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The effects of alterations within the 3' untranslated region of the pyruvate kinase messenger RNA upon its stability and translation in *Saccharomyces cerevisiae*

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ABSTRACT

A 53 basepair deletion was constructed within the 3' untranslated region (3' UTR) of the yeast pyruvate kinase (PYK) gene borne upon a centromeric plasmid. Various modular assemblies of the pUC13 polylinker DNA (single unit = 44 bp) were used to replace the deleted region, and the effects of these modifications upon both transcript stability and translation ascertained in yeast. The use of a differential probing stratagem, based on the hybridisation of specific oligonucleotides to either pUC13 polylinker or unaltered PYK 3' UTR sequences, allowed for discrimination between mutant (plasmid borne) and wild-type (chromosomal) PYK transcripts. In no construct was there any significant alteration in mRNA stability, but translation of the PYK mRNA was severely curtailed by truncation of the 3' UTR or the presence of a strong hairpin-loop structure in the 3' UTR. A specific mutation in the N-terminal coding sequences, which created a premature termination codon in both a 3' 'tagged' PYK plasmid and a PYK/LacZ fusion gene, aborted the translation of a majority of their transcripts but left their chemical half-lives unaltered. This observation is at variance with some previously published data (Losson & Lacroute (1979) Proc Natl Acad Sci USA 76, 5134; Pelsey & Lacroute (1984) Curr Genet 8, 277), but is consistent with our own earlier observation that there is no obvious link between ribosome loading and mRNA stability in yeast (Santiago et al. (1986) Nucleic Acids Res 14, 8347). Possible reasons for this disparity are discussed.

INTRODUCTION

The primary sequence of a gene contains a myriad of control signals that determine the levels of transcription, RNA processing, translation and mRNA stability. Some portions of the 3' untranslated region (3' UTR) are purported to show a general level of sequence conservation (1-3) which dictate the sites for polyadenylation and transcriptional termination. Considerable evidence exists for the presence of specific nucleotide sequences which strongly influence the stability of gene transcripts 4-11) and these can reside within the 3' UTR of the mRNA molecule (12-14). Previously, manipulations within the 3' UTR of structural genes in S.cerevisiae have led to variable alterations to mRNA length, polyadenylation, stability and translatability depending upon the gene used and type of alteration made (15-

17). These observations were based upon indirect measurements of mRNA translation and stability unlike the direct assays applied in this study.

It has been argued that the stability of an RNA molecule may be intrinsically linked to its translation, in both prokaryotes (18-21) and eukaryotes (22, 23). Certainly, in the case of histone mRNA in S.cerevisiae, translation is thought to destabilise the transcript (24, 25). Positioning of premature termination codons near the 5' end of the URA1 and URA3 genes of yeast (22, 23) can decrease the steady-state levels of these mRNAs by as much as 5-fold by severely diminishing their chemical half-lives. Such an effect is tempered by moving the premature termination codon further away from the site of translation initiation, and can be alleviated by extragenic nonsense suppression. These studies suggested that, during translation, ribosomes might passively protect an mRNA from degradation. However, they are contradicted by studies of the CYC1 mRNA (15) and a wider analysis of ribosome loading and stability (26). This apparent incongruity has been studied further using a differential probing system that is capable of distinguishing between similar levels of transcript from plasmid-borne or chromosomal PYK genes.

MATERIALS AND METHODS

Strains

S.cerevisiae DBY746 (His3, Leu2, Trp1, Ura3) and Escherichia coli C1400 (SupE, SupF, Hsd5, Met$^-$, RecA, lambda L512) were employed as the hosts for all basic and modified E.coli/yeast shuttle vectors.

RNA Preparation

Total RNA was isolated from S.cerevisiae by the method described by Lindquist (27).

Blotting and Hybridisation

RNA analysis, by both northern blotting and dot matrix techniques, has been fully described previously (26, 28). Oligonucleotides for hybridization to specific regions of various mRNAs were synthesized in the Department of Biochemistry (Glasgow University). 5' end-labelling of oligonucleotides was accomplished using the standard reaction conditions described for polynucleotide kinase (29). At various stages, nick-translated probes of virtually the whole E.coli B-galactosidase gene (3kb BamHI fragment from pMC1871) and the S.cerevisiae PYK gene (511bp XbaI/BglII fragment from pSPK2; ref. 26) were synthesized using standard methodology (30). Conditions for reproducible attainment of maximal hybridization with minimal background for

