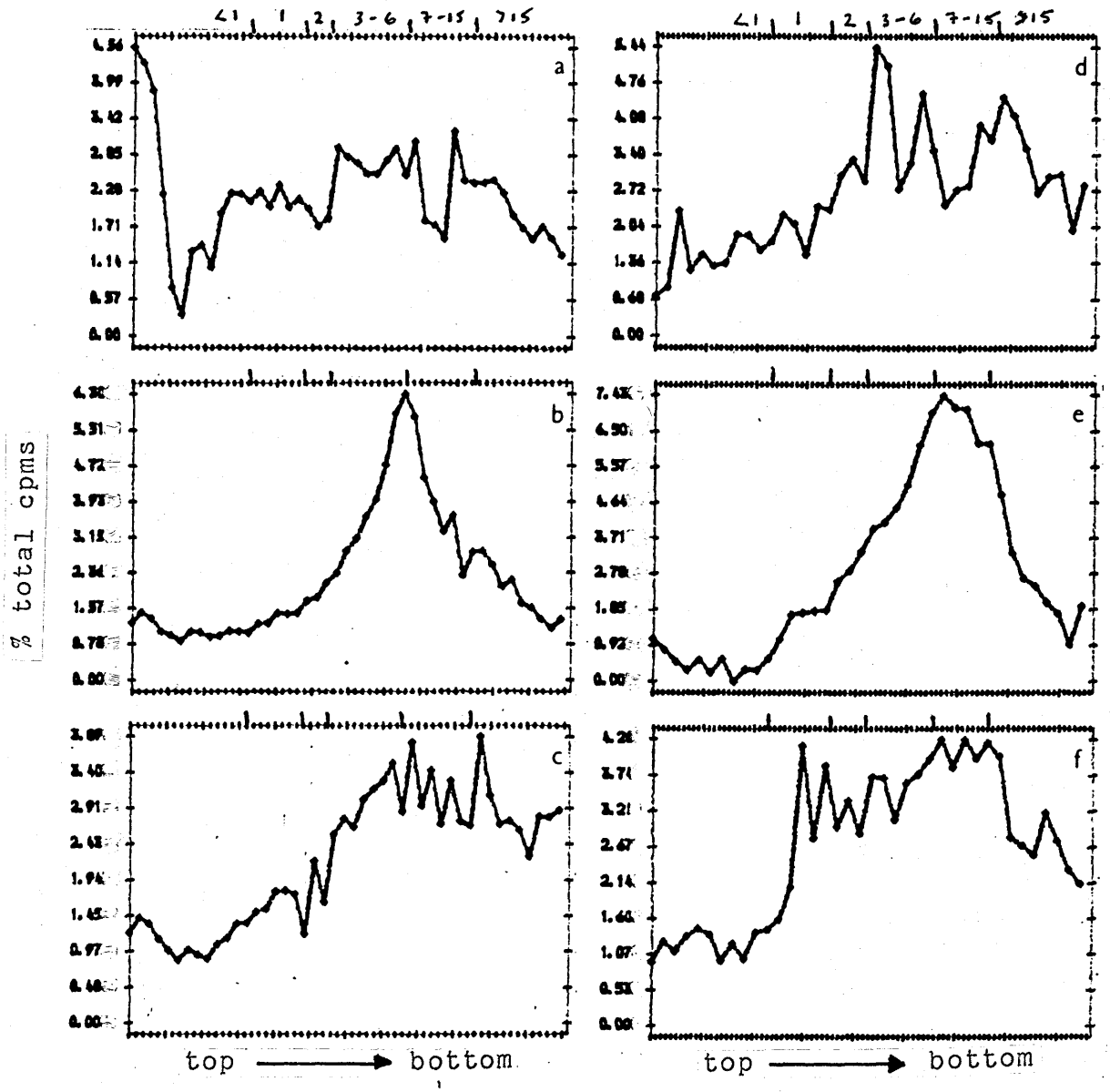


Figure 4.2.6

Distribution of the PYK, G3PD and RP1 mRNAs across polysome gradients prepared from transformants WT1 and WT2. (data from Paul Moore)

	Strain	mRNA	TCF		Strain	mRNA	TCF
a	WT1	PYK	14650	d	WT2	PYK	15246
b	WT1	G3PD	3768	e	WT2	G3PD	12655
c	WT1	RP1	4835	f	WT2	RP1	2814

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 15 ribosomes respectively.

mRNA	Strain	Polysome fraction (% total cpms)				PYK mRNA (% total)
		0-2	3-6	7-15	>15	
PYK	DBY746	23	25	39	13	0.6 ± 0.2
PYK	WT1	51	22	14	13	9.8 ± 0.8
PYK	WT2	42	32	10	15	5.7 ± 1.8
G3PD	DBY746	14	25	48	13	
G3PD	WT1	13	34	38	16	
G3PD	WT2	18	28	35	18	
RP1	DBY746	26	32	21	10	
RP1	WT1	22	31	25	22	
RP1	WT2	33	28	28	11	

Table 4.2.11

Distribution of the PYK, G3PD, and RP1 mRNAs across polysome gradients prepared from host (DBY746; my data) and transformed (WT1 & WT2; Paul Moore's data) strains.

centromeric sequences from chromosome IV and the selectable marker HIS3. It was decided to transform DBY746 with both YCpPKG2 (HIS3) and pLD1(35) WT (LEU2). The rationale behind the experiment was as follows. On the analysis of polysome gradients from double transformants which contain high PYK mRNA levels, if a decrease was observed in the ribosome loading on the PYK mRNA (generated from pLD1(35)WT), but not in that of the β-galactosidase/PYK fusion mRNA (generated from YCpPKG2), this would imply that the dosage compensation mechanism does not act solely on the 5'-(or 3'-)UTR of the PYK mRNA. Conversely, if the ribosome loading of the β-galactosidase/PYK fusion mRNA and the PYK mRNA were both adversely affected by high PYK mRNA levels, this would imply that a cis-acting element(s) in the PYK 5'-(or 3'-)UTR was responsible for, or was responding to, the dosage compensation mechanism.

Initially, attempts were made to transform a strain containing YCpPKG2 with pLD1(35) WT, but these proved unsuccessful. Therefore, DBY746 was transformed with both plasmids simultaneously and double transformants selected by selection for a LEU^+, HIS^+, Trp^-, Ura^- phenotype. After an incubation of approximately two weeks, two transformants displaying the correct phenotype had grown up. These transformants were grown in selective liquid culture and polysome preparations performed as described previously (section 2.3), with one slight difference in that some of the sphaeroplasts were used to estimate the β-galactosidase

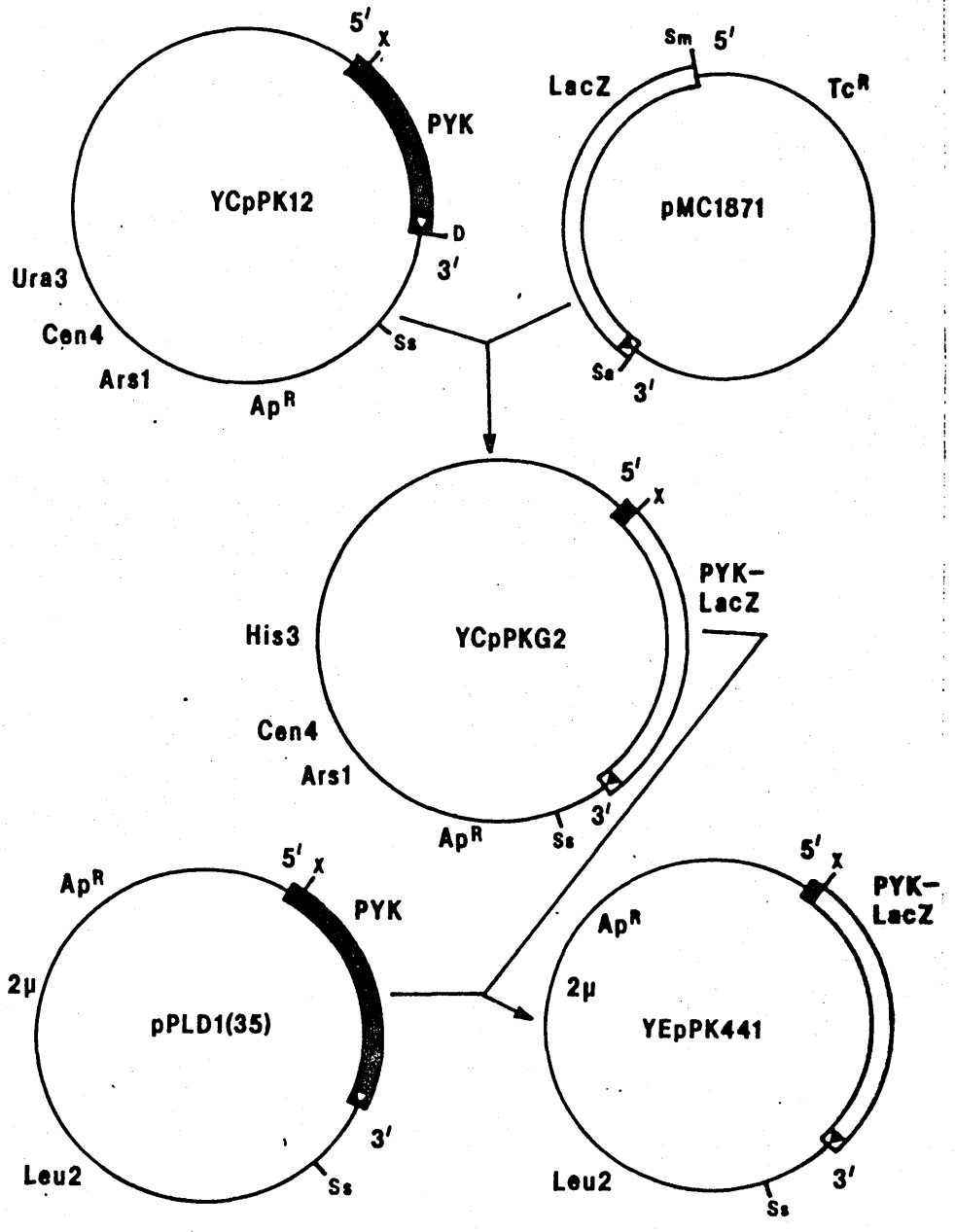


Figure 4.2.7

Simplified representation of the construction of single and multicopy yeast plasmid vectors containing the β-galactosidase gene of <u>E.coli</u> under <u>PYK</u> transcriptional control. The restriction sites designated Ss (SstI), X (XbaI), D (DraI) and Sm (SmaI) are not unique within the plasmids shown. Partial digestion, Klenow-filling and purification of various fragments from agarose gels were carried out by Dr.I.J.Purvis to insert the β-galactosidase coding sequence behind the translation initiation codon of PYK in both single and multicopy yeast plasmid vectors.

activities of the cultures (section 2.10). The results obtained from polysome gradients are presented in Fig. 4.2.8 and Fig. 4.2.9, and are summarised in Table 4.2.12; Table 4.2.13 shows the results of the β-galactosidase assays. The first point to note is that although the gradients are not identical, examination of the distributions of the control mRNAs (actin, G3PD and RP1) shows that the the gradients GW5 and GW6 are quite similar. Secondly, there does not appear to be any significant difference between the distribution of the PYK mRNA in gradients GW5 and GW6, although they are different from the host strain (see e.g. Fig. 4.2.4). Thirdly, the β-galactosidase/PYK fusion mRNA appears to be sedimenting on larger polysomes in the gradient from transformant GW5 than that for GW6. This difference in ribosome loading of the β-galactosidase/PYK mRNA in the two transformants appears to be confirmed by the difference in the β-galactosidase enzyme activity of the two transformant cultures.

4.3 Discussion

The initial observation that some form of dosage compensation mechanism appears to operate at the translational level on PYK expression, has recently been confirmed by Paul Moore in this laboratory.

An attempt by the author to repeat this observation was unwittingly defeated by careful experimental design. The levels of PYK mRNA in the transformants used for the experiment were accurately measured before polysome

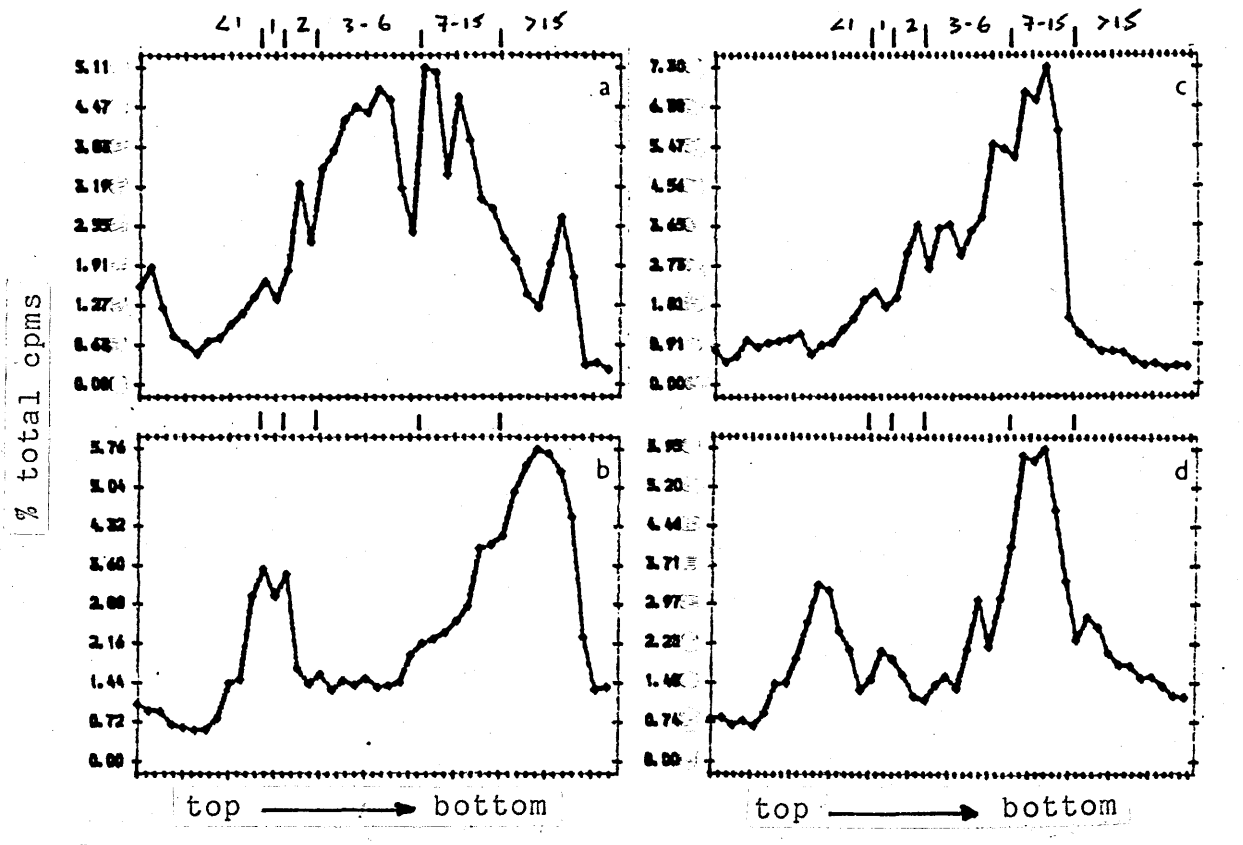


Figure 4.2.8

Distribution of the PYK and β-gal mRNAs across polysome gradients prepared from transformants GW5 and GW6 (see also Fig. 4.2.9 and Table 4.2.12)

	Strain	mRNA	TCF		Strain	mRNA	TCF
a	GW5	PYK	100977	c	GW6	PYK	60136
b	GW5	β-GAL	14289	d	GW6	β-GAL	7595

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 15 ribosomes respectively.

