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Translation and stability of an *Escherichia coli* β-galactosidase mRNA expressed under the control of pyruvate kinase sequences in *Saccharomyces cerevisiae*

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ABSTRACT

Plasmids were assembled in which the coding region of the pyruvate kinase (PYK) gene of Saccharomyces cerevisiae was replaced by that of the B-galactosidase (LacZ) gene from Escherichia coli. Analysis of the resultant, chimaeric transcripts from low copy number, centromeric plasmids indicated that this substitution caused a dramatic reduction in the steady-state level of the messenger RNA (mRNA). This fluctuation cannot be wholly accounted for by the 2-fold decrease in mRNA stability observed. This is consistent with the existence of a transcriptional Downstream Activation Site (DAS) within the PYK coding region, analogous to the DAS reported within the yeast phosphoglycerate kinase gene (PGK; Kingsman, S M et al. (1985) Biotech. Gen. Eng. Rev. 3, 377). At these low levels of heterologous gene expression, comparison of the distribution of PYK and PYK/LacZ transcripts across polysome gradients revealed no significant effect mediated by their striking disparity in codon usage. Nevertheless, upon increasing B-galactosidase mRNA levels, via manipulation of plasmid copy number, a distinct decline in ribosome loading was observed for the heterologous PYK/LacZ transcript which was not mirrored by either endogenous PYK transcripts or other yeast mRNAs of high (Ribosomal protein 1) or moderate (Actin) codon bias. However, high levels of the PYK/LacZ mRNA did affect the translation of an endogenous mRNA with poor codon bias (TRP2). The possible basis for this phenomenon is discussed.

INTRODUCTION

S. cerevisiae is widely used as a host for the expression of 'foreign' genes (1,2). Not only is the coupling of heterologous genes to yeast transcriptional and translational control modules of potential commercial value, but it is also a powerful tool in the experimental dissection of those control mechanisms. As the protein product of the E. coli LacZ gene (B-galactosidase) is produced in a functional form in yeast and is easily quantified, this gene has been commonly used in such examinations of transcriptional control from hybrid constructs (3,10).

Sequences involved in promoter modulation are not necessarily limited to the 5' flanking regions of a gene, as witnessed by the enhancer sequences of

various mammalian genes (11). In S. cerevisiae, the expression of foreign proteins using the promoter from the glycolytic gene phosphoglycerate kinase (PGK) has often led to manifestly lower levels of product than expected. This suppression of protein synthesis reflects a low steady-state level of the heterologous mRNA (2,12). Conflict arises when formulating an explanation for this phenomenon. Three models have been proposed (25). Two of these are based upon the destabilization of mRNA associated with deleting all, or a significant part, of the PGK coding region. This effect is thought to be mediated either by removal of a stabilizing sequence or secondary/tertiary structure from the mRNA, or by prevention of a protection event normally linked with synthesis of the PGK protein itself (12,25). Alternatively, it is suggested that an unspecified 'enhancer-like' unit (the Downstream Activation site) exists within the coding sequence which positively influences PGK promoter activity (1). This paper analyses the presence of a functionally analogous system in another glycolytic gene, using a B-galactosidase insertion between the initiation and termination codons of PYK. Such a construct also facilitates the study of the influence that different heterologous mRNA levels may have on translation. There is a strong correlation between the relative abundances of yeast tRNA isoacceptors and the use of their respective codons in abundant mRNA species (13,14). Alterations in the rate of protein synthesis caused by codon usage effects should be related to the level of transcript present, whilst variations due to codon context or structural constraints should be independent of mRNA concentrations. In the case of the B-galactosidase mRNA in yeast, such is the codon bias (codon bias index = -0.05) that increasing concentrations of this mRNA may seriously upset the intracellular tRNA balance and hence impinge upon the translation of endogenous mRNAs. This possibility is tested in this study.

MATERIALS AND METHODS

Strains

The S. cerevisiae and E. coli strains used have been described in the preceding, associated paper.

Growth Rates and Plasmid Stability

The growth rates for S. cerevisiae strains, either containing or lacking a recombinant plasmid, were determined using the absorbance at 600nm of cultures in YPD (1% Yeast extract, 2% Bacto-peptone, 2% Glucose) or GYNB (0.65% yeast nitrogen base (without amino acids), 2% Glucose, 50ug/ml of

appropriate amino acid supplements). All cultures were evaluated for total viable cells and the proportion of these cells maintaining the plasmid marker by plating onto the relevant selection agar at the time of sampling for a particular experiment.