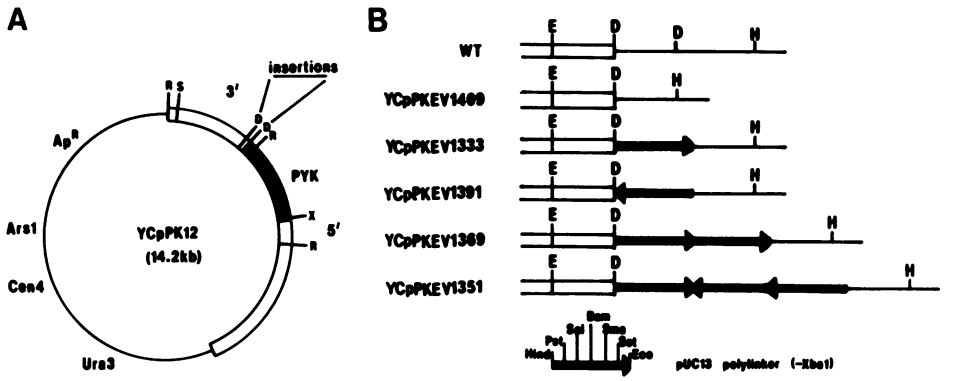

Figure 1: Construction Of Plasmid-borne PYK Genes With Modified 3'UTR Regions. A) Progenitor plasmid YCpPK12. The restriction sites shown, R(EcoRI), S(SstI), D(DraI) and X(XbaI) are not unique within the plasmid. Partial digestion, and purification of designated DNA fragments from agarose gels were necessary in the construction of the 3'UTR modifications shown in B). Here the different 3'UTR regions produced by deletion of DraI fragment (YCpPKEV1409) and insertion of polylinker DNA (➡shows orientation) are shown; E (EcoRI) D (DraI), H(HpaI).

all oligonucleotide and nick-translated probes were determined experimentally. The general procedures for blotting, filter preparation, washing and autoradiography were as described before (26, 28).

mRNA Stability & Translation

The use of 1,10-phenanthroline as a transcriptional inhibitor for measurements of mRNA stability has been fully described elsewhere (28), as has the technique of sucrose density gradient centrifugation of post-mitochondrial supernatants used for analysis of yeast polysomes (26).

Plasmid Constructions

The replacement of the 3' UTR of PYK was undertaken using the plasmid YCpK12 (Figure 1A), an *E.coli*/*S.cerevisiae* shuttle vector based upon the centromeric vector YCp50 (31). By a series of complicated manipulations the 53 bp DraI fragment in the 3' UTR of the PYK gene was deleted and replaced by single or multiple inserts of pUC13 polylinker DNA (a 44bp unit previously modified by S1 nuclease treatment to remove the internal XbaI site). The resultant 3' constructs are shown in Figure 1B. The assembly of an *E.coli* B-galactosidase (LacZ) gene under transcriptional control of the PYK locus is described in detail in the following paper. In this construct, the PYK coding region was replaced with that from the LacZ gene, the translational fusion occurring after the second codon of the PYK gene. In a further set of constructs, the reading frames of both the PYK/LacZ fusion gene and one of

OLIGONUCLEOTIDE DESIGNATION	SEQUENCE	SPECIFICITY
038	5'-TAATATCTTCATTCAATCATGATTC-3'	wt PYK
027	5'-TTTATATAAAATTTAAACGGTAGAGAC-3'	YCpPKEV1409
040	5'-CCCGGGGATCCTAGTCGACCTGCCAGCCCA-3'	YCpPKEV1333,1369&1351
037	5'-GCAGGTCGACTAGGATCCCCGGGCGAGCTCG-3'	YCpPKEV1351&1391

Table 1: Design And Specificity of Oligonucleotides Required For Differential Probing. 038 is complementary to part of the 3' region deleted from the wild-type PYK mRNA to generate the mutants. 027 was designed to hybridize with mRNA1409, the mutant carrying the 53 base deletion, and is complementary to the sequences in PYK which flank either side of the deletion. 037 and 040 were designed to hybridize with opposite strands of the modified polylinker. For example, an mRNA carrying a single copy of the polylinker will hybridize with only one of these oligonucleotides, depending upon the orientation of the insert.

the 3'-tagged PYK constructs (1333; Figure 1B) were disrupted by Klenow-filling the unique XbaI site spanning second and third codons of each gene.