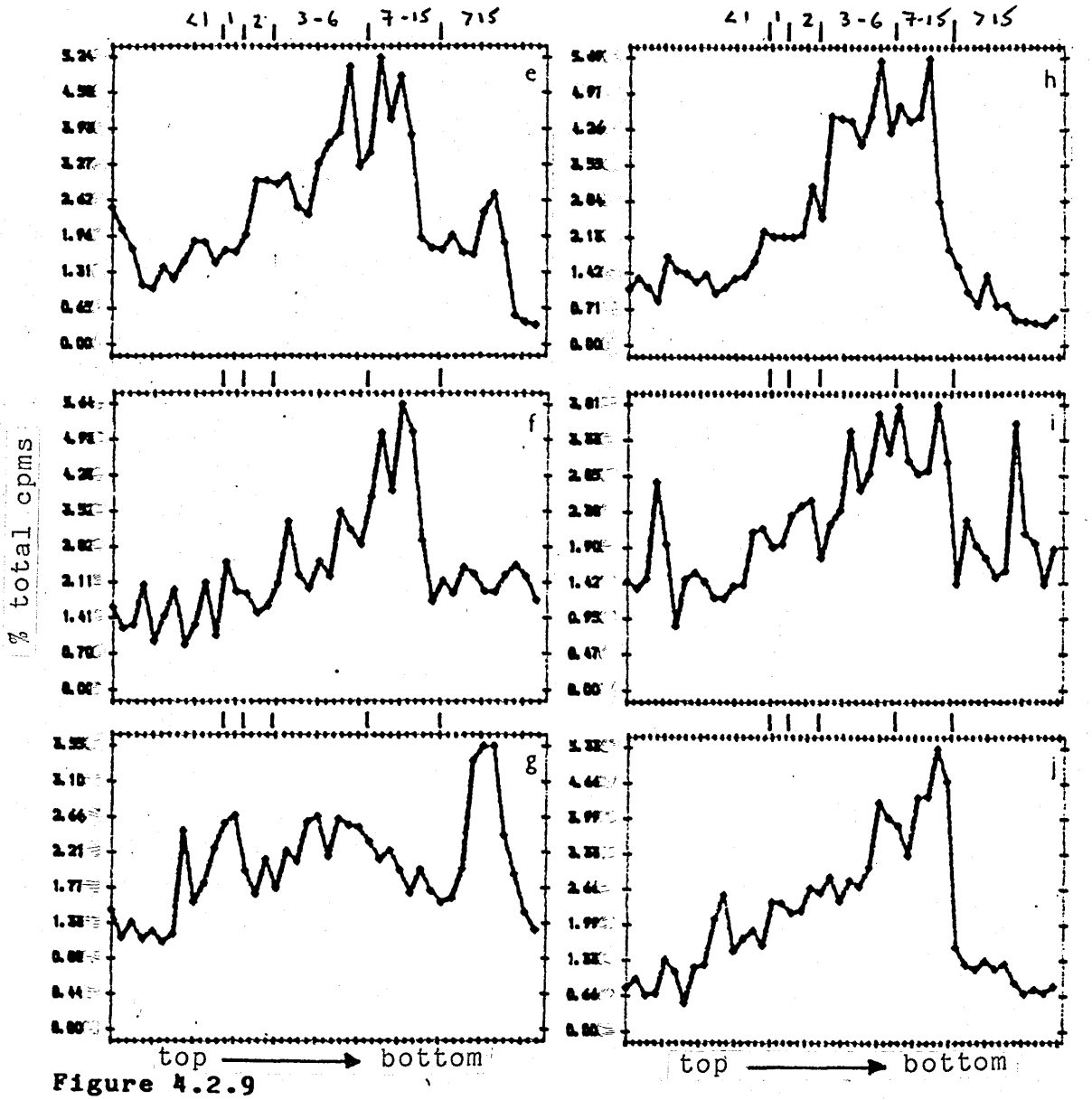


Figure 4.2.9

Distribution of the actin, G3PD and RP1 mRNAs across polysome gradients prepared from transformants GW5 and GW6.

(see also Fig. 4.2.8 and Table 4.2.12)

	Strain	mRNA	TCF		Strain	mRNA	TCF
e	GW5	ACTIN	83340	h	GW6	ACTIN	28380
f	GW5	G3PD	29677	i	GW6	G3PD	25302
g	GW5	RP1	5689	j	GW6	RP1	14298

The lines above the graph mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 & 15 ribosomes respectively.

mRNA	Strain	Polysome fraction (% total cpms)				mRNA (% total)
		0-2	3-6	7-15	>15	
PYK	DBY746	23	25	39	13	0.6 ± 0.2
PYK	GW5	20	38	31	11	2.9 ± 0.4
PYK	GW6	23	35	39	4	5.4 ± 2.5
B-GAL	DBY746	NA	NA	NA	NA	NA
B-GAL	GW5	26	11	21	42	0.072 ± 0.05
B-GAL	GW6	33	14	38	15	0.079 ± 0.06
ACTIN	DBY746	23	23	40	14	
ACTIN	GW5	27	39	25	9	
ACTIN	GW6	27	39	28	6	
G3PD	DBY746	14	25	48	13	
G3PD	GW5	22	34	34	9	
G3PD	GW6	34	29	21	16	
RP1	DBY746	26	32	31	10	
RP1	GW5	37	39	20	4	
RP1	GW6	32	31	30	6	

Table 4.2.12

Distribution of the PYK, B-gal, actin, G3PD and RP1 mRNAs across specific regions of polysome gradients prepared from host (DBY746) and doubly-transformed (GW5 & GW6) strains.

NA: not applicable

Strain	*PYK* mRNA (% total mRNA)	β-gal enzyme level[a] (10^3 x tetramers/viable cell)
GW5	2.9 ± 0.4	41.9
GW6	5.4 ± 2.5	7.4

Table 4.2.13

β-galactosidase enzyme levels of yeast transformants GW5 and GW6

[a]values adjusted to similar culture densities

preparations were begun, in order to select transformants with widely differing PYK mRNA levels. Since the problem of "wind-down" of PYK mRNA levels had not been diagnosed at the start of the experiment, no attempt was made to minimise the storage time between transformation and polysome analysis. Unfortunately, this meant that at the time of the polysome preparations, the levels of PYK mRNA in the transformants studied were considerably lower than had previously been measured and thus the experiment failed to address the problem for which it was designed.

The results of the β-galactosidase/PYK fusion experiment are of some interest, but it must be stressed that since there appeared no real difference in the ribosome loadings of the PYK mRNA in the two transformants, the data must be treated with extreme caution. Why a difference in the ribosome loading of the fusion mRNA should occur, and not occur on the PYK mRNA is the subject of some interest. Obviously a significant decrease in the ribosome loading on the PYK mRNAs was expected since the double transformants in this experiment had elevated levels of this mRNA (Table 4.2.13). It is not possible to make any firm conclusions until the experiment is repeated and different levels of PYK mRNA are shown to also affect the translation of this mRNA. In a large proportion of the systems which exhibit translational control that have been examined to date, this control is thought to be mediated at the level of initiation of translation (Hamilton et al, 1982; Thireos et al, 1984;

Warner et al, 1985; Benedetti & Baglioni, 1986; Werner et al, 1987). Hence it is attractive to speculate that the apparent inhibition of the translation of the β-galactosidase/PYK fusion mRNA at high PYK mRNA levels is mediated by sequences in the PYK 5'-UTR. However, it is possible that either the 5'-UTR, the 3'-UTR, or both regions of the PYK mRNA could be involved in the translational control mechanism, since both are present in the β-galactosidase/PYK construct. Translational control mediated by 3'-UTRs has been shown to occur in at least two cases: the translation of human β-interferon mRNA (Kruys et al, 1987) and the introduction of stable secondary structure into the 3'-UTR of the PYK gene affected the translation of its corresponding mRNA (Purvis et al, 1987a). Therefore, an effect of the PYK 3'-UTR in this translational dosage compensation mechanism cannot be excluded. It is most likely that the translational control mechanism observed acting on the PYK mRNA is doing so via a transient inhibition of translation initiation.

The translational control mechanisms of the CPA1 gene of S.cerevisiae (Werner et al, 1987), the GCN4 gene of S.cerevisiae (Thireos et al, 1984) and of Avian retroviral mRNAs (Katz et al, 1986) all operate via the presence of small upstream open-reading frames (upstream ORFs) in the 5'-leader sequences of these mRNAs. No such upstream ORFs are present in the 5'-leader of the PYK mRNA (Burke et al, 1983).

The TCH1 mRNA of S.cerevisiae, which encodes RP1 (also

referred to as ribosomal protein L3; Schultz & Friesen, 1983) is subject to translational control (Pearson *et al*, 1982; Warner *et al*, 1985). Cells containing 3.5 times the normal levels of translatable RP1 mRNA synthesised no more than 1.2 times as much RP1 protein as control cells (Pearson *et al*, 1982). This discrepancy could not be attributed to a decrease in protein stability (Pearson *et al*, 1982). These findings were confirmed by Warner and co-workers (1985) who showed from polysome analyses that in cells carrying excess copies of the *TCM1* gene there was a shift of the *TCM1* mRNA from larger to smaller polysomes. They suggested that this reduction in polysome size was most probably occurring via a transient inhibition of peptide initiation (Warner *et al*, 1985). However, this remains to be confirmed. Similar results were also reported for overexpression of the *CYH2* gene of *S.cerevisiae* and the translation of its mRNA (Warner *et al*, 1985). In each case, elevating the levels of one mRNA did not appear to have a corresponding effect on the translation of other mRNAs (Warner *et al*, 1985). In this study, a similar result has been observed with the expression of the *PYK* gene; elevated levels of the *PYK* mRNA did not appear to affect the translation of the control mRNAs actin and G3PD. Furthermore, the inhibition of *PYK* mRNA translation cannot be occurring via the same mechanism which affects RP1 translation since the distribution of this mRNA is also unaffected in these experiments (probe RP1).

Therefore, the possibility remains that we may be

observing the effect of an mRNA discriminatory factor which is specifically required for the efficient translation of the PYK mRNA. If the levels of PYK mRNA are increased, this factor becomes limiting for translation and, therefore, the rate of initiation from the PYK mRNA is decreased relative to other mRNAs. Alternatively, it may be that the PYK enzyme itself feedback inhibits the translation of its own mRNA. (The mechanism for this is unknown, but the enzyme might bind to the 5'-UTR of its own mRNA and thereby inhibit scanning along it.) An increase in the levels of PYK mRNA would lead to an increase in the levels of the enzyme with a corresponding increase in the level of inhibition of translation of the PYK mRNA. In fact, the PYK enzyme could itself be viewed as an mRNA discriminatory factor!

4.4 Future Experiments

It is necessary to repeat the experiment involving analysis of the double transformants containing both YCpPKG2 and pLD1(35)WT. It is necessary to test rigorously for a possible effect on PYK mRNA translation as well as for the one on the β-galactosidase/PYK fusion mRNA. It remains a distinct possibility that, for some unknown reason, the initial experiment described here has generated a misleading result. As described above, such an experiment is important in defining the cis-acting sequences within the PYK mRNA that are functionally important for the dosage compensation effect.

Further characterisation of the dosage compensation

mechanism is currently being attempted by expressing a *pyk* mRNA containing an N-terminal premature stop codon at high levels in DBY746. (This is currently being done by Paul Moore in this laboratory.) Should translation of the wild-type *PYK* mRNA be affected by this, this would imply that translational elongation of the *PYK* coding sequence was not necessary for the proper functioning of the dosage compensation mechanism. Therefore, this experiment will test the feasability of the two models described above, i.e. the likelihood that the mechanism involves competition between *PYK* mRNAs for a limiting factor at the level of translational initiation, or alternatively, that the mechanism might be operating via some feedback mechanism of the protein onto the mRNA. If this were the case, translation of the wild-type *PYK* mRNA would remain unaffected by high levels of non-translatable *pyk* mRNA.

Should the occurrence of a possible mRNA discriminatory factor be deemed likely, *in vitro* protein binding studies and mRNA competition studies could then be attempted using a yeast *in vitro* translation system (Tuite & Plesset, 1986), in an attempt to isolate this factor, or factors.

CHAPTER 5

RESULTS AND DISCUSSION:

THE EFFECTS OF MANIPULATIONS ON THE 5'-END OF THE *PYK* GENE ON ITS TRANSLATION *IN VIVO*

5.1 Introduction

At the outset of this project, G.D.Searle made available a number of plasmids containing oligonucleotide insertions immediately 3' to the ATG translation initiation codon of the PYK gene. The control plasmid pLD1(35)WT was a derivative of pAYE4(34), in which deletions totalling approximately 1kb had been made to generate a plasmid in which the Xba1 site immediately 3' to the ATG translation initiation codon was unique (Fig. 5.1.1). Into this unique Xba1 site, four different double-stranded oligonucleotide insertions had been made, each totalling 42bp. Two of these insertions were designed to analyse the effects of codon bias on the translation of the PYK mRNA (pLD1(35)1C & 2D), and analyses of these are described in section 5.2. The other two insertions were designed to analyse the effects of secondary structure involving the 5'-UTR of the PYK mRNA on its translation (pLD1(35)3K & 4H), and analyses of these are described in section 5.3. Robert Cafferkey of G.D.Searle had already sequenced the constructs to ensure that the inserted sequences were correct, and that there were no multiple insertion events. The plasmids have a 2-micron replication origin and thus should be maintained at high copy numbers upon transformation into yeast. Transformants with different levels of PYK mRNA arise depending on their plasmid copy number. This was particularly useful to Searle since, as a biotechnology company, they were interested at that time in any possible problems concerned with overexpression of heterologous sequences using PYK gene sequences.

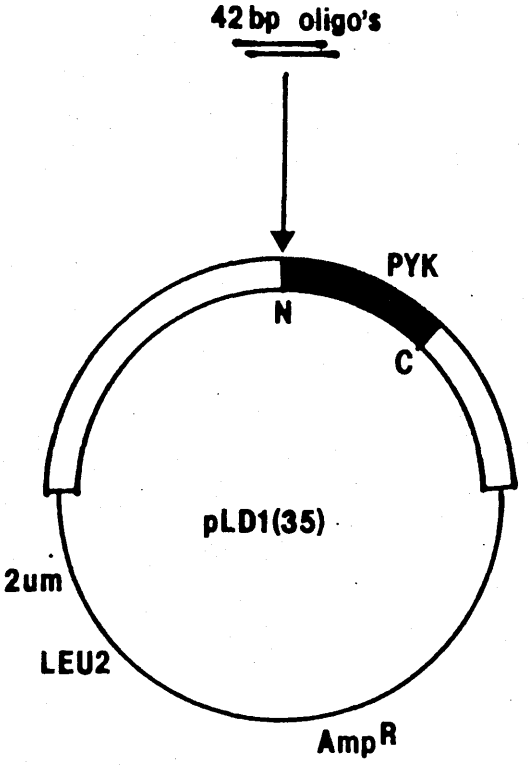


Figure 5.1.1

Plasmid pLD1(35) WT. The arrow indicates the site of oligonucleotide insertions into the PYK gene to generate plasmids pLD1(35) 1C, 2D, 3K and 4H.

An initial experiment in which single transformants containing each of the plasmids were analysed and compared with the untransformed host strain (DBY746) was described in the previous chapter. Also described in Chapter 4 were experiments which strongly suggested that the PYK gene of S.cerevisiae is subject to some form of translational dosage compensation. Clearly, dosage compensation effects will complicate translational analyses of the PYK insertion mutants. Therefore, the general approach in this area of work has been to attempt to rule out any effects due to dosage compensation. Hence, transformants exhibiting similar levels of PYK mRNA were selected for polysome analysis. Transformation was followed by growing each of the yeast transformants up in small-scale culture, isolating RNA from thes cultures, and measuring the abundance of the PYK mRNA in each. Initially, it had been hoped to assess the effects of the oligonucleotide insertions by expressing them at high levels and simply measuring the distribution of all PYK mRNA on polysome gradients. In this way, the relative effect of wild-type mRNA transcribed from the chromosomal locus would be minimised. This would have reduced the complication of analysing the translation of a mixed population of mutant and wild-type PYK mRNAs in each transformant. Unfortunately, due to problems of obtaining and maintaining high levels of PYK expression (caused by the "wind-down" effect) and to the phenomenon of dosage compensation, this approach could no longer be considered

suitable. Therefore, oligonucleotide probes were developed to distinguish between mutant and wild-type PYK mRNAs. These are described in the following sections.