Enzyme Assay

The level of B-galactosidase in different plasmid-containing yeast strains was established by the widely used chromatogenic conversion of ortho-nitrophenyl-B-D-galactopyranoside (15). Routinely, cells were grown to mid-logarithmic growth phase (A_{600} = 0.6) after sub-culturing from minimal selective medium (approximately 10 divisions after relief from selection). Then 40ml of culture was centrifuged at 5000xg for 5 minutes and resuspended in 1ml of assay buffer (100mM Na_2PO_4, 1mM $MgSO_4$, 100mM B-Mercaptoethanol, 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 exactly 1 minute. 1ml of the lysate was centrifuged at 12,000xg for 10 minutes and dilutions of the supernatant used for enzyme assays. The protein content of the lysates was checked by the Bradford assay. Relative enzyme activity is expressed as functional B-galactosidase tetramers per plasmid-bearing *S. cerevisiae* cell.

RNA Estimations

RNA was prepared using the procedures of Lindquist (16). Qualitative analysis was performed by northern blotting (18), and quantitative evaluation via dot blotting followed by hybridization with probes of known specific activities using 18S rRNA as an internal control (18).

Poly(U) Sepharose Column Chromatography

The presence or lack of a poly(A) tail upon specific mRNA species was analysed using poly(U) Sepharose column chromatography as described previously (17). The relative abundance of specific mRNAs in poly(A)+ and poly(A)- RNA was again measured by dot blotting.

Half-life and Polysome Distribution

The techniques used match those described previously (18,19).

Plasmid Construction

Virtually the complete *E. coli* B-galactosidase coding region was placed between PYK initiation and termination codons on the plasmid YCpPK12 by first deleting most of the PYK coding sequence and replacing it with pUC13 polylinker DNA (Figure 1). It was into this 'expression cassette' that the 3kb SmaI/SalI fragment from pMC1871 (20), containing the LacZ coding region, was cloned. Using partial digestion with XbaI and SstI, the whole PYK/LacZ

chimaera was transferred to the multicopy plasmid pJBD207 (21). Thus both centromeric (YCpPKG2; low copy) and 2u-based (YEpPKG441; high copy) plasmids were constructed that contain B-galactosidase sequences under PYK transcriptional and translational control.

Plasmid Copy Number

Quantitative analysis of plasmid copy-numbers in preparations of total yeast DNA was carried out using a dot blot matrix. Identical filters, containing serial dilutions of EcoR1-digested DNA in duplicate, were hybridized with [^{32}P]-probes of known specific activities for the Actin, HIS3 and LacZ genes. Following autoradiography, the amount of hybridization was quantified by cutting out each dot and subjecting it to scintillation counting. This allowed accurate determination of the relative levels of chromosomal (Actin and HIS3) and plasmid DNA sequences (LacZ). The correct hybridization and wash conditions were experimentally defined for each probe by Southern blotting.

Probe Synthesis

The following fragments were gel-purified for nick-translation; the 3kb BamHI fragment from pMC1871 (20) containing the whole B-galactosidase coding sequence, the 511bp XbaI/BglII fragment from pSPK2 containing a portion of the PYK coding region, the 1.7kb BamHI fragment of pMA700 (22) containing the HIS3 gene, and the 915bp EcoRV fragment from pAS1 (24) containing a piece of the TRP2 gene. The whole of plasmid pYA301 (23) was used as a probe for actin mRNA, and an 18S rRNA probe was generated using pSP65R (18). The mRNA for ribosomal protein 1 (Rp1) was quantified using a 5' end-labelled oligonucleotide (34-mer).

RESULTS

PYK/LacZ Expression

Upon transformation of *S. cerevisiae* DBY746 with plasmids YCpPKG2 and YEpPK441 (Figure 1), colonies showing both the correct auxotrophic markers and the ability to produce functional B-galactosidase were selected. The large majority of colonies derived from YCpPKG2 displayed similar levels of enzyme activity, but a wide range of activities was observed from transformants containing the multicopy plasmid YEpPKG441. One isolate from the population of YCpPKG2 transformants (LACJ), and two from the YEpPKG441 transformants (LAC7 and LAC8) were chosen for more detailed examination due to their widely differing levels of expression of the foreign sequences (Table 1). Under the conditions of study, the chromosomal PYK locus produced