RESULTS

Differential Probing Stratagem

In order to distinguish between PYK transcripts of plasmid or chromosomal origin it was necessary to synthesize oligonucleotides specific for the 3' UTR of either mutant or wild-type mRNA species. Table 1 shows the sequence and specificity of these oligonucleotides. Northern blot evaluation of RNA from the relevant plasmid-containing yeast strains (Figure 2) implies

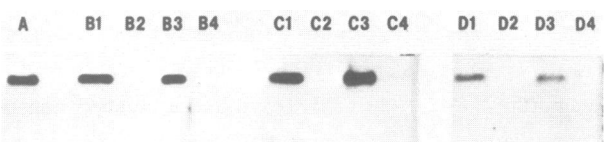

Figure 2: Differential Hybridization of Oligonucleotides To Wild-type And Mutant PYK mRNAs. **A)** A northern blot of RNA from DBY746 hybridized with the oligonucleotide probe specific for wild-type PYK mRNA (038). **B)** RNA from DBY746 carrying 1) YCpPKEV1333, 2) YCpPKEV1351, 3) YCpPKEV1369 and 4) YCpPKEV1391, probed with oligonucleotide 040 (see Table 1). **C)** RNA from DBY746 carrying 1) YCpPKEV1351, 2) YCpPKEV1369, 3) YCpPKEV1391 and 4) YCpPKEV1409, probed with oligonucleotide 037. **D)** RNA from DBY746 carrying 1) YCpPKEV1351, 2) YCpPKEV1369, 3) YCpPKEV1409 and 4) YCpPKEV1391, probed with oligonucleotide 027.

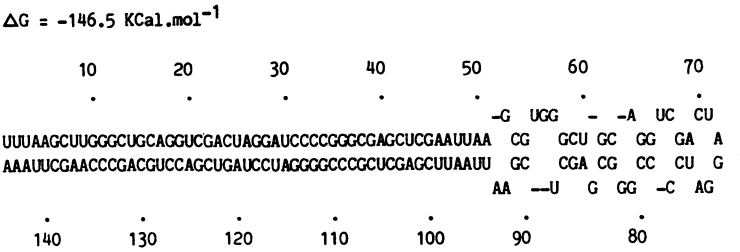

Figure 3: Diagramatic Representation of the Putative Hairpin Loop In The 3' UTR of the PYK mRNA derived from YCpPKEV1351. Analysis was undertaken using the computer program 'FOLD' (36).

that, using the oligonucleotides specific for either the 3' UTR of wild-type or mutant transcripts, it is possible to distinguish between 'tagged' plasmid and chromosomally derived mRNAs in the same RNA isolate. The secondary structure formed by the triple insert of pUC13 DNA in YCpPKEV1351, is so stable (ΔG = -146.5 KCal.mol^{-1}; Figure 3) as to be refractory to the normal denaturing conditions used for northern blotting. This means that the 'tagged' transcript from YCpPKEV1351 only hybridises strongly to one (037) of the two pUC13-specific oligonucleotides (040 and 037) that would normally be capable of binding to the sequences of the triple insert. Also, there is binding of the deletion- specific oligonucleotide (027) to PYK transcripts from YCpPKEV1351 as the formation of strong hairpin and loop causes a spatial alignment of 3' UTR sequences theoretically over 130bp apart (Figures 2 & 3).

mRNA Half-Life Measurements

The chemical stability of PYK transcripts *in vivo* has been previously measured using nick-translated probes (28). Analysis of both modified and wild-type PYK mRNAs using oligonucleotide hybridization is shown in Table 2. At least 4 independent measurements of mRNA stability were made for each type of PYK transcript, the resultant half-lives being normalised to the stability of 18s rRNA (28). There is no significant difference in the stability of wild-type chromosomally-derived PYK transcripts when different probing systems are used (nick-translation versus 3' UTR-specific oligonucleotides; Table 2) indicating that the mRNA turnover rate of the whole mRNA matches that of the 3' UTR. It is also obvious that the removal of the 3' UTR sequences preceding the proposed polyadenylation and transcription-termination sequences and replacement with pUC13 sequences of similar length but higher G:C content, has no significant effect upon PYK mRNA stability. The presence of a strong secondary structure in the 3' UTR does not increase

Table 2: Stability Of Wild-Type And Mutant PYK mRNAs.

mRNA	HALF-LIFE (min.)	NUMBER OF MEASUREMENTS
WT - GENE PROBE	59.8±7.8	6
WT - OLIGO PROBE	61.7±8.1	7
YCpPKEV1333 →	60.3±3.7	4
YCpPKEV1391 ←	65.0±7.8	4
YCpPKEV1351 →◄←	64.3±13.9	4
YCpPKEV1369 →→	49.3±5.8	4

the stability of the PYK mRNA. However, doubling the insert size in this region may slightly decrease the mRNA half-life, a change which could be explained by an increased susceptibility to endonucleolytic attack.