5.2 The Influence of Codon Bias on the Translation of the PYK mRNA

5.2.1 Experimental Design

The purpose of this set of experiments was to determine whether the codon usage of a gene can affect its efficiency of translation, as measured by the ribosome loading of its mRNA determined from polysome analysis.

The two Searle plasmids pLD1(35)1C & 2D contained PYK genes mutated by the insertion of the oligonucleotide sequences described in Fig. 5.2.1. The inserted sequences were designed to encode almost identical amino acid sequences. The only difference between the two inserted amino acid sequences is the conservative substitution of a threonine residue for a serine residue at amino acid position 16. This was done in order to maintain the Xba1 site at the 5'-end of the inserted DNA unique. The insertion of similar amino acid sequences was important since it excluded possible secondary effects, however unlikely, such as some feedback mechanism from slightly differing proteins to the PYK mRNA. Also, the inserted amino acid sequence contains no particular bias toward basic, acidic, polar or uncharged amino acids (Fig. 5.2.1). Fig. 5.2.1 also illustrates the dramatic difference in the codon bias of the two inserted sequences. The sequence

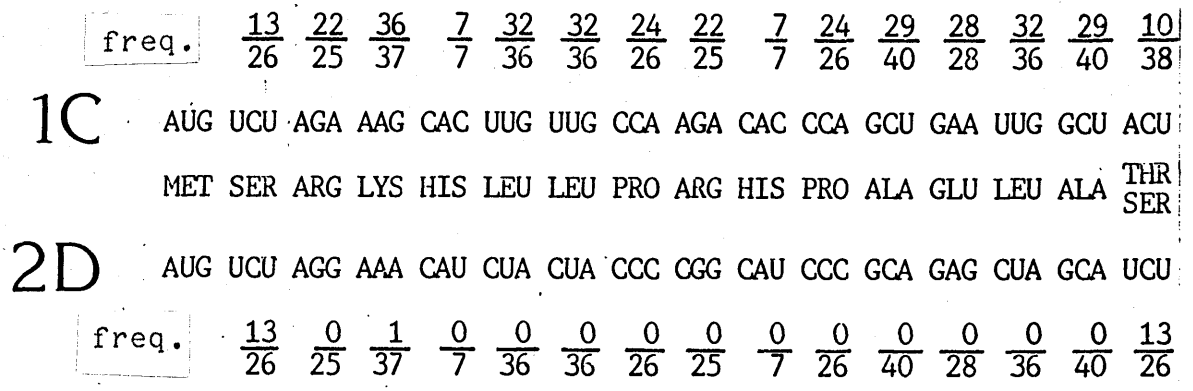


Figure 5.2.1

Sequences of the two oligonucleotide insertions in plasmids pLD1(35) 1C and 2D. The diagram also shows the frequency of occurrence of each of the codons used in the normal PYK mRNA.

contained in plasmid 1C has very good codon bias i.e. all the codons utilised fall into the subset of "preferred" codons (Bennetzen & Hall, 1982). In contrast, the sequence contained in plasmid 2D has very poor codon bias i.e. none of the codons utilised fall into the subset of "preferred" codons (Bennetzen & Hall, 1982). This bias is well illustrated by the frequency with which each of these codons are utilised in the normal PYK gene (Fig. 5.2.1).

The inserted sequences were also carefully designed to ensure that the predicted secondary structure in this region should not be radically different for the two mutant PYK mRNAs. Fig. 5.2.2 illustrates the computer-predicted secondary structures for the first 130 bases of the mutant PYK mRNAs (using the programme "FOLD"; Zuker & Stiegler, 1981). The predicted secondary structures and their free energies of formation are not radically different.

The sequence environment around the AUG translation initiation codon was not altered in either mutant. Experiments have demonstrated that the nucleotide at +4 is one of those which is important in determining translational efficiency (Kozak, 1982; Kozak, 1986a). A bias toward certain sequences in this region has recently been observed, especially in efficiently expressed yeast genes, and it is thought that these sequences might also regulate the efficiency of translation (Hamilton et al, 1987). Sequences 5' to the initiation codon were identical in the two constructs. Also, the first codon after the AUG and

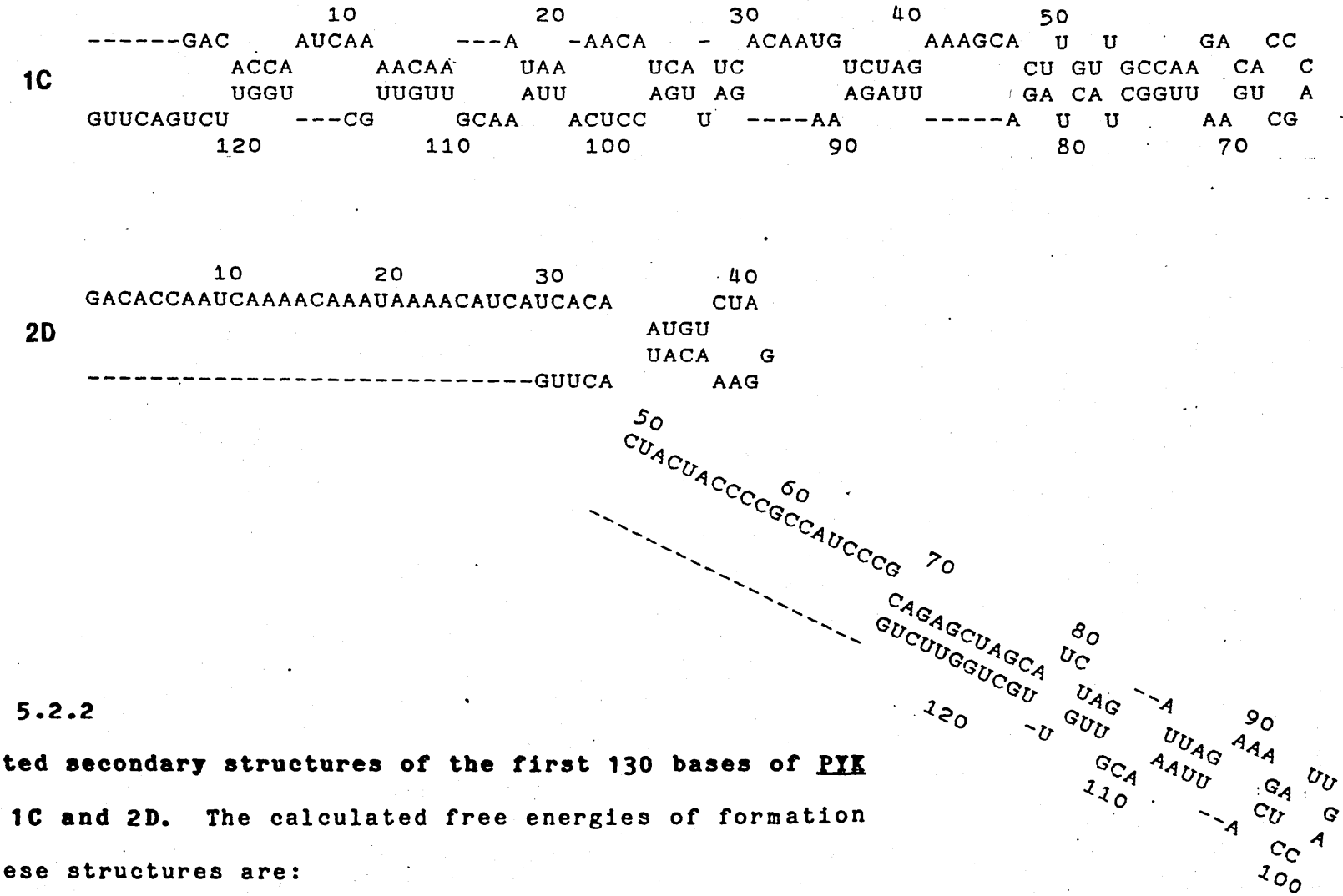


Figure 5.2.2

Predicted secondary structures of the first 130 bases of <u>PYK</u> mRNAs 1C and 2D. The calculated free energies of formation for these structures are:

1C -18.0 kcal mol^{-1}

2D -15.0 kcal mol^{-1}

especially the nucleotide at +6 were also identical.

5.2.2 Results

DBY746 was transformed with the plasmids pLD1(35)1C and pLD1(35)2D. Eleven transformants cotaining 1C, and six containing 2D were isolated, and their auxotrophic markers checked. These transformants were grown up in small (5ml, YPG) cultures, RNA extracted and the abundance of PYK mRNA estimated for each (Table 5.2.1). A range of PYK mRNA abundances was observed, and it was decided to compare transformants with relatively high, but similar, PYK mRNA levels (1CM6 & 2DN1) in which dosage compensation might be expected to affect translation of the PYK mRNAs to similar extents, and transformants with similar and relatively low PYK mRNA levels (1CN2 & 2D8) in which the effects of dosage compensation should be reduced. It is worth bearing in mind that the nature of the inserts might be exerting differential effects on the expression of the mRNAs, and hence might be influencing the dosage compensation mechanism to differing extents. At high levels of expression of the PYK mRNA with poor codon bias, there may be a significant drain on the pools of rare amino-acyl tRNAs in the cell. This might have an effect on the translation of other poorly biased mRNAs and hence be detrimental to the cell. Therefore, increased selection pressure might be operating for decreased expression of pLD1(35)2D compared with pLD1(35)1C.

The four transformants selected 1CM6, 1CN2, 2DN1 and

Strain	PYK mRNA (% total mRNA)	Strain	PYK mRNA (% total mRNA)
1CA	3.7	1CN1	3.8
1CB	4.1	1CN2	1.3
1C2	1.9		
1CM1	6.8	2D5	2.9
1CM2	5.7	2D8	1.4
1CM3	2.3	2DM1	7.9
1CM4	5.7	2DM2	5.4
1CM5	2.5	2DM3	3.6
1CM6	4.2	2DN1	4.6

Table 5.2.1

Preliminary measurements of PYK mRNA abundance in transformants containing plasmids pLD1(35) 1C or 2D.

2D8 were subjected to polysome analysis (chapter 2.3). Briefly, following an initial period of growth in minimal medium, the transformants were grown for a limited number of generations in rich medium before the cells were harvested and their cell walls removed. The sphaeroplasts were allowed to recover (Chapter 2.3), and then a proportion were stored under ethanol (for RNA extraction at a later date), whilst the remainder were lysed, and their post-mitochondrial supernatants subjected to sucrose density gradient centrifugation. These gradients were fractionated, the RNA denatured, and a proportion of each fraction dot-blotted onto nitrocellulose to be challenged with radioactive probes specific for various mRNAs.

The data from gradients 1CM6 and 2DN1 are presented in Fig. 5.2.3, Fig. 5.2.4 and Table 5.2.2. Although differences can be observed between the actin mRNA profiles for the two transformants, these differences are consistent between all the control mRNA profiles (actin, G3PD and RP1). There is consistently more material sedimenting in the 3-6 ribosome region of gradient 1CM6 in comparison to gradient 2DN1. Once again, this minor difference in the general profile of the gradient, might reflect slight variability in the physiological state of the transformants at the time of polysome analysis. Alternatively, these minor differences may be due to minor differences in the preparation of the sucrose gradients. If allowance is made for differences between gradients, there does not appear to be any

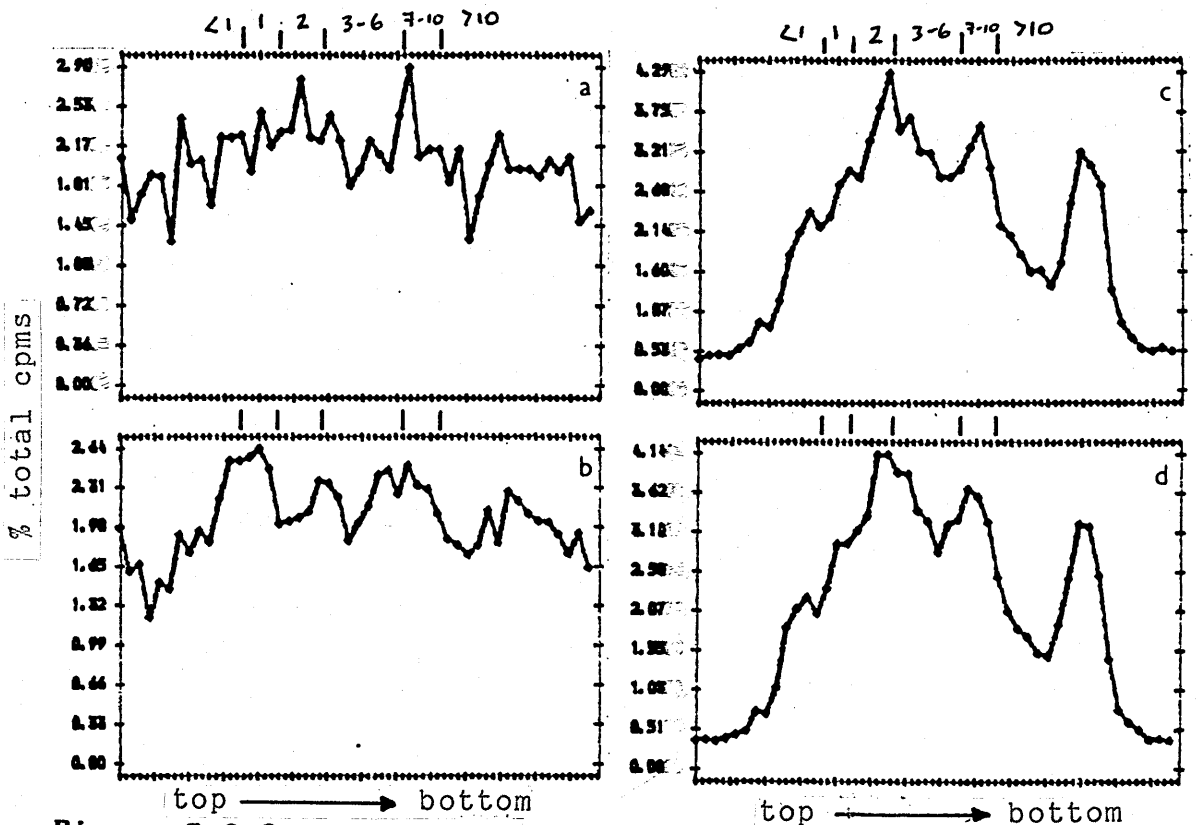


Figure 5.2.3

Distribution of the wild-type and mutant PYK mRNAs across polysome gradients prepared from transformants 1CM6 and 2DN1. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF
a	1CM6	PYK(038)	1959
b	1CM6	PYK(223)	2601
c	2DN1	PYK(038)	7966
d	2DN1	PYK(224)	13953