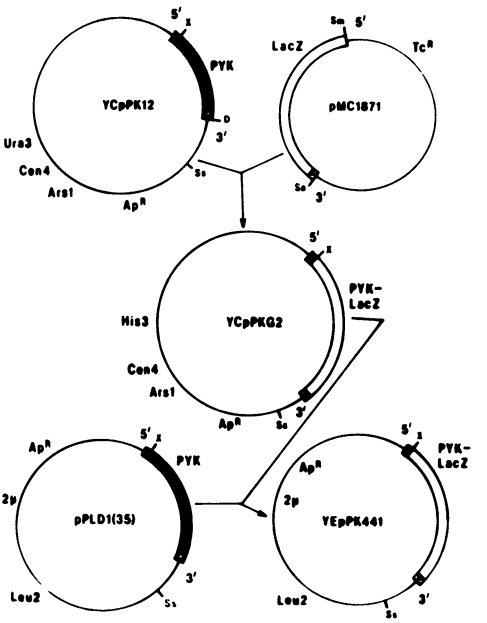

FIGURE 1: Simplified Representation of the Construction of Single and Multicopy Yeast Plasmid Vectors Containing the LacZ gene of E.coli under PYK Transcriptional Control. The restriction sites designated Ss (SstI), X (XbaI), D (DraI) and Sm (SmaI) are not unique within the plasmids shown. Partial digestion, Klenow-filling and purification of various fragments from agarose gels were necessary for insertion of the B-galactosidase coding region behind the initiation codon of PYK in both single and multicopy yeast plasmid vectors. The arrow within the coding regions (PYK, PYK/LacZ) indicates direction of transcription.

0.6 ± 0.12% of total mRNA, whereas the levels of the PYK/LacZ mRNA ranged from 0.04 ± 0.01% in YCpPKG2 transformants to 0.96 ± 0.05% in the strain LAC8. As the YCpPKG2-bearing yeast cells have, in general, a single copy of the PYK/LacZ gene the ratio of steady-state mRNA level from the wild-type PYK gene to that from the PYK/LacZ gene is 31:1 in molar terms. However, as the stability of the chimaeric transcript is approximately half that of PYK mRNA (Table 2), this would indicate a ratio of approximately 15:1 in the rates of synthesis of the PYK and PYK/LacZ mRNAs, respectively. Even when the copy number of the fusion gene exceeds 50 per haploid genome (LAC8) the steady-state molar level of transcript does not exceed that of mRNA produced from the single chromosomal locus of PYK. The production of enzymatically functional B-galactosidase increases with escalating levels of mRNA (Table

TABLE 1: Collated Data of B-Galactosidase Expression in a Variety of Plasmid Constructs.

STRAIN	LACJ	LAC7	LAC8
PLASMID	YCpPKG2	YEpPKG441	YEpPKG441
PLASMID COPY NUMBER	1-2	9-11	50-70
STABILITY (%)[a]	100	>95	75-85
B-GALACTOSIDASE mRNA LEVEL (% of Total mRNA)	0.04±0.01	0.20±0.01	0.96±0.05
B-GALACTOSIDASE ENZYME LEVEL (10^4xTetramers/Viable Cell)	1.27±0.11	3.61±0.25	12.3±1.70
DOUBLING TIME (min.) RICH MEDIUM	120	125	140
DOUBLING TIME (min.) MINIMAL MEDIUM	162	430	>600

(a) The stability of plasmids is represented by the percentage of cells still bearing the selectable marker and heterologous gene after 10 generations of growth in non-selective media.

1). However, it is not a parallel response. The elevated production of B-galactosidase appears to have a deleterious effect upon the doubling time of the host yeast cell, this being particularly prevalent when cells are grown in minimal medium (Table 1). Relief from this growth constraint can be achieved either by plasmid-loss, decrease in copy number, or internal DNA rearrangements in the plasmid which cause inactivation of PYK/LacZ gene expression. All three occurrences have been observed especially in the case of LAC8 (results not shown).

TABLE 2: Relationship between mRNA Stability and Abundance.

STRAIN	LACJ		LAC8	
PROBE	ABUNDANCE (% total mRNA)	STABILITY (mins)	ABUNDANCE (% total mRNA)	STABILITY (mins)
Lac Z	0.04 ± 0.01	27.4 ± 3.4	0.96 ± 0.05	27.6 ± 4.7
PYK	0.61 ± 0.18	57.0 ± 11.5	0.63 ± 0.07	54.0 ± 6.5
pAT153	<0.01	ND	0.08 ± 0.00	9.0 ± 1.0
Rp1	ND	ND	ND	10.0 ± 0.00

(ND = not done)

TABLE 3: Poly(U) Sepharose Elution of RNA from the Strain LAC8.