mRNA Distribution Upon Polysome Gradients

The distribution of the specific mRNAs across polysome gradients was determined either using nick-translated PYK probes or the specific 3' UTR oligonucleotides (Table 1). The gradients from all plasmid-bearing yeast strains showed very similar absorption profiles at 260nm (Figure 4). The proven ability to probe identical filters from a fractionated polysome gradient with oligonucleotides capable of defining mutant or wild-type mRNAs, removes the difficulties inherent in the comparison of different gradients since the wild-type PYK mRNA can be used as an internal control in each case. It is clear (Figure 4) that in all cases other than YCpPKEV1351 and YCpPKEV1409 the distribution of modified PYK transcripts across the polysome gradients closely matches that of the wild-type mRNA. When the hairpin-loop structure is present in the 3' UTR a serious curtailment of ribosome loading occurs. If this structure is removed, leaving one unit of the polylinker (by deleting a large region of the pUC13 insert using the 3 internal Sal I sites), normal translation of the mutant PYK transcript is resumed (results not shown). Therefore, the reduced translatability of the PYK mRNA from YCpPKEV1351 is due to the 3' insertion, and not due to an undefined secondary mutation elsewhere in the mRNA. A severe effect upon ribosomal loading is also observed for the PYK mRNA containing the 3' UTR deletion (YCpPKEV1409; Figure 4).

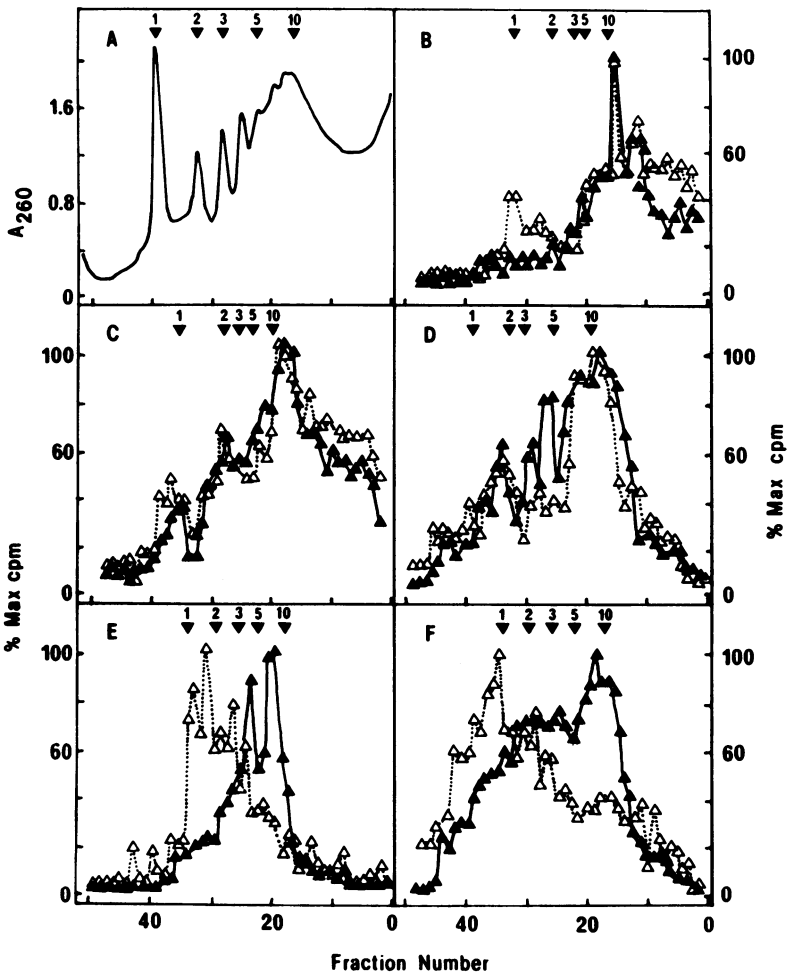

Figure 4: Comparison Of The Distribution Of Wild-type And Modified PYK mRNAs Upon Polysome Gradients. A polysome gradient (**A**) from strains bearing the different plasmid constructs was divided into 48 equal fractions and used to prepare dot blots on nitrocellulose filters. Different PYK mRNAs were quantified using the appropriate, specific oligonucleotide; ▲ chromosomal PYK mRNA, and △ plasmid-borne 3'UTR-modified PYK mRNA. The different gradients shown are polysome gradients from DBY746 carrying each modified PYK gene: **B** = YCpPKEV1333; **C** = YCpPKEV1391; **D** = YCpPKEV1369; **E** = YCpPKEV1351 and **F** = YCpPKEV1409.