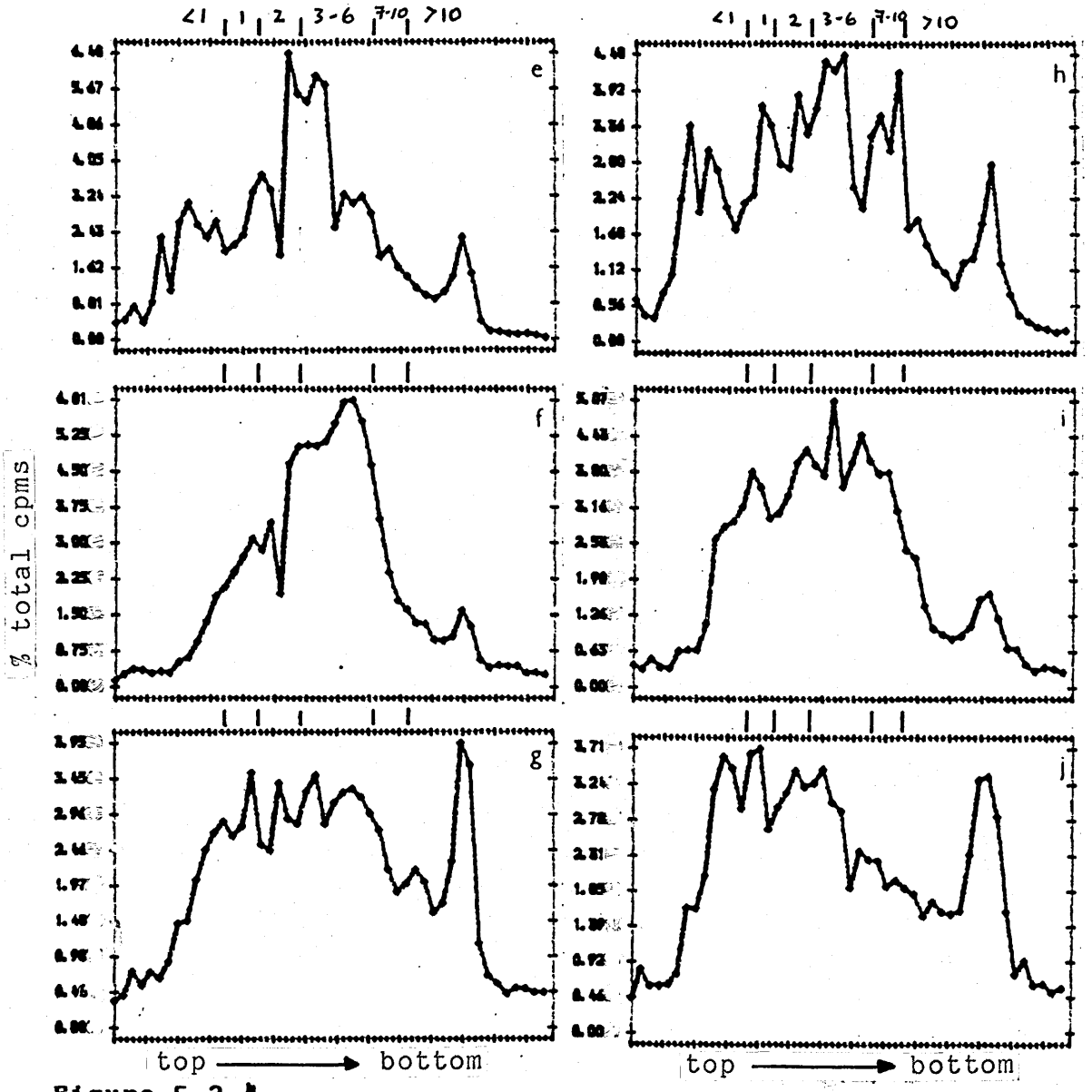


Figure 5.2.4

Distribution of the mRNAs actin, G3PD and RP1 across polysome gradients of transformants 1CM6 and 2DN1. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF		Strain	mRNA	TCF
e	1CM6	ACTIN	82577	h	2DN1	ACTIN	79893
f	1CM6	G3PD	25340	i	2DN1	G3PD	22480
g	1CM6	RP1	7032	j	2DN1	RP1	7460

mRNA	Strain	Polysome fraction (% total cpms)					PYK mRNA (% total)
		<1	1-2	3-6	7-10	>10	
PYK(038)	1CM6	28	23	23	12	17	1.1 ± 0.4
PYK(038)	2DN1	12	27	28	14	19	0.6 ± 0.1
PYK(223)	1CM6	24	23	22	10	21	
PYK(224)	2DN1	11	26	29	14	21	
ACTIN	1CM6	19	26	33	15	7	
ACTIN	2DN1	21	25	24	16	14	
G3PD	1CM6	7	26	36	24	8	
G3PD	2DN1	14	28	25	20	13	
RP1	1CM6	17	27	24	18	15	
RP1	2DN1	21	29	21	11	18	

Table 5.2.2

Distribution of the PYK (total and mutant), actin, G3PD and RP1 mRNAs across polysome gradients prepared from host (DBY746) and transformant (1CM6 and 2DN1) strains.

significant difference between the PYK profiles of the two transformants (Fig.5.2.3). Similarly, an examination of gradients 1CN2 and 2D8 (Fig 5.2.5, Fig. 5.2.6 and Table 5.2.3) reveals that for the control mRNAs actin, G3PD, and RP1, consistently more material sediments in the 7-10 ribosome region of 1CN2 than does in 2D8. Once again, when differences between the gradients have been taken into account, there appears to be no appreciable difference between the PYK profiles of the two gradients. The peaks observed near the bottom of the gradients are due to material dislodged from the bottom during fractionation, and should be ignored. Thus it appears that under these conditions there is no significant difference in the translation of the PYK 1C and 2D mRNAs.

The explanation for this lack of difference becomes clear when the PYK mRNA abundances of the various transformants are estimated using RNA extracted from sphaeroplasts stored immediately prior to lysis of the remainder for centrifugation on sucrose density gradients. The results of this analysis are presented in Table 5.2.4. It should be stressed that in all the transformant cultures, the LEU2 marker was present in at least 94% of the cells at the time of sphaeroplasting. Thus it appears that whilst most of the cells have not lost the plasmid, there has been a "wind-down" in PYK mRNA levels as observed previously (chapter 4). It was not considered that these PYK mRNA abundances were sufficiently high as to render negligible the contribution of the chromosomally encoded PYK mRNA to

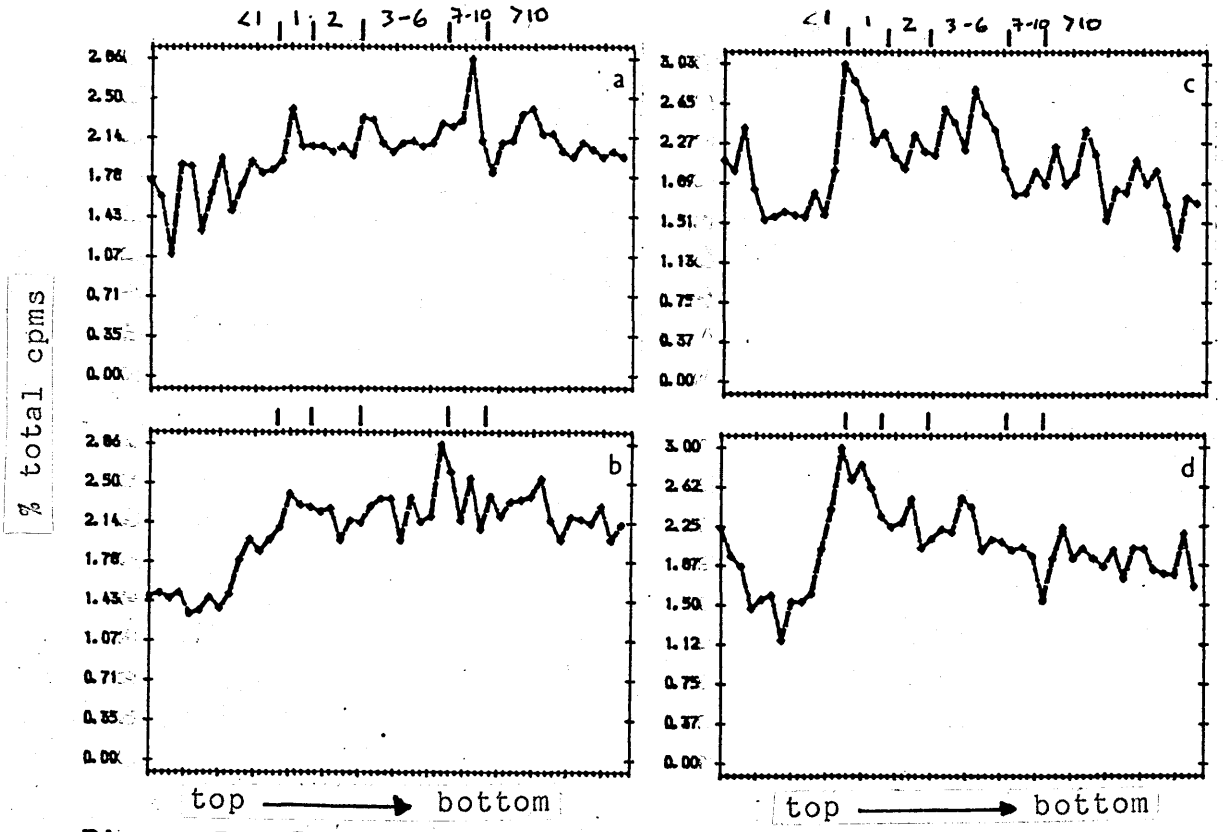


Figure 5.2.5

Distribution of the wild type and mutant PYK mRNAs across polysome gradients prepared from transformants 1CN2 and 2D8. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF
a	1CN2	PYK(038)	1892
b	1CN2	PYK(223)	2701
c	2D8	PYK(038)	1932
d	2D8	PYK(224)	2801

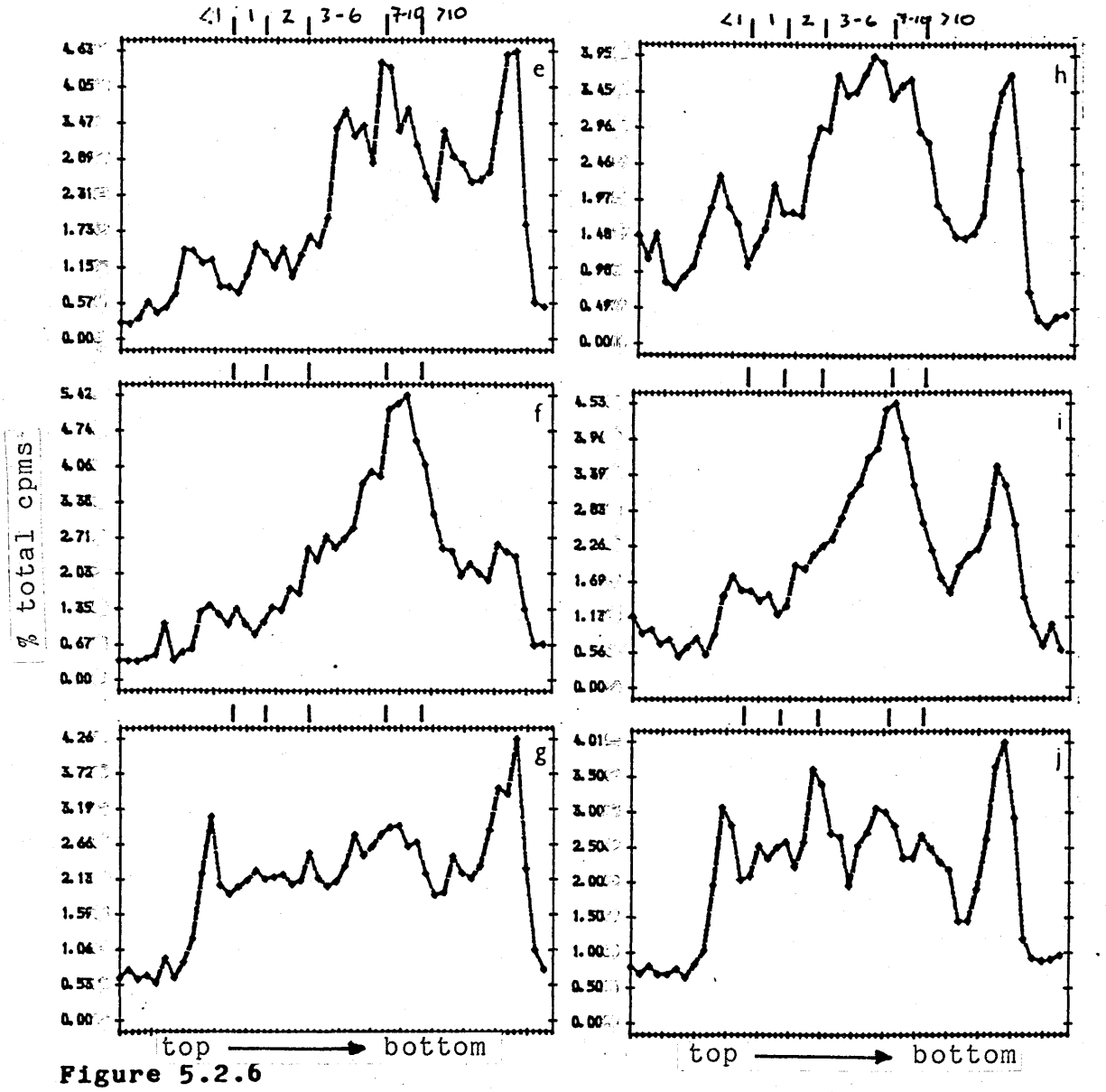


Figure 5.2.6

Distribution of the actin, G3PD and RP1 mRNAs across polysome gradients prepared from transformants 1CN2 and 2D8. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF		Strain	mRNA	TCF
e	1CN2	ACTIN	83823	h	2D8	ACTIN	59119
f	1CN2	G3PD	24242	i	2D8	G3PD	17184
g	1CN2	RP1	4683	j	2D8	RP1	4627

mRNA	Strain	Polysome fraction (% total cpms)					PYK mRNA (% total)
		<1	1-2	3-6	7-10	>10	
PYK(038)	1CN2	27	22	23	12	17	1.5 ± 1.1
PYK(038)	2D8	19	25	25	6	24	1.6 ± 0.5
PYK(223)	1CN2	15	26	25	12	22	
PYK(224)	2D8	24	30	20	7	19	
ACTIN	1CN2	10	10	23	27	31	
ACTIN	2D8	18	18	34	15	16	
G3PD	1CN2	12	9	21	31	27	
G3PD	2D8	10	16	36	24	15	
RP1	1CN2	14	21	19	20	25	
RP1	2D8	15	26	30	13	16	

Table 5.2.3

Distribution of the PYK (total and mutant), actin, G3PD and RP1 mRNAs across polysome gradients prepared from host (DBY746) and transformant (1CN2 and 2D8) strains.

Strain	PYK mRNA (% total mRNA)	
	Initial value	Value at time of polysome analysis
1CN2	1.3	1.5 ± 1.1
1CM6	4.2	1.1 ± 0.4
2DN1	4.6	0.6 ± 0.1
2D8	1.4	1.0 ± 0.5

Table 5.2.4

Abundance of the PYK mRNA measured in transformant strains (1CN2, 1CM6, 2DN1 & 2D8) before storage, and after storage prior to polysome analysis.

the polysomal distribution of the PYK mRNA as a whole. Therefore, oligonucleotides were designed which would only hybridise to PYK transcripts from the plasmid locus. The sequences of these two oligonucleotides are given in Table 5.2.5. It was essential to ensure that the oligonucleotides were specific to the mutant PYK mRNAs, and did not cross-hybridise to the wild-type PYK mRNA under the conditions employed in the experiment. Therefore, a Northern blot of RNA isolated from the host strain (DBY746) and from the mutant (1C or 2D) was challenged with radiolabelled oligonucleotides 223 (specific for 1C mRNA) and 224 (specific for 2D mRNA). The resulting autoradiograph is shown in Fig. 5.2.7, and proves that the oligonucleotides are specific for their respective mutant PYK mRNAs.

The four gradients were then probed with an oligonucleotide (038, Table 2.6.4.1; Purvis et al, 1987b) to show the distribution of all PYK mRNAs in the cell (mutant and wild type), and probed with an oligonucleotide specific for the mutant PYK mRNA concerned (223 or 224). The level of PYK mRNA in transformant 2DN1 was of some concern, since this is a similar level to that of the host strain (DBY746). However, it has been shown in analogous experiments that at such low levels of total PYK expression, this expression is composed of equal amounts of chromosomally-encoded and plasmid-encoded sequences (Purvis et al, 1987b). The results generated by probing these gradients with the various PYK probes described are shown in Fig. 5.2.3 and

Designation	Specificity	Sequence (5' - 3')	Hybridisation conditions
223	1C	CCAATTCAGCTGGGTGTCTTGGCAA	30% formamide 37°C
224	2D	GCTAGCTCTGCGGGATGGCGGGGTA	40% formamide 37°C
038[1]	all PYK mRNAs	TAATATCTTCATTCAATCATGATTC	20% formamide 37°C
220	only wt PYK	ATCTTTCTAATCTAGACATTGTGATG	35% formamide 37°C

Table 5.2.5

Sequences and hybridisation conditions of the oligonucleotide probes used in this study. ([1] from Purvis *et al*, 1987; this oligonucleotide hybridises to the 3'-UTR of the *PYK* mRNA, all the others span the AUG translation initiation codon at the 5'-end of the *PYK* mRNA.