PROBE	PERCENTAGE OF mRNA	
	Poly(A)-	Poly(A)+
rRNA	95	5
PYK	11	89
LacZ	11	89
pAT153	14	86

Poly(A) Tail

The replacement of the PYK coding region with that of B-galactosidase resulted in a transcript of around 3.2kb as visualized by northern blotting (results not shown). If RNA from a yeast strain bearing a high copy number plasmid is challenged on northern blots with a probe synthesized from a plasmid carrying E. coli B-lactamase sequences (pAT153), then an additional transcript of about 2.3kb is seen (data not shown). This suggests a single

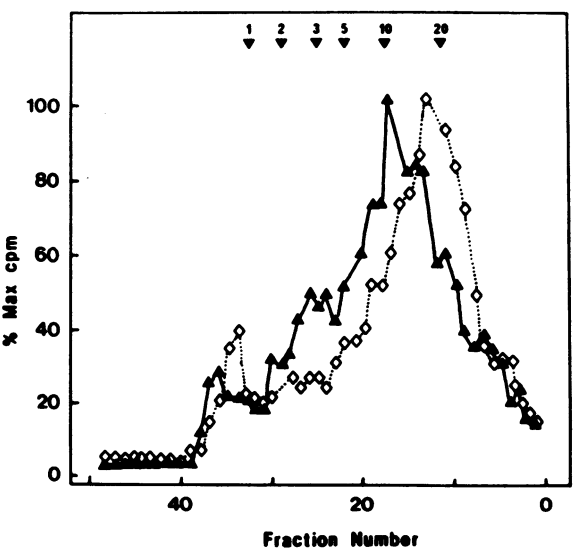

FIGURE 2: Distribution of PYK/LacZ and PYK mRNAs upon a Polysome Gradient. Chromosomal PYK (▲) and PYK/LacZ (◇) mRNAs were identified using specific nick-translated probes on identical filters of a fractionated gradient from the yeast strain LACJ. The arrows at the top of the diagram represent the position of polysomes with 1,2,3,5,10 or 20 ribosomes, respectively.

TABLE 4: Distribution of mRNAs upon Polysome Gradients from Strains producing different amounts of B-galactosidase.

mRNA (codon bias index)	STRAIN	POLYSOME FRACTION (% total cpm) 0-2	2-6	6-15	>15	PEAK VALUE OF RIBOSOME LOADING
	764	NA	NA	NA	NA	NA
Lac Z	LACJ	10	14	24	52	19
(-0.05)	LAC7	7	25	61	6	14
	LAC8	18	30	39	13	12
	746	13	27	55	5	10
PYK	LACJ	3	32	52	13	10
(0.95)	LAC7	16	33	46	5	10
	LAC8	22	29	49	5	10
	746	36	29	31	4	6
Rp1	LACJ	44	32	22	2	6
(0.92)	LAC7	40	28	30	2	6
	LAC8	30	31	32	7	7
	746	23	30	32	15	7
Actin	LACJ	18	34	37	11	7
(0.79)	LAC7	18	34	33	15	7
	LAC8	19	28	34	18	8
	746	ND	ND	ND	ND	ND
TRP2	LACJ	12	27	46	15	10
(0.13)	LAC7	21	34	33	12	5
	LAC8	31	35	23	11	5

Polysome gradients from strains 746 (plasmid-free), LACJ (YCpPKG2) LAC7 and LAC8 (both YEpPK6441) were fractionated and probed with sequences specific for B-galactosidase, PYK, actin, Rp1 and TRP2 mRNAs. The distribution of each mRNA is described as the percentage of total cpm in fractions bearing 0-2, 2-6, 6-15 and >15 ribosomes. (NA = not applicable; ND = not done)

defined region for transcriptional termination. Also, the results from poly(U) Sepharose chromatography indicate that both transcripts are polyadenylated in yeast (Table 3).

mRNA Distribution upon Polysome Gradients

In yeast strains showing low levels of B-galactosidase mRNA and enzyme activity (LACJ) the mRNA bears almost twice the number of ribosomes on average than the progenitor PYK mRNA although both have the same 5' untranslated region and environment around the initiation codon (Figure 2). However, the length of the B-galactosidase coding region is twice that of the PYK mRNA. Thus this result is to be expected if the rates of initiation and elongation of the two mRNAs are the same. This, in turn, would indicate that the vastly different codon usages of B-galactosidase (codon bias index in

yeast = -0.05), and PYK (codon bias index = 0.95), has no significant influence upon the rate of translational elongation when the heterologous mRNA is at low abundance. A clear decrease in the ribosome loadings of the B-galactosidase mRNA is seen when the levels of this mRNA are increased. This is most clearly illustrated when the gradients are divided into regions bearing 0-2, 2-6, 6-15 and greater than 15 ribosomes (Table 4). This decreased ribosome loading may at least partially explain the non-linear response between increasing mRNA levels and enzyme activity (Table 1). The diminution of ribosome loading on the PYK/LacZ mRNA is not paralleled in endogenous yeast transcripts of high (PYK = 0.95; Rp1 = 0.93) or moderate (actin = 0.79) codon bias. However, increasing levels of PYK/LacZ mRNA do affect the translation of an endogenous mRNA with poor codon bias (TRP2 = 0.13).