Premature Termination Analysis

A significant alteration was observed in the distribution of mutant pPKEV1351 PYK transcripts upon polysome gradients relative to the wild-type PYK mRNA, and yet there was no apparent effect upon the chemical half-life of

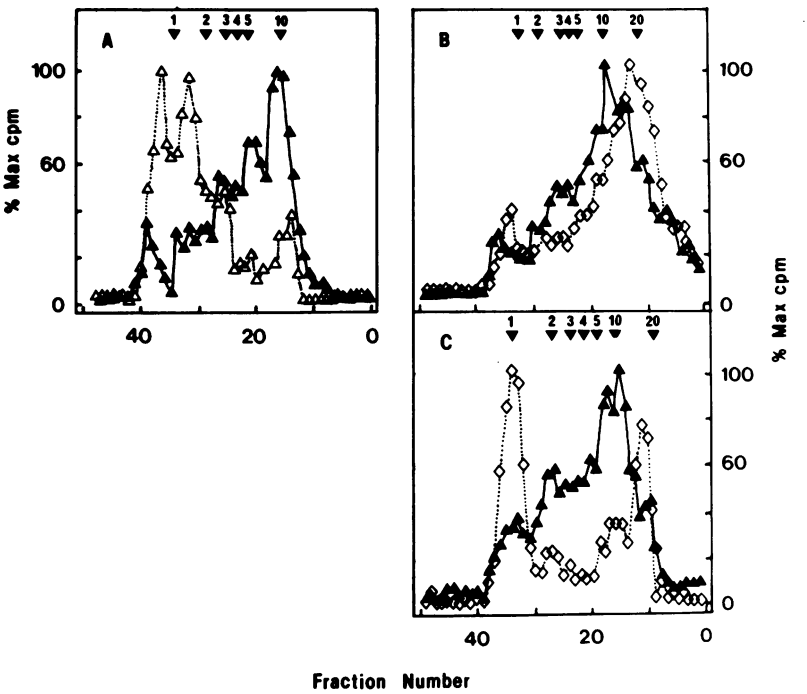

Figure 5: Polysome Gradient Distribution of PYK and PYK/LacZ mRNAs. With or Without Premature Termination Codons Near the Initiation Codon. A) The distribution of a PYK mRNA with the XbaI site at the 5' end of the coding region 'Klenow-filled' to create a premature termination codon (YCpPKEV1333D; △), is compared to that of the wild-type, chromosomal PYK mRNA (▲). In gradients B and C, a PYK/LacZ mRNA (◇) before (B) or after (C) insertion of the XbaI-derived termination codon is compared with the chromosomal PYK mRNA (▲). The arrows at the top of each profile correspond to the approximate position of peaks with 1 to 20 ribosomes as determined from the A260 profile of the gradients.

the mutant mRNA. Therefore, the general applicability of models for eukaryotic mRNA degradation that are based upon ribosomal potection is again queried. Further investigation was undertaken by creating a premature termination codon near the N-terminus of the coding regions of both a 3' tagged PYK transcription unit (pPKEV1333) and a PYK/LacZ fusion gene. The internal comparison of the polysomal distributions for the relevant mRNAs shows a near-complete abandonment of translation for both 5' altered mRNAs with respect to wild-type PYK transcripts (Figure 5). A slight complication arises due to a degree of natural frameshift suppression, particularly in the case of the B-galactosidase transcript. However, even here less than 30% of the mRNA carrying the premature termination codon is fully translated. Such

Table 3: Comparison Of Ribosome Loading With mRNA Half-Life.

PLASMID (PROBE USED)	POLYSOMAL DISTRIBUTION (% Total cpm) 0-2	2-6	6-15	>15	mRNA HALF-LIFE (mins)
YCpPKEV1333D (038)	26	15	10	47	61.7±8.1
YCpPKEV1333D (040)	61	20	16	3	45.0±7.0
YCpPKG2 (LacZ)	10	14	24	52	27.4±3.4
YCpPKG2D (LacZ)	48	11	18	23	26.6±3.5

Each of the gradients shown in Figure 5 were divided into fractions representing 0-2, 2-6, 6-15 or >15 ribosomes. The percentage of an mRNA present in each of these fractions was determined by cutting and weighing the mRNA distribution profile.

sequence-specific frameshift suppression has already been reported in S.cerevisiae (32). Interestingly, the mRNA stabilities show little change associated with this loss of ribosomal protection (Table 3 & Figure 5).