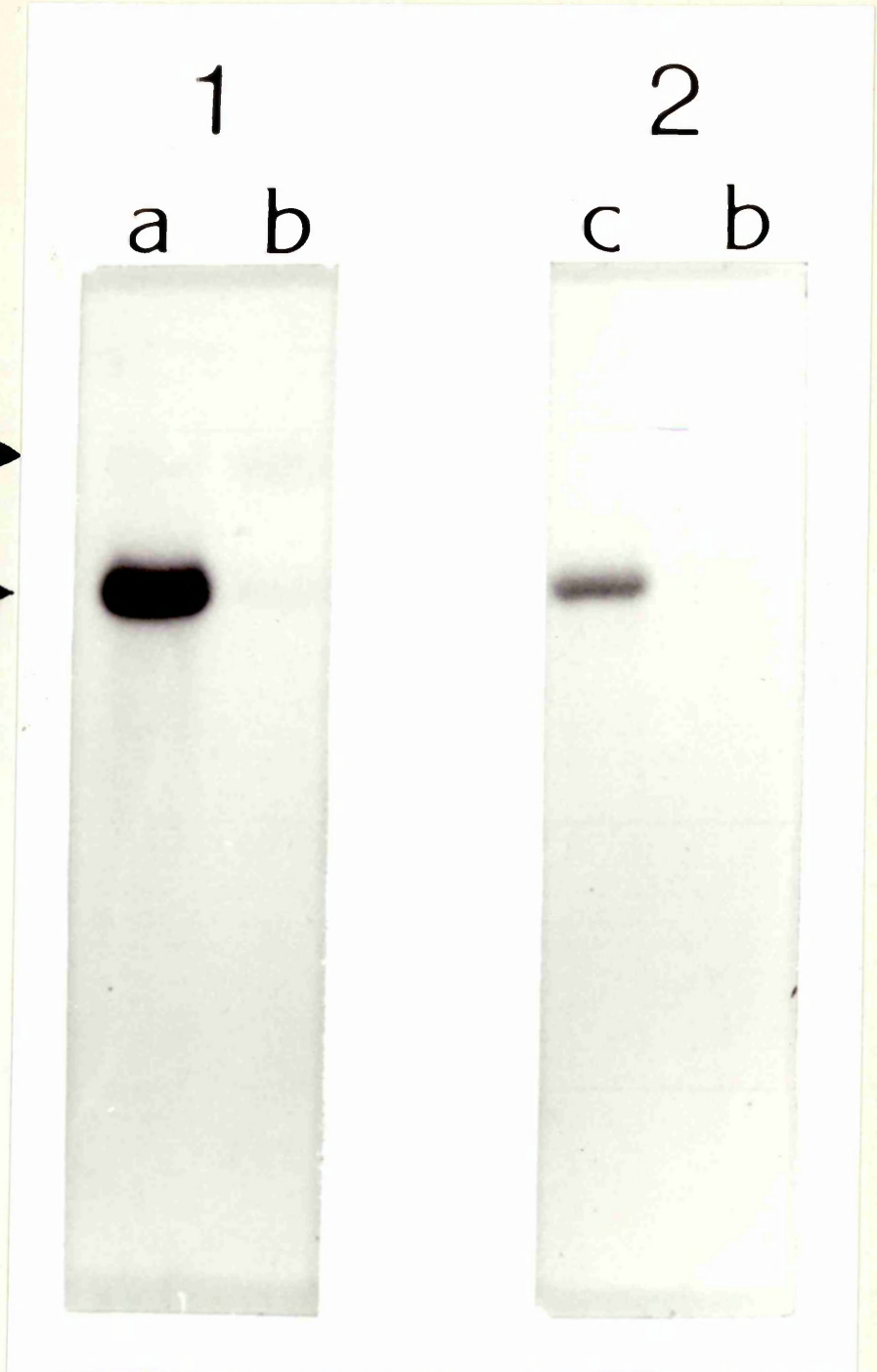


Figure 5.2.7

Northern blot to test the specificities of the two PYK oligonucleotide probes 223 and 224.

1: RNA from 2DN1 (a) and DBY746 (b) probed with end-labelled oligonucleotide 224;

2: RNA from 1CM6 (c) and DBY746 (b) probed with end-labelled oligonucleotide 223.

Fig. 5.2.5. From these data we can conclude that there is no significant difference in the distribution of the wild-type and mutant PYK mRNAs on these polysome gradients. That is, at low levels of expression, the insertion of thirteen rare codons immediately 3' to the translation initiation codon of the PYK gene appears to have no significant effect on the translation of its mRNA.

An experiment to investigate the influence of codon bias on translation was also conducted in conjunction with Dr.I.J.Purvis of this laboratory. A multicopy (2-micron based) and a single-copy (centromeric-based) plasmid were constructed, in which the coding region of the E.coli β-galactosidase gene was used to replace the coding region of the PYK gene of S.cerevisiae. These plasmids, YEpPK441 and YCpPKG2, and their derivation are illustrated in Fig. 4.2.7. These plasmids were transformed into DBY746 and three transformants containing different levels of the β-galactosidase/PYK fusion mRNA were selected for polysome analysis. The data from the polysome analyses are summarised in Table 5.2.6. A probe for the TRP2 mRNA was also used, since this mRNA has a low codon bias (codon bias index = 0.13; Bennetzen & Hall, 1982) in contrast to the other mRNAs used previously in this study which have relatively high codon biases (codon bias indices; actin = 0.79, RP1 = 0.92, PYK = 0.95; Bennetzen & Hall, 1982). The codon bias of the β-galactosidase/PYK fusion mRNA in S.cerevisiae was calculated as -0.05% (Purvis et al, 1987c).

At low levels of the β-galactosidase/PYK fusion mRNA,

Table 5.2.6

Distribution of the PYK, β-gal/PYK fusion, actin, RP1 and TRP2 mRNAs across specific regions of polysome gradients prepared from host (DBY746) and transformed (LAC J, YCpPKG2; LAC7 & LAC 8; YEpPK6441 - see Fig. 4.2.7) strains. (from Purvis et al, 1987c) NA: not applicable; ND: not done

mRNA	Strain	Polysome fraction (% total cpms)				Abundance fusion mRNA (% total)
		0-2	3-6	7-15	>15	
PYK	DBY746	13	27	55	5	
PYK	LAC J	3	32	52	13	
PYK	LAC 7	16	33	46	5	
PYK	LAC 8	22	29	49	5	
β-GAL	DBY746	NA	NA	NA	NA	
β-GAL	LAC J	10	14	24	52	0.04 ± 0.01
β-GAL	LAC 7	7	25	61	6	0.20 ± 0.01
β-GAL	LAC 8	18	30	39	13	0.96 ± 0.05
ACTIN	DBY746	23	30	32	15	
ACTIN	LAC J	18	34	37	11	
ACTIN	LAC 7	18	34	33	15	
ACTIN	LAC 8	19	28	34	18	
RP1	DBY746	36	29	31	4	
RP1	LAC J	44	32	22	2	
RP1	LAC 7	40	28	30	2	
RP1	LAC 8	30	31	32	7	
TRP2	DBY746	ND	ND	ND	ND	
TRP2	LAC J	12	27	46	15	
TRP2	LAC 7	21	34	33	12	
TRP2	LAC 8	31	35	23	11	

the ribosome loading of the fusion mRNA is approximately twice that of the progenitor PYK mRNA. Since the fusion mRNA is approximately twice the length of the PYK mRNA, similar rates of initiation and elongation on the two mRNAs could account for this result. Hence, it may be concluded that the vastly different codon usages of the two mRNAs (PYK and β-galactosidase/PYK fusion) does not significantly affect the rate of translation elongation when the heterologous mRNA is expressed at low levels. However, this is not the case when the heterologous mRNA is expressed at elevated levels. An increase in the level of the fusion mRNA leads to a diminution in the ribosome loading of this mRNA. This diminution in ribosome loading is not mirrored in endogenous transcripts of relatively high codon bias (Actin, RP1 and PYK), but increased levels of the heterologous transcript do lead to a diminution in the ribosome loading of an endogenous transcript of low codon bias (TRP2).

5.2.3 Discussion

There arises the intriguing question of why in one case codon bias does not appear to influence translation (cf 1C & 2D) and in another case it does appear to exert a significant influence (β-galactosidase/PYK fusion).

Theoretical analyses have suggested that non-preferred codons, which correspond to relatively low abundance tRNAs, are translated more slowly than preferred codons (Bennetzen

& Hall, 1982). This is probably due to the increased time taken to select low abundance tRNAs compared to that for high abundance tRNAs (Pedersen, 1984; Robinson et al, 1984; Bonekamp et al, 1985). Therefore, it is argued that codon bias has evolved to maximise translation, by reducing the drainage on tRNA pools (Holm, 1986). Whilst Bulmer (1987) argues that tRNA abundance and codon usage co-evolved. He has argued that dual evolutionary pressures both to utilise abundant tRNAs (and thereby prevent excessive drainage of tRNA pools) and of codon usage upon tRNA abundance, are sufficient to account for the observed division of yeast genes into two major groups: those expressed at high levels with good codon bias, and those of low expression with poor codon bias (or random codon usage) (Bulmer,1987). Under normal circumstances, differential rates of gene expression are not thought to be due to variable codon usage (Holm, 1986). However, non-preferred codons might limit gene expression if their utilisation exceeds normal levels such as the expression of heterologous sequences in an organism (Holm, 1986; Varenne & Lazdunski, 1986). However, codon bias is thought to have little effect on the expression of certain heterologous sequences in yeast (Kingsman et al, 1985); Kniskern et al, 1986).

Why should there be this apparent discrepancy between theoretical prediction and experimental observation? Firstly, this may be the product of indirect experimental observation. Kniskern and co-workers (1986) did not directly measure translation in their experiments and, since

the Hepatitis B antigen protein is stable in yeast, the high protein levels they observed may have been due to protein accumulation. Secondly, the levels of heterologous mRNA might not have been sufficient to stress the translation system to the point where dramatic effects due to codon bias are oberved. When heterologous protein synthesis is directed by the PGK promoter of yeast, it is thought that the levels of heterologous mRNA produced are too low to cause problems associated with poor codon bias (Kingsman et al, 1985). These low levels of heterologous expression are thought to be due to the disruption of a transcriptional Downstream Activation Site (DAS), thought to be located in the coding region of the PGK gene (Kingsman et al, 1985; Mellor et al, 1987).

It may not only be the absolute levels of poorly biased mRNAs that is important, but also the position in which non-preferred codons occur along an mRNA (Varenne & Lazdunski, 1986). Robinson and co-workers (1984) introduced a sequence of four arginine codons into the reading-frame of the CAT gene of E.coli. These codons were recognised in one construct by an abundant tRNA, and in another by a rare tRNA. After inducing the CAT gene with the preferred arginine codons, linked to the TRP promoter, CAT was the major cellular protein produced. The CAT gene containing four rare arginine codons synthesised only one-third the amount of CAT protein compared to that produced from a CAT gene containing four preferred arginine codons. The pool of

rare tRNAs seemed to prove insufficient to support increased expression of the construct containing non-preferred arginine codons after induction (Robinson *et al*, 1984). Thus, to see *dramatic* effects caused by codon bias, it seems necessary to stress the translation system of the cell, by greatly increasing the levels of an mRNA with poor codon bias and/or by increasing the presence of strings of a particularly unfavourable codon. Alternatively, growth in minimal medium, rather than rich medium, may increase the effects of codon bias on translational elongation since intracellular pools of amino acids (and therefore possibly of charged tRNAs) may be lower under these conditions. Certainly, the radical effect of overexpression of the B-galactosidase/PYK gene upon the growth of the host yeast cell in minimal medium would be consistent with this supposition (Purvis *et al*, 1987c). Unfortunately, it was not feasible to test this directly since it was not technically possible to perform polysome analyses in minimal medium using our procedures (Chapter 2.3).

There was no observable effect of codon bias when *PYK* mutant 2D was analysed and compared with *PYK* mutant 1C. This was almost certainly because the *PYK* mRNAs concerned were not being expressed at high enough levels to cause translational stress by draining the pools of rare tRNAs. Although mRNA from mutant 2D was being expressed up to a level of approximately 0.5% total mRNA, only a short region (13 codons) of this mRNA is poorly biased. This short region did not seem to cause a sufficiently large drainage

of the rare tRNA pools to see, in turn, a significant effect upon polysomal distribution. Slight effects of codon bias upon translation of PYK 2D mRNA may not have been resolved using our methodology.

Conversely, it has been shown that increasing the level of a β-galactosidase/PYK fusion mRNA resulted in a decrease in the ribosome loading of this mRNA. The translation of high levels of this heterologous mRNA, an mRNA with extremely poor codon bias in S.cerevisiae (-0.05%; Purvis et al, 1987c), is thought likely to cause a severe depletion in the amino-acyl tRNAs corresponding to what are normally rarely-used codons (Holm, 1986). This severe depletion in rare tRNA pools may be leading to an increase in ribosome "stalling" during elongation (Parker et al, 1978) and hence be the cause of a decrease in ribosome loading. Similarly, the TRP2 mRNA has a poor codon bias, and hence depletion of rare amino-acyl tRNAs would also affect this mRNA, causing ribosome "stalling" and premature termination (Parker et al, 1978).

The inefficient translation of heterologous mRNAs due to their poor codon bias in S.cerevisiae has also been suggested by other workers. A yeast PGK-human interferon-alpha fusion expressed in S.cerevisiae does not produce as high a level of protein as might be expected using this system (Tuite et al, 1982). Various reasons were suggested why this might be so, one of which was that the codon bias of human interferon-alpha is not maximal for yeast (Tuite et

al, 1982). However, this lack of expression has now almost certainly been attributed to low RNA levels caused by deletion of the transcriptional activator sequence from the PGK coding sequence (Kingsman et al, 1985; Mellor et al, 1987). Compagno and co-workers (1987) fused the coding sequence of the zein gene from Zea mays to the hybrid yeast promoter GAL1-10/CYC1. They demonstrated that the increase in rate of heterologous protein synthesis was not related to the level of the zein fusion mRNA. They concluded that the poor codon bias of the zein fusion mRNA in S.cerevisiae (0.2) was probably inhibiting translation of this mRNA. A very similar hypothesis has been proposed by Chen and Hitzeman (1987) to explain the reduced accumulation of human 3-phosphoglycerate kinase in S.cerevisiae.

In conclusion, it appears that expression of a heterologous sequence at relatively high levels in yeast is limited, at least in part, at the level of translational elongation when the codon bias of that mRNA is poor for yeast. An experiment designed to assess the effects of specific changes in the codon bias of the PYK gene failed to reveal any effect, probably because the mRNA concerned carried too few non-preferred codons and was being expressed at too low a level. The "wind-down" in PYK mRNA levels described in the previous chapter appears to have contributed to these relatively low levels of PYK overexpression.

5.2.4 Future Experiments

Possibly the most obvious experiment is to attempt to express the mutant PYK mRNAs 1C and 2D at much higher levels. It has already been demonstrated that levels up to 7.9% total mRNA are possible (Table 5.2.1). A rapid screening of fresh transformants would be essential for this approach to work. It has been suggested that high levels of the PYK 2D mRNA might be selected against since the discharging of rare tRNA pools might have a deleterious effect on the translation of other cellular mRNAs. Therefore, some thought might be given to putting these sequences under the control of an inducible promoter e.g. GAL1/10. In addition, it would be interesting to analyse the translation of the PYK mRNAs from 1C and 2D in cells grown in minimal medium.

We have shown that expression of an heterologous mRNA at high levels leads to a reduced ribosome loading for this mRNA on polysome gradients. Whilst this is almost certainly due to the influence of codon bias, it cannot be ruled out that this is occurring due to some inherent property of this heterologous mRNA. Therefore, it might be useful to fuse the coding sequence of a yeast gene with poor codon bias (e.g. TRP2 = 0.19) behind the translation start site of the PYK gene, and assess the translation of this relatively homologous fusion construct.

It has been suggested that it is not merely the presence of poor codon usage, but possibly the distribution of poor codons along an mRNA which influence its efficiency

of translation (Robinson et al, 1984; Varenne & Lazdunski, 1986; Purvis et al, 1987a). This hypothesis could be tested by inserting a short sequence immediately 3' to the translation start site of the PYK gene, which encodes a string of, say, five amino acids encoded by either poor or good codons. If the translation of the mRNA containing the poorly biased insert was shown to be adversely affected, attempts could be made to alleviate this. One possible approach would be to overexpress the tRNA gene which recognises the rare codon to increase its abundance in the cell, and to overexpress its tRNA-synthetase gene as well (to ensure adequate charging of the excess tRNA molecules). A second approach would be to carry out site-directed mutagenesis on the anticodon of an abundant tRNA, such that the new anticodon recognises a previously non-preferred codon.