mRNA Half-Lives

The measurement of B-galactosidase mRNA stability in strains LACJ and LAC8 shows that, for this mRNA, there is no link between half-life and abundance (Table 2). The mRNA stabilities observed for B-galactosidase (relatively stable), and Rp1 and B-lactamase (relatively unstable) all reinforce our previous evidence for dual mRNA populations in S.cerevisiae (18) and suggest that heterologous sequences respond to the mRNA turnover pathways in a similar manner to homologous mRNAs. Also the halving of the stability of the PYK/LacZ mRNA relative to the PYK mRNA can be conveniently explained by the doubling in mRNA length, if one accepts an inverse relationship between mRNA length and half-life described previously by Santiago and co-workers (18).

DISCUSSION

The existence of a Downstream Activation Site (DAS) within the coding sequence of the PGK gene in S. cerevisiae is a source of controversy (25). A similar pattern of observations to that recorded for the PGK gene accrues upon the deletion of the coding sequence of another glycolytic gene (PYK) and its replacement with heterologous DNA. Although the 5' and 3' non-coding sequences of the PYK gene remain essentially intact, the steady-state level of the B-galactosidase mRNA is approximately 15-fold lower than one would expect from a fully functional PYK promoter. It is not until the plasmid copy number exceeds 50 per haploid genome that the levels of heterologous mRNA roughly equivalent to those of the PYK mRNA are achieved. Hitzemann and co-workers have argued (25) that the equivalent deficiencies in PGK mRNA

levels are due to the loss of a CIS-acting sequence which stabilizes the mRNA (this stabilization being mediated by the synthesis of the enzyme). This clearly does not occur for PYK. Although the half-life of the B-galactosidase mRNA is lower than that of the PYK mRNA, this cannot account for the dramatic decrease in steady-state level of the heterologous mRNA. The reduced half-life may well be associated with the increase in mRNA length (18). This data reinforces a model which predicts that an internal sequence (Downstream Activation Site) influences the rate of transcription of the PYK gene (1).

The analysis of the B-galactosidase activities in strains LAC5, LAC7 and LAC8 shows that an increase in mRNA levels does not produce a proportional increase in the level of functional protein. Although this may be due to protein inactivation by agglomeration as observed in PGK overexpression (26), or to increased protein turnover, the results from polysome gradients indicate that there may be a reduced translation rate for this mRNA species. Increasing the levels of B-galactosidase mRNA has a significant effect upon its ribosomal loading. There is a strong correlation between the codon usage in efficiently expressed yeast genes and the relative abundances of the isoaccepting tRNAs (13,14). Therefore, the translation of high levels of the B-galactosidase mRNA, an mRNA with extremely poor codon bias, may lead to a severe depletion of amino-acyl tRNAs which correspond to 'rarely-used' codons (27). This in turn would cause a significant increase in the time taken to translate these codons, and could ultimately result in ribosome stalling and premature termination in extreme cases. This premature termination would account for the reduced ribosome loading on the B-galactosidase mRNA in strain LAC8.

A severe depletion of 'rare' amino-acyl tRNAs would be expected to affect the translation of all mRNAs with poor codon bias. Therefore, it is not surprising that the ribosome loading on the TRP2 mRNA (codon bias index = 0.13) is markedly reduced in the strains LAC7 and LAC8 (Table 4), while the mRNAs with high degrees of codon bias (PYK, Rp1 and actin) are unaffected. Hence, the significant alteration in growth rate, particularly in minimal medium (Table 1), caused by over-expression of B-galactosidase may be due to the presence of a forced imbalance in tRNA abundance (and hence problems with translation elongation in general). Alternatively, the reduced growth rates may be due to the presence of high levels of a functional foreign enzyme that damages the internal physiological balance of the yeast cell.

It has been shown in the associated paper that premature termination of

translation of the B-galactosidase mRNA has no significant effect upon its stability. Here it is confirmed that mRNA half-life is independent of ribosome loading, and that the half-life of the B-galactosidase mRNA is not influenced by its abundance. No change in the stability of the B-galactosidase mRNA is observed when the level of this mRNA is elevated 15-fold (Table 2).

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