DISCUSSION

The ability to distinguish mutant from wild-type mRNAs in the same population has been the corner-stone of this study. In the case of important metabolic genes such as PYK, deletion of the chromosomal copy or the use of multicopy plasmid vectors to over-express the gene may have serious physiological effects upon the host which might cause unknown secondary effects on the parameters of interest. The use of centromeric-based plasmids, which theoretically ensure similar levels of chromosomal and plasmid-derived transcripts, should alleviate such problems.

Alterations to the 3' UTR of the PYK gene of S.cerevisiae have resulted in a range of plasmid-borne genes carrying foreign DNA sequences substituting for the normal region that separates translation termination from the proposed polyadenylation and transcription termination signals. The insertion of sequences of similar length but different base composition has no gross effect upon transcriptional termination (Figure 2), polyadenylation (data not shown), mRNA stability (Table 2) or ribosome loading (Figure 4). This is also the case when a tandem array of pUC13 polylinker DNA is present that increases the spacing between the termination codon and the PYK 3' UTR signal

sequences. The positioning of a very strong secondary structure between the translational termination codon and the poly(A) tail has little influence upon transcript stability, unlike the REP sequences of E.coli (33). Perhaps the theoretical protection against 3' - 5' exonuclease activity afforded to prokaryotic transcripts by such secondary structures is already supplied by the poly(A) tail in eukaryotic mRNAs. However, the hairpin-loop produced by the triple polylinker insert in the mRNA from YCpPKEV1351 does seriously alter the ribosomal loading pattern. The presence of a strong secondary structure has been shown to have a deleterious effect upon translational initiation or elongation depending upon its position within the transcript (34, 35). Why a strong secondary structure located at the 3' end of the transcript should have such a major influence upon translation is unclear. However, Figure 3 shows that the termination codon actually lies within the stem structure. Therefore, the possible inability of the transcript to terminate translation correctly may ultimately prevent initiation of translation upon such defective mRNA molecules via some unknown feed-back mechanism. This explanation does not account for the reduced ribosome loading observed for the mRNA from YCpPKEV1409 (Figure 4). The oligonucleotide (027), purportedly specific only for this deletion transcript, also hybridises with the PYK transcript from plasmid YCpPKEV1351. Therefore, it is feasible that the hairpin-loop formation in mRNA 1351 brings together the sequences found at the 3' region of the deletion transcript 1409. Hence the juxtapositioning of these sequences, normally well separated in the 3' UTR, may actually be the cause of poor ribosomal loading rather than the formation of strong secondary structure. The basis for this is unclear.

Using a 'tagged' PYK transcription unit and the PYK/LacZ gene fusion, it has been clearly demonstrated that the presence of a premature stop codon only a few base pairs after the initiation codon has no significant influence upon the stability of that transcript although translation is dramatically attenuated. These observations reinforce our own previous conclusions (26) as well as the work of Zaret and Sherman upon CYC1 mutations (15) that there is no general relationship between ribosome loading and mRNA stability. These data do not seem to be consistent with the observations of Lacroute and co-workers on the URA1 and URA 3 genes (22/23). There are, however, several possible explanations for this apparent discrepancy. It is possible that the decrease in mRNA stability associated with a loss of ribosomal protection may be an inherently constant value which is independent of the mRNA species or

its normal half-life. This would account for the relatively dramatic effect of premature termination upon the unstable URA mRNAs and for the relatively insignificant effect of premature termination on long-lived mRNAs such as PYK. Alternatively, the basis for the observed discrepancy may lie within the separation of S.cerevisiae mRNAs into stable and unstable populations as reported earlier (28). As the URA and PYK mRNAs belong to different populations, a forced alteration in translation may produce disparate effects. For example, members of the unstable mRNA population (as represented by the URA3 transcript) may contain specific potential targets (7,13,37) for mRNA degradation which are absent from members of the stable mRNA population. Clearly if this were the case, premature termination codons in unstable mRNAs would cause deprotection of such targets. Since such targets would not exist in stable mRNAs, premature termination codons would have no significant effect upon the stability of these mRNAs. Further analysis of the factors that determine mRNA stability will clarify this situation.

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