5.3 The Influence of Secondary Structure on Translation of the PYK gene.

5.3.1 Experimental Design

The purpose of this experiment was to test whether secondary structure formation involving the 5'-UTR of the PYK mRNA had any effect upon its translation as determined by polysomal analysis.

G.D.Searle had made available two plasmids containing PYK genes mutated by the insertion of 42 base-pair double-stranded oligonucleotides immediately 3' to the ATG

translation start site. The sequences of the two insertions (pLD1(35)3K & 4H) are given in Fig. 5.3.1. The inserted amino acid sequence is identical in both cases. This was an important control, since it enabled the exclusion of any possible secondary effects, such as some unknown feedback mechanism from the altered protein upon the translation of the PYK mRNA. Also, the inserted amino acid sequence is not heavily biased towards any particular group of amino acids (acidic, basic, polar etc.). Fig. 5.3.1 also illustrates the codon bias of the two inserted sequences. It should be noted that the insert contained in plasmid 3K has the poorer codon bias of the two.

The two inserted sequences were designed to form different levels of secondary structure with the 5'-UTRs of their mRNAs. The results of an optimal computer folding of the first 130 bases of these mRNAs, obtained using the computer programme "FOLD" (Zuker & Stiegler, 1981) are presented in Fig. 5.3.2, along with the predicted folding of the first 130 bases of the wild type PYK mRNA (Fig.5.3.3). It is clear from the computer analysis that mutant 4H should form a much more thermodynamically stable secondary structure involving its 5'-UTR than should mutant 3K (the hairpin loop involving nucleotides 1-87 of the PYK 4H mRNA has a predicted free energy of -35.8 kcal mol^{-1}). Once again, the nucleotides considered important in determining the efficiency of the AUG translation start site remained unchanged (see chapter 5.2.1 for details).

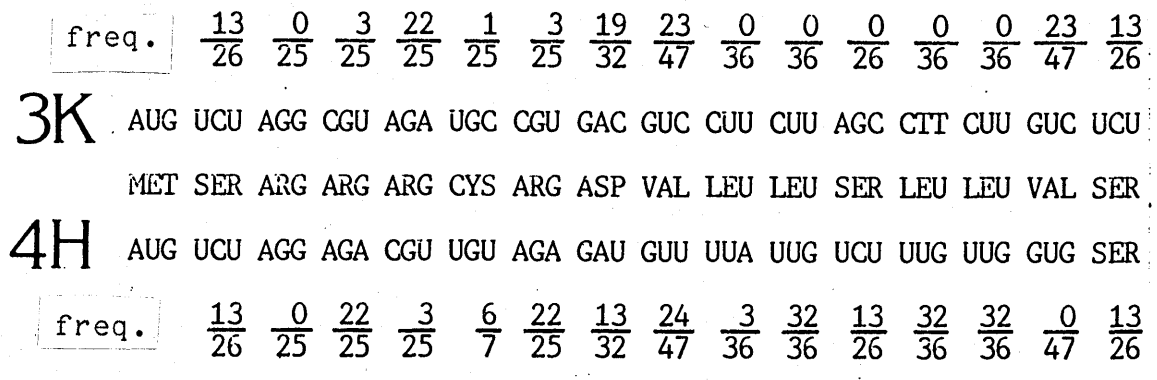


Figure 5.3.1

Sequences of the two oligonucleotide insertions in plasmids pLD1(35) 3K and 4H. The diagram also shows the frequency of occurrence of each of the codons used in the normal PYK mRNA.

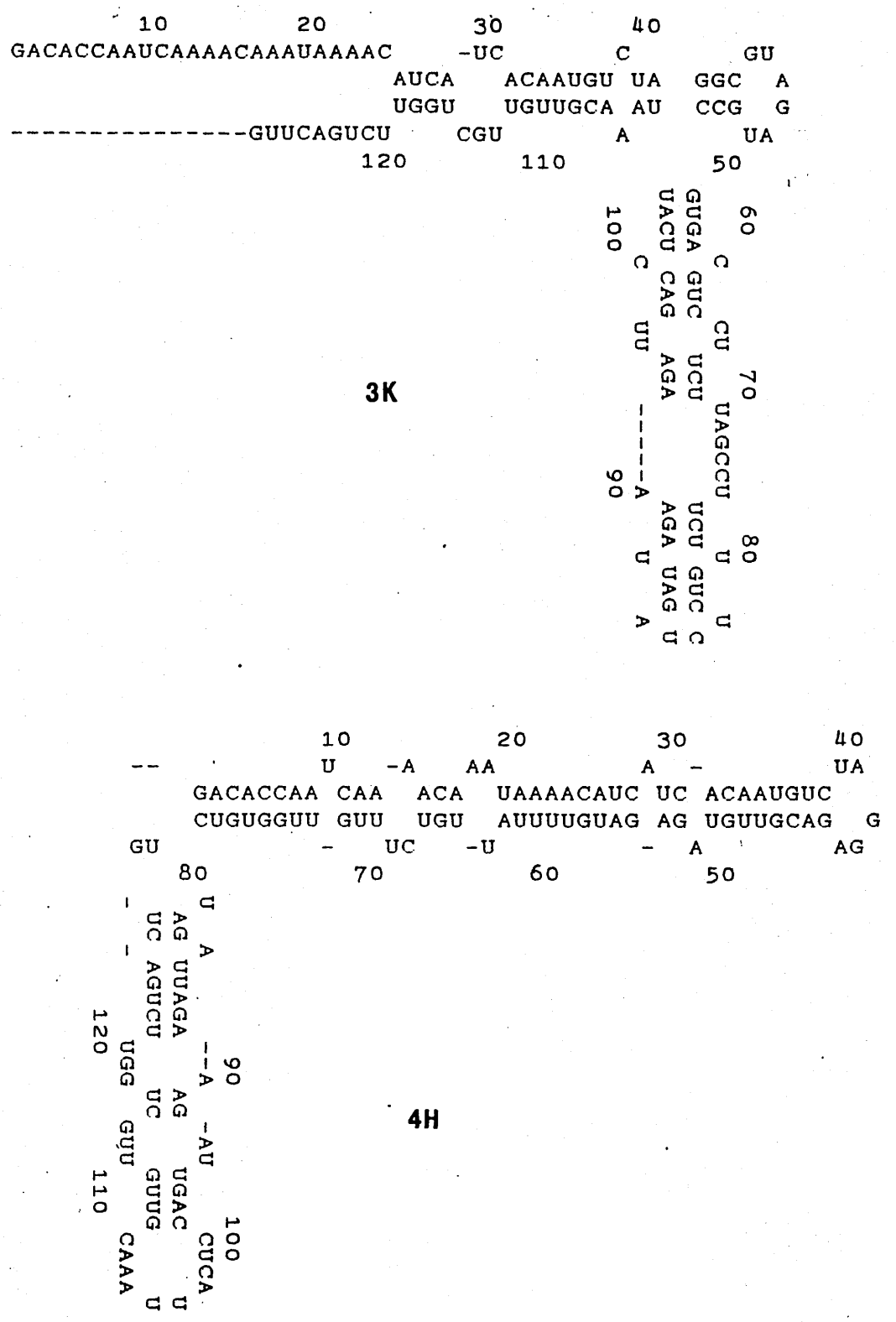


Figure 5.3.2

Predicted secondary structures of the first 130 bases of <u>PYK</u> mRNAs 3K and 4H. The calculated free energies of formation for these structures are:

3K -20.7 kcal mol^{-1}

4H -36.6 kcal mol^{-1}

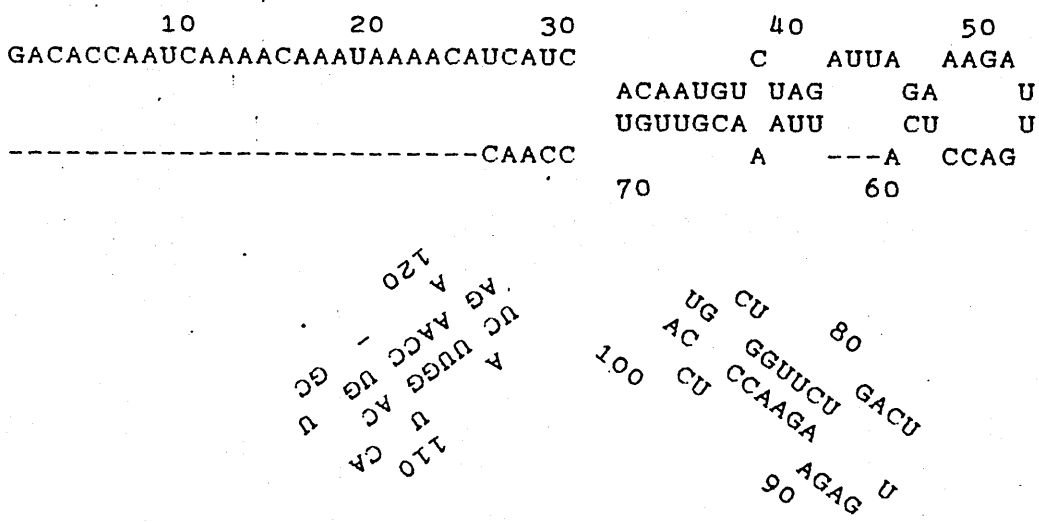


Figure 5.3.3

Predicted secondary structure of the first 130 bases of the wild type <u>PYK</u> mRNA. The calculated free energy of formation for this structure is -15.5 kcal mol^{-1}.

5.3.2 Results

The phenomenon of "wind-down" in PYK expression (discussed in chapter 4) had been observed previous to the start of this experiment. Therefore, it was decided to make the step from transformation to polysome analysis as rapid as possible. Six transformants of 3K and six of 4H were isolated, and an attempt made to estimate the PYK mRNA levels in these strains very rapidly. This rapid analysis was only performed once to save time, and hence the data was not particularly accurate (Table 5.3.1), but it was sufficient to suggest that transformants 3K4, 3K6, 3K7, 4H3, 4H5 and 4H6 contained high PYK mRNA levels, and hence might reward further study. These transformants were quickly subjected to polysome preparations.

The abundance of the PYK mRNA contained in these transformants was remeasured using RNA isolated from sphaeroplasts stored immediately prior to lysis of the remainder for the polysome preparations. The estimated PYK mRNA levels of these transformants are given in Table 5.3.2.

As described in chapter 5.2, problems of "wind-down" in PYK expression leading to relatively low PYK mRNA abundances, had emphasised the need for probes specific for the mutant mRNAs. For this experiment, a decision had already been made to utilise specific oligonucleotide probes, before the level of PYK mRNA was known. In retrospect this was probably not necessary since relatively high PYK mRNA levels were obtained!

Strain	PYK mRNA (% total mRNA)	Strain	PYK mRNA (% total mRNA) *
3K1	22	4H1	46
3K3	3	4H2	81
3K4	26	4H3	82
3K5	4	4H4	64
3K6	28	4H5	98
3K7	25	4H6	90

Table 5.3.1

Preliminary measurements of PYK mRNA abundance from transformants with pLD(1)35 3K and 4H.

* Values are high, probably due to dilution error in standards.

Design of the specific oligonucleotide probes was complicated by data which indicated that strong hairpin loops are not efficiently denatured under the conditions normally employed for Northern blotting (Purvis et al, 1987b). Since it had been predicted that the 5'-UTR of the 4H PYK mRNA would form a relatively strong hairpin (Fig. 5.3.2), it was feared that hybridisation to this mRNA might not be efficient. However, the free energy of formation of this loop is considerably lower than that in the experiment of Purvis and co-workers (1987b). Therefore, it is possible that this problem of inefficient hybridisation might not have arisen. Be that as it may, it was decided to adopt a different strategy to that adopted in the analysis of the 1C/2D mutants. This approach involved estimating the polysome distribution of total PYK mRNA using the oligonucleotide probe 038 (Table 2.6.4.1). The same gradients were also challenged with a probe specific for the wild-type PYK mRNA. This probe, designated 220, was designed to hybridise across the translation start codon and into the coding region of the wild type PYK mRNA and, therefore, should not hybridise efficiently, under the appropriate conditions, to the mutated PYK mRNAs which contain extra sequences inserted immediately 3' to the translation start site. An experiment was performed to confirm that oligonucleotide 220 did hybridise to wild type PYK sequences specifically. A Southern blot was prepared of sets of three lanes of plasmids pLD1(35)WT, 3K and 4H, restricted with EcoR1. Each set of lanes was then probed with radiolabelled

oligonucleotide 220 with different concentrations of formamide in the hybridisation fluid (i.e. at different effective temperatures of hybridisation). After washing the filters under standard conditions (Chapter 2.8.5), the resulting autoradiograph is shown in Fig. 5.3.4. On the basis of this autoradiograph, a concentration of 35% formamide was adopted for all hybridisations involving oligonucleotide 220, since, under these conditions, there was no detectable cross-hybridisation between the 220 probe and 3K or 4H sequences.

Due to the possible complications of translational dosage compensation, transformants with similar PYK mRNA levels were selected for comparison. Therefore, gradients 3K7 and 4H5 were compared, as were 3K4 and 4H6 (Table 5.3.2). An examination of 3K7 and 4H5 (Fig. 5.3.5, Fig. 5.3.6 and Table 5.3.3) revealed the following. The actin mRNA profiles showed that in gradient 3K7 more material was sedimenting in the region of the gradient where 7 or more ribosomes sediment than in the corresponding region of gradient 4H5. Gradient 4H5 contained a greater proportion of material sedimenting in the pre-monosome area of the gradient than did gradient 3K7. Examination of the profiles for the other two control mRNAs, G3PD and RP1, revealed a similar pattern to that observed for the actin mRNA: a greater proportion of the control mRNAs were distributed nearer the bottom of gradient 3K7, and in a relatively large monosome peak for gradient 4H5. When these differences were

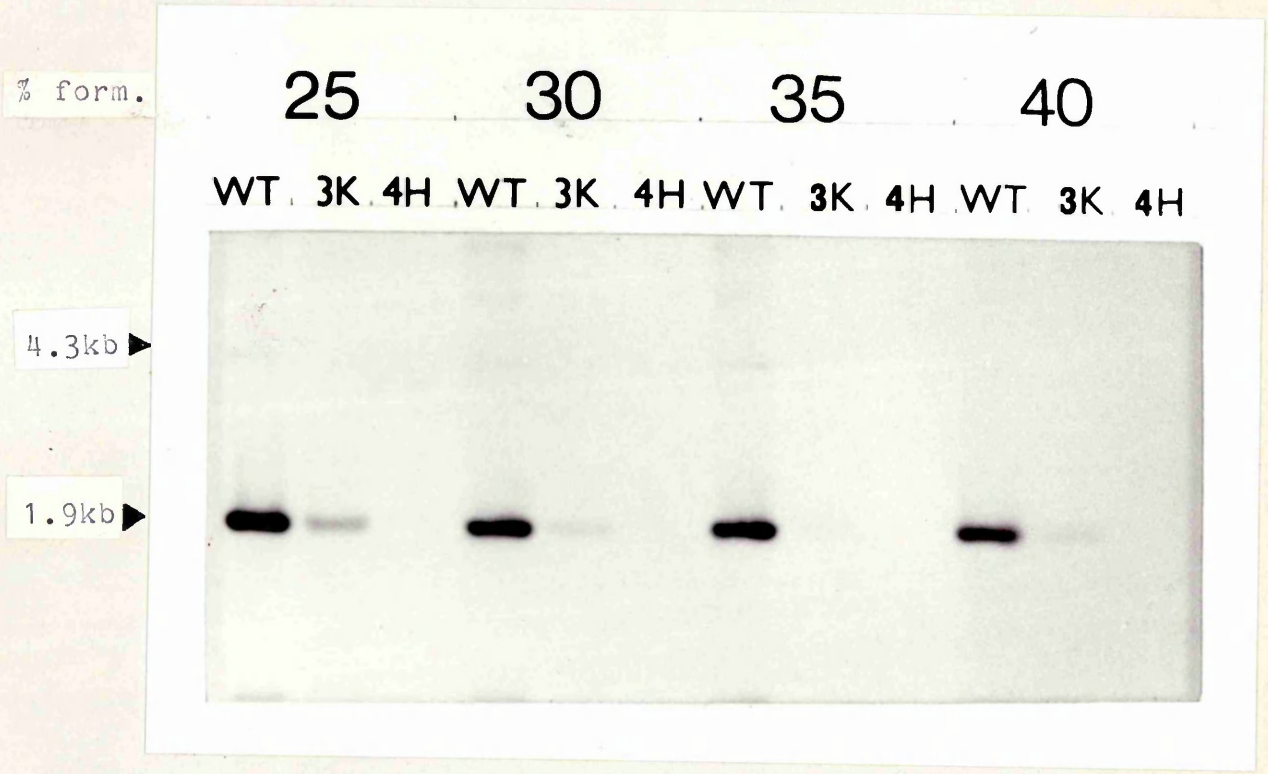


Figure 5.3.4

Southern blot to test specificity of wild type PYK oligonucleotide probe 220. Plasmids pLD1(35) WT, 3K and 4H were restricted with EcoR1 and hybridised with end-labelled oligonucleotide 220 at different concentrations of formamide (25%, 30%, 35% and 40%).

Strain	PYK mRNA (% total mRNA)	
	Initial value	Value at time of polysome analysis *
3K4	26	7.0 ± 1.2
3K7	28	11.6 ± 1.0
4H5	98	10.2 ± 1.2
4H6	90	6.0 ± 1.0

Table 5.3.2

Abundance of the PYK mRNA measured in transformant strains (3K4, 3K7, 4H5 & 4H6) before storage, and after storage prior to polysome analysis.

* see Table 5.3.1

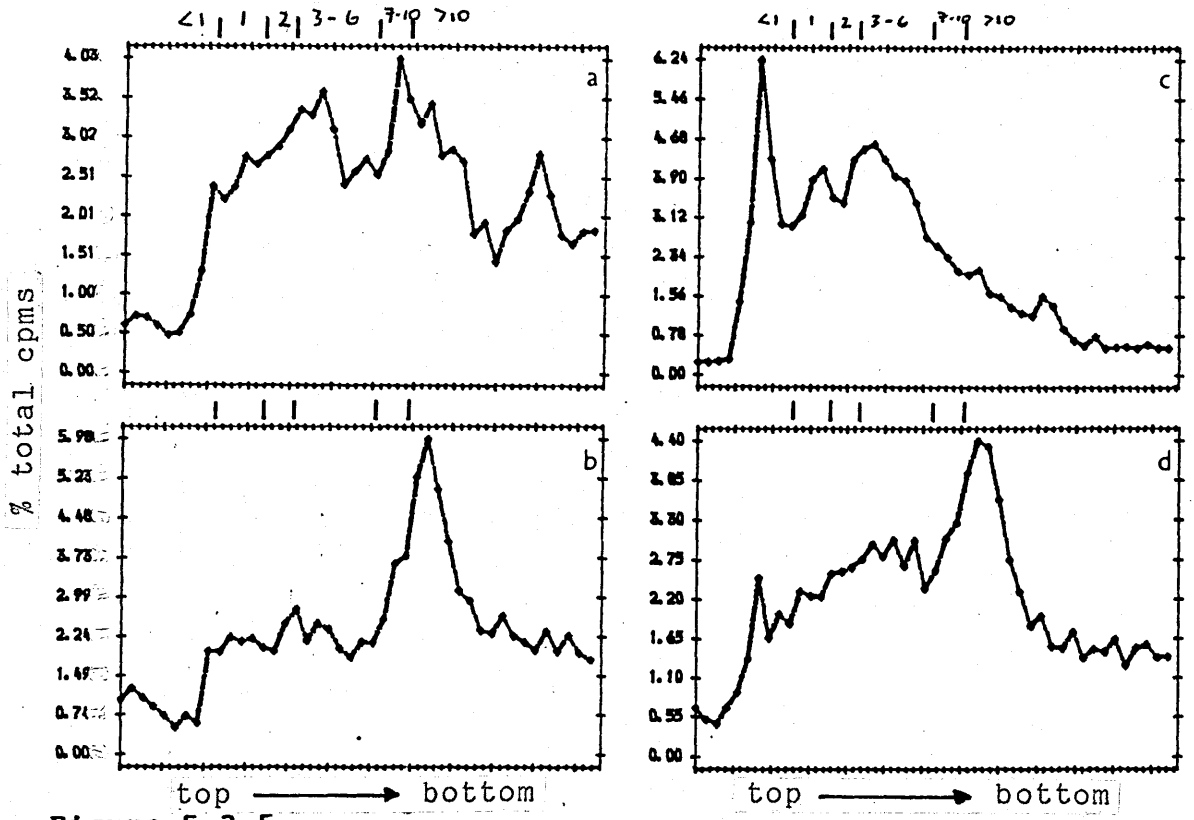


Figure 5.3.5

Distribution of the wild-type, and total, PYK mRNAs across polysome gradients prepared from transformants 3K7 and 4H5. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF
a	3K7	PYK(038)	9477
b	3K7	PYK(220)	13031
c	4H5	PYK(038)	23209
d	4H5	PYK(220)	15462

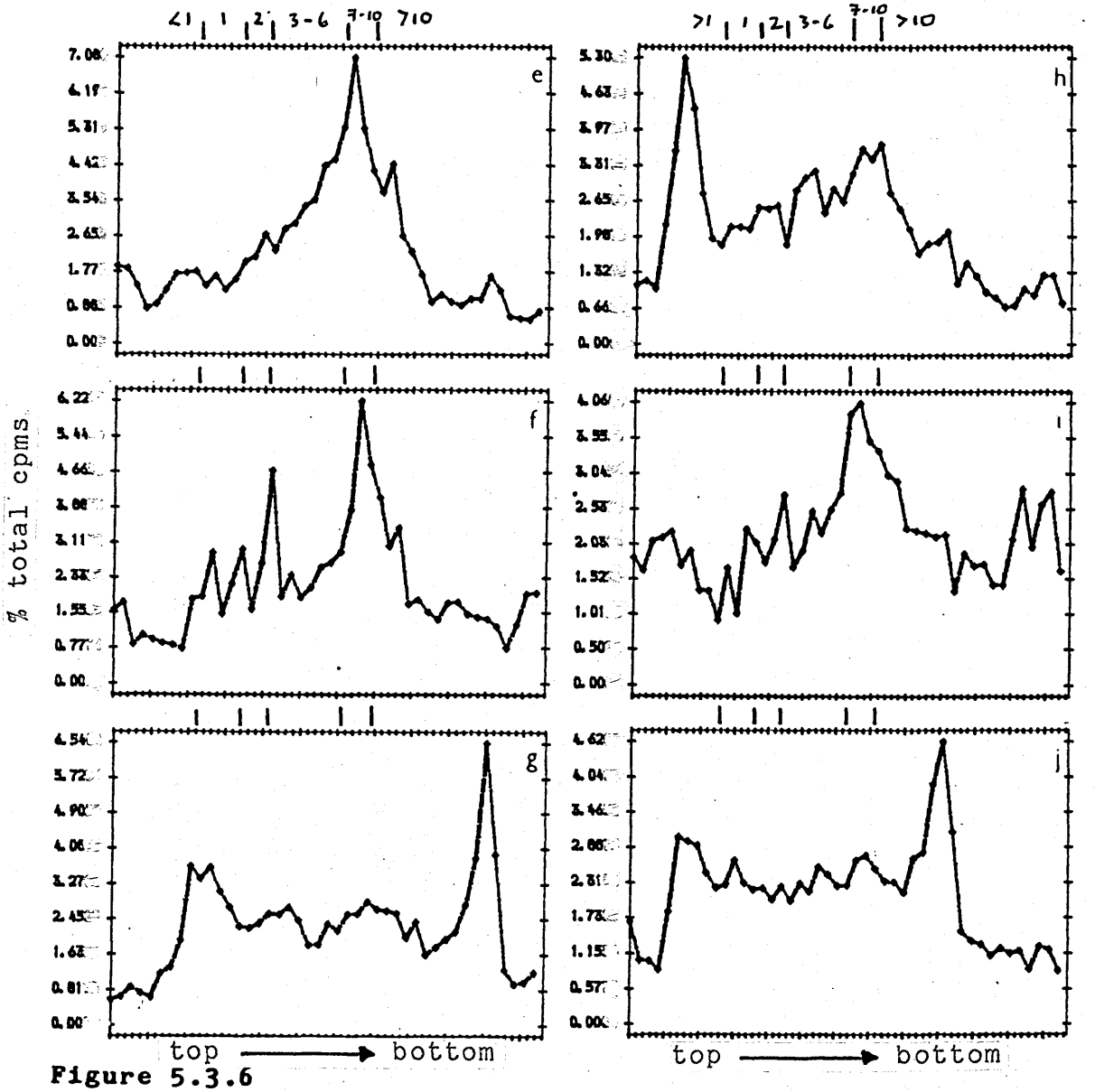


Figure 5.3.6

Distribution of the mRNAs actin, G3PD and RP1 across polysome gradients of transformants 3K7 and 4H5. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF		Strain	mRNA	TCF
e	3K7	ACTIN	15266	h	4H5	ACTIN	17209
f	3K7	G3PD	19615	i	4H5	G3PD	33192
g	3K7	RP1	9837	j	4H5	RP1	9998

mRNA	Strain	Polysome fraction (% total cpms)					PYK mRNA (% total)
		<1	1-2	3-6	7-10	>10	
PYK(038)	3K7	5	25	28	12	30	11.6 ± 1.0
PYK(038)	4H5	22	28	29	8	13	10.2 ± 1.2
PYK(220)	3K7	9	20	21	11	39	
PYK(220)	4H5	11	19	24	12	34	
ACTIN	3K7	11	12	30	22	25	
ACTIN	4H5	28	16	21	13	22	
G3PD	3K7	6	19	24	20	31	
G3PD	4H5	19	13	20	21	27	
RP1	3K7	11	29	22	10	28	
RP1	4H5	22	20	21	12	26	

Table 5.3.3

Distribution of the PYK (total and wild type), actin, G3PD and RP1 mRNAs across specific regions of polysome gradients prepared from transformants 3K7 and 4H5.

taken into account, the profiles for probes 038 (total PYK mRNA) and 220 (wild type PYK mRNA alone) revealed a dramatic difference between the two gradients. In both gradients, the wild type PYK mRNA has a similar ribosome loading (this is most evident from an examination of Table 5.3.3). However, a comparison of the distribution of total PYK mRNA in the cell (i.e. both chromosomal and plasmid-derived transcripts) shows that for 3K7 a significant proportion of the mRNA molecules carry 7 or more ribosomes. Conversely, in gradient 4H5, the majority of the PYK mRNA molecules contain fewer than 7 ribosomes. Within the same gradient, the wild-type PYK mRNA (220 oligonucleotide probe) transcribed from the chromosomal locus is translated significantly more efficiently. Therefore, the secondary structure involving the 5'-UTR of the 4H PYK mRNA appears to be significantly inhibiting the initiation of translation from this molecule. It should be mentioned that the wild type PYK mRNA in transformant 3K7 appears to have a somewhat better ribosome loading than that for all the PYK mRNA molecules in the cell.

The data for gradients 3K4 and 4H6 are given in Fig. 5.3.7, Fig. 5.3.8 and Table 5.3.4. The amount of material sedimenting at, or near, the bottom of gradient 3K4 is greater than for the 4H6 gradient. This was due to material dislodged from the bottom of the tube during fractionation and should be discounted for the purposes of comparison. The actin mRNA shows a similar pattern of ribosome distribution above the monosome peak in both gradients, but

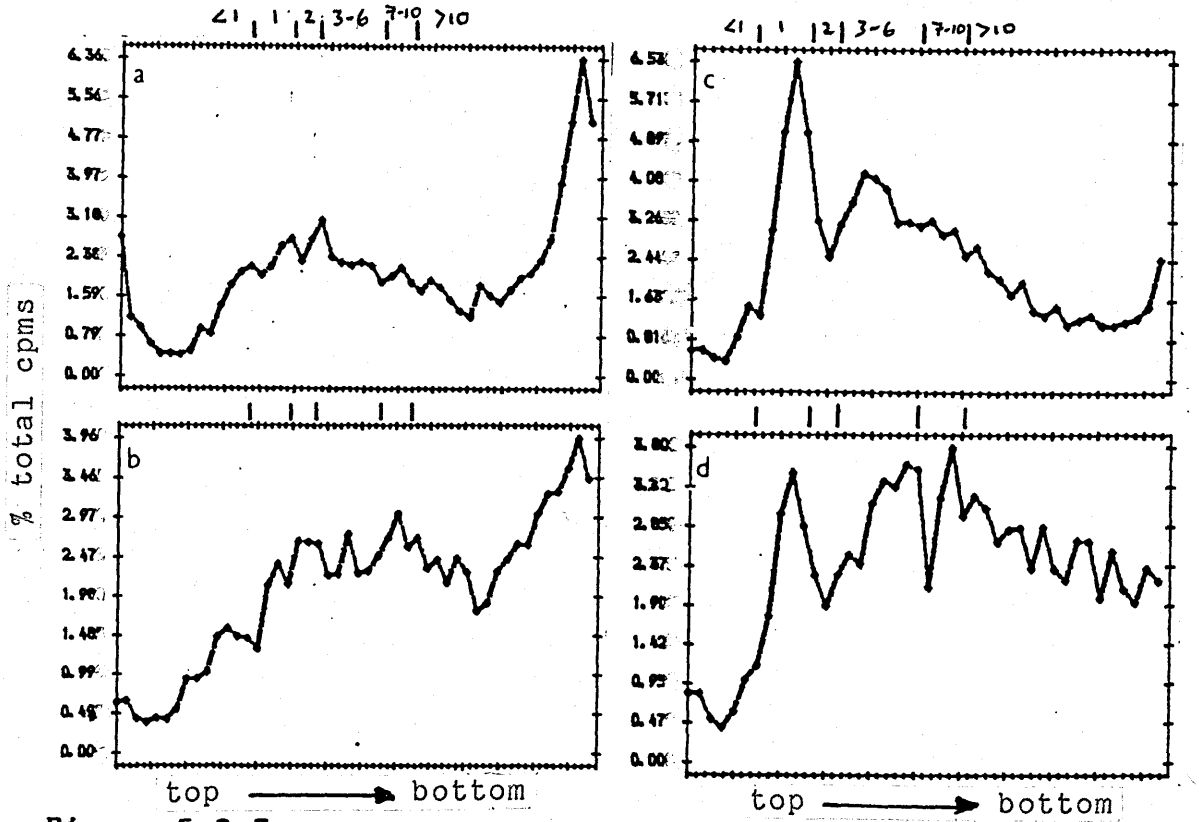


Figure 5.3.7

Distribution of the wild type, and total, PYK mRNAs across polysome gradients prepared from transformants 3K4 and 4H6. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF
a	3K4	PYK(038)	8615
b	3K4	PYK(220)	13375
c	4H6	PYK(038)	17626
d	4H6	PYK(220)	10957

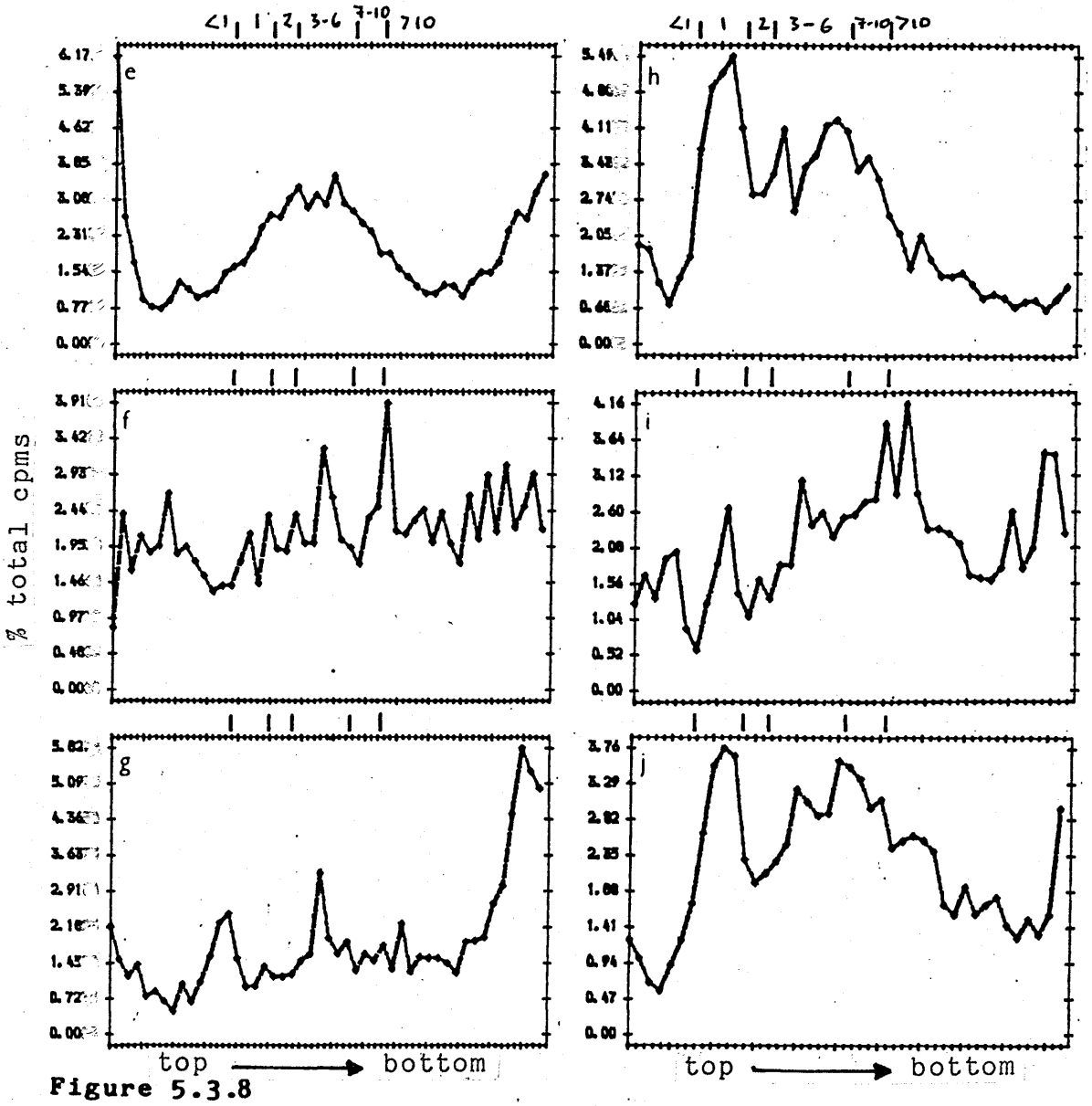


Figure 5.3.8

Distribution of the actin, G3PD and RP1 mRNAs across polysome gradients prepared from transformants 3K4 and 4H6. The lines above the graphs mark the maximum limit of sedimentation of polysomes carrying <1, 1, 2, 6 and 10 ribosomes respectively.

	Strain	mRNA	TCF		Strain	mRNA	TCF
e	3K4	ACTIN	29838	h	4H6	ACTIN	56275
f	3K4	G3PD	37579	i	4H6	G3PD	25510
g	3K4	RP1	17678	j	4H6	RP1	10340

mRNA	Strain	Polysome fraction (% total cpms)					PYK mRNA (% total)
		<1	1-2	3-6	7-10	>10	
PYK(038)	3K4	21	29	27	9	15	7.0 ± 1.2
PYK(038)	4H6	3	36	32	12	16	6.0 ± 1.0
PYK(220)	3K4	15	24	25	14	21	
PYK(220)	4H6	4	28	33	15	20	
ACTIN	3K4	20	21	34	12	13	
ACTIN	4H6	7	35	30	14	13	
G3PD	3K4	26	14	20	8	32	
G3PD	4H6	15	14	23	14	34	
RP1	3K4	25	16	20	9	30	
RP1	4H6	5	28	26	16	25	

Table 5.3.4

Distribution of the PYK (total and wild type), actin, G3PD and RP1 mRNAs across polysome gradients prepared from transformants 3K4 and 4H6.

the monosome peak in gradient 4H6 is greatly exaggerated in comparison to that of 3K4. Similar patterns of ribosome distribution are observed for the other two control mRNAs G3PD and RP1. The profiles for gradient 3K4 appear somewhat "flattened" due to the presence of the large amount of material hybridising near the bottom of the gradient. The pattern of distribution of the wild type PYK mRNA appears to follow that of the control mRNAs, i.e. a larger monosome peak in gradient 4H6 but, an otherwise similar pattern of distribution for the two gradients. When the pattern of distribution of the wild-type and mutant PYK mRNA molecules together is compared, it becomes apparent that, despite the differences between the gradients, in gradient 3K4 a larger proportion of the polysomal PYK mRNA is sedimenting with more than 6 ribosomes compared with gradient 4H6. Also, the majority of PYK mRNA molecules in gradient 4H6 are sedimenting with fewer than 7 ribosomes on them. Therefore, a similar conclusion may be drawn from a comparison of gradients 3K7 and 4H5, and 3K4 and 4H6. In each case, relative to the control mRNAs, the PYK 4H mRNA is loaded with fewer ribosomes than the PYK 3K mRNA. Therefore, secondary structure involving the 5'-UTR of the PYK mRNA appears to significantly inhibit initiation of translation on this mRNA.

5.3.3 Discussion

In this set of experiments, higher levels of PYK mRNA were recorded than in the analogous experiments involving 1C and 2D (chapter 5.2). It is most likely that this difference is not due to the biological influence of the inserted sequences, since in both cases the PYK mRNAs carrying the control and experimental inserts were expressed at similar levels. The difference is more probably due to the speed with which the 3K/4H experiments were performed, in comparison to those with 1C/2D. Therefore, the potential for "wind-down" in PYK mRNA levels observed in the case of the 1C/2D experiments was much reduced in the 3K/4H experiments by the rapidity of the analysis.

These experiments have demonstrated that the presence of more extensive secondary structure involving the 5'-UTR of the 4H PYK mRNA leads to a decrease in the ribosome loading of this mRNA, most probably due to a transient inhibition of the initiation of translation from this mRNA. This work is in accord with the results of a number of other workers. Pelletier and Sonenberg (1985a) demonstrated that the introduction of hairpin loops, by the insertion of linker sequences into the 5'-UTR of the HSV TK gene, resulted in a decrease in the efficiency of translation of its mRNA, both *in vivo* (COS-1 or L-cells) and *in vitro* (rabbit reticulocyte lysates). Kozak (1986b) demonstrated that the introduction of a hairpin loop (ΔG = -30 kcal mol^{-1}) either into the 5'-UTR, or involving the AUG start codon of the proinsulin gene, does not decrease the yield of

proinsulin *in vivo* (COS-1 cells). However, the introduction of a more stable hairpin structure (ΔG = -50 kcal mol^{-1}) decreased the yield of proinsulin by 85-95%, irrespective of its position in the 5'-UTR. She argues that during initiation of translation, migrating 40S subunits can presumably melt moderately stable duplexes, but stall, or are at least slowed down, by more stable structures (Kozak, 1986b). Lawson and co-workers (1986) generated secondary structures in the 5'-leader sequences of rabbit globin and reovirus mRNAs by hybridisation *in vitro* with short (15-20 bases) complementary DNA molecules. Their effects upon translation were measured in wheatgerm and reticulocyte lysate *in vitro* translation systems. Their results indicated that structures involving the first 15 bases 3' to the cap inhibited translation, but structures more than 15 bases 3' to the cap did not. This decrease was not due to inhibition of cap recognition by eIF-4F (Lawson *et al*, 1986). However, it does suggest that secondary structure right at the 5'-end of an mRNA might be inhibiting formation of a stable mRNA.eIF-4F complex, and that more distal secondary structures are capable of being melted by migrating 40S subunits. Baim and co-workers (1985b) showed that the efficiency of translation of the *CYC1* mRNA of *S.cerevisiae* could be reduced if the AUG initiation codon was located in, or close to, base-paired regions. They also showed that the formation of a hairpin loop (ΔG = -19.6 kcal mol^{-1}) twelve bases 3' to the AUG initiation codon was

sufficient to inhibit elongation *in vivo* (Baim *et al*, 1985b). Similarly, Chaney and Morris (1979) suggested that secondary structure in the mRNA of the MS2 coat protein impeded movement of ribosomes during elongation in MS2-infected *E.coli*. This gave rise to the accumulation of nascent peptide chains of discrete size.

Zaret and Sherman (1984) showed that *CYC1* mutants with abnormal sequences in their 3'-UTRs alter the efficiency of translation of this mRNA. It was suggested that the 3'-UTR could be enhancing or inhibiting translation by affecting the secondary structure of the mRNA, and thereby altering the rates of initiation or elongation. Spena and co-workers (1985) showed that *in vitro*, the translational efficiency of zein mRNA from *Zea mays* was reduced by hybrid formation between the 5'- and 3'-UTRs. Konings and co-workers (1987) predicted the possible secondary structure formation on a number of eukaryotic mRNAs and suggested that in the majority of these, there may occur some interaction between the 5'- and 3'-UTRs. This might help explain how the 3'-UTRs of mRNAs can inhibit their translation, as in the case of certain *CYC1* mutants (Zaret & Sherman, 1984). We have also shown in this laboratory that the introduction of a stable hairpin into the 3'-UTR of the *PYK* mRNA results in a decrease in the ribosome loading of this mRNA, but the basis for this is unclear.

In the experiments involving the *PYK* mutants 3K and 4H, it was observed that the ribosome loading of the wild type *PYK* mRNA transcribed from the chromosomal locus was greater

in both cases, than that of the total PYK mRNA. It must be stressed that this difference is much more marked in the 4H mutants than in the 3K mutants. Whilst this result can be explained in terms of increased secondary structure involving the 5'-UTR of the 4H PYK mRNA, what of the 3K PYK mRNA? Fig. 5.3.2 and Fig. 5.3.3 illustrate the predicted secondary structure at the 5'-ends of the PYK 3K and wild type mRNAs. There is some difference in both the structures and the free energies of secondary structure formation for these two mRNAs. The initiation codon of the PYK 3K mRNA is involved in more extensive secondary structure than that of the wild type PYK mRNA. This may be causing a slight decrease in the initiation rate of the PYK 3K mRNA. Furthermore, the sequence inserted into PYK mutant 3K contains a number of poor codons (Fig. 5.3.1), and it is possible that a slower elongation rate through this region is also having a slight effect on the initiation rate of this mRNA. However, in the light of the codon bias analysis described in section 5.2, the author would not favour the latter explanation.

5.3.4 Future experiments

It has been shown that the introduction of a stable secondary structure involving the 5'-UTR of the PYK mRNA results in a decrease in the ribosome loading of this mRNA, most probably caused by a reduced rate of translational initiation. Since *in vitro* translation systems are now

available for S.cerevisiae (Tuite & Plesset, 1986), investigations into how secondary structure influences translation could now be attempted. The results of Lawson and co-workers (1986) suggest that secondary structure might influence the formation of the first initiation complex involving the 5'-cap, whilst other results suggest that secondary structure inhibits 40S migration along the 5'-leader (Baim et al, 1985b; Pelletier and Sonenberg, 1985a; Kozak, 1986b). It is likely that the effect of mRNA secondary structure formation upon translation is dependent upon the location of the secondary structure within the mRNA. Ribosome binding studies using short stretches of mRNA may allow the distinction to be made as to how important the influence of secondary structure is on these stages of translation initiation. Furthermore, the introduction of stable secondary structure at an internal site in the PYK gene may allow the demonstration of inhibition of translation elongation by the specific accumulation of a PYK nascent peptide chain of discrete size.

CHAPTER 6

CONCLUDING REMARKS

A method has been described for the analysis of the translation *in vivo* of specific mRNAs in *S.cerevisiae*. The method involves fractionation of post-mitochondrial supernatants by sucrose density gradient centrifugation. Treatment of these post-mitochondrial supernatants with RNase A, EDTA, and cycloheximide, prior to centrifugation, has shown that this method is specific for the separation of yeast polysomes. RNA was shown to be intact across the polysome gradients by Northern blotting. The distribution of specific mRNAs across these polysome gradients was then determined by fractionating the gradients, preparing dot blots, and hybridising the blots with probes specific for individual mRNAs.

The method has been used in two collaborative experiments. The first established that no simple relationship could exist between mRNA stability and translation in *S.cerevisiae* (Santiago *et al*, 1987). In this experiment, the distribution across a polysome gradient of 10 specific mRNAs of known half-life was determined. No obvious correlation was observed between the ribosome loading on these mRNAs and their half-lives.

The second collaborative experiment established that specific manipulations within the 3'-UTR of the *PYK* gene could affect the translation of its mRNA in *S.cerevisiae* (Purvis *et al*, 1987b). Here, specific modifications were made to the 3'-UTR of the *PYK* gene *in vitro* and the effects of these changes upon the translation of the mutant *PYK* mRNAs determined *in vivo*, using the method of polysome

analysis. Whilst certain mutations in the 3'-UTR had no significant effect upon the translation of the PYK mRNAs produced, two others seemed to reduce the efficiency with which the mutant mRNAs were translated. The biological basis for this observation is unknown.

In a further set of experiments, the wild type PYK gene was transformed into yeast on the multicopy vector pJDB207. Transformants appeared to contain variable levels of PYK mRNA, and this seemed to have a deleterious effect upon their growth. The translation of the wild type PYK mRNA was analysed in transformants carrying different levels of this mRNA, ranging from wild type levels (0.6% of total mRNA) to approximately 9% of total mRNA. These experiments showed that the PYK gene of S.cerevisiae appears to be subject to a dosage compensation mechanism which operates at the translational level; when present at elevated levels, the PYK mRNA carries significantly fewer ribosomes. This translational control mechanism does not appear to influence the translation of the actin or G3PD mRNAs, nor can the mechanism be the same as that which operates on the RP1 mRNA, since the translation of this mRNA is similarly unaffected.

Experiments were devised to analyse the effects of poor codon bias on the translation of the PYK mRNA. The insertion of 13 non-preferred codons into the N-terminus of the PYK coding region appeared to have no effect on the translation of this mRNA, within the limits of the analysis.

(This mutant PYK mRNA was compared with a control, mutant PYK mRNA which carried an equivalent insertion of 13 codons encoding a similar amino acid sequence, but with preferred codons. Translation of both of these mutant PYK mRNAs was also compared with other internal controls: the actin, G3PD, and RP1 mRNAs.) This lack of effect was attributed to the relatively low abundance of the mutant PYK mRNA, combined with the relatively small number of non-preferred codons inserted into the PYK gene. However, a B-galactosidase/PYK fusion mRNA, containing almost the entire E.coli B-galactosidase coding sequence (codon bias index in yeast = -0.05%), showed a decrease in its ribosome loading when the level of the fusion mRNA was increased. The translation of TRP2, an mRNA with poor codon bias (codon bias index = 0.13%) was also affected, whereas the translation of other mRNAs with good codon bias (actin, G3PD and RP1 mRNAs) was seemingly unaffected. Therefore, it was concluded that codon bias does appear to significantly influence the translation of an mRNA, but only under "extreme circumstances" (for example, overexpression of an mRNA with extremely poor codon bias).

Experiments were also performed to assess the effects of secondary structure formation at the 5'-end of the PYK mRNA upon its translation. Two mutant PYK genes were constructed (at G.D.Searle) by the insertion of 42 bp into the N-terminus of the coding region. While one insertion was designed to increase the possible secondary structure formation at the 5'-end (as predicted by computer analysis),

the other insertion was designed to encode the same amino acid sequence, but cause a relatively low amount of secondary structure formation. Increasing the secondary structure formation at the 5'-end of the PYK mRNA caused a dimunition in its ribosome loading, whilst wild-type PYK mRNA and control, mutant PYK mRNA appeared unaffected. Therefore, it was concluded that excessive secondary structure formation at the 5'-end of yeast mRNAs appears to inhibit their translation, presumably at the level of initiation.

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