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Research Article

The transcriptional inhibitor thiolutin blocks mRNA degradation in yeast

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Abstract

Thiolutin is commonly used as a general inhibitor of transcription in yeast. It has been used to calculate mRNA decay rates by stopping the transcription and then determining the relative abundance of individual mRNAs at different times after inhibition. We report here that thiolutin is also an inhibitor of mRNA degradation, and thus its use can lead to miscalculations of mRNA half-lives. The inhibition of mRNA decay seems to affect the mRNA degradation pathway without impeding poly(A) shortening, given that the decay rate of total poly(A) amount is not reduced by thiolutin. Moreover, the thiolutin-dependent inhibition of mRNA degradation has variable effects on different functional groups of genes, suggesting that they use various degradation pathways for their mRNAs. Copyright © 2007 John Wiley & Sons, Ltd.

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Introduction

The process of eukaryotic gene expression involves several regulated steps at the mRNA level. mRNA is transcribed by RNA polymerase II and exported to the cytoplasm. There, the turnover rate of mRNA is regulated by different pathways. In yeast, two main routes for mRNA degradation exist. In both decay pathways, the degradation of mRNA is initiated by the shortening of the poly(A) tail at the 3′ end (reviewed in Parker and Song, 2004). Then, either the cap structure of the 5′ end is removed and the transcript degraded by a 5′ → 3′ exonuclease or, alternatively, the mRNA is degraded by a series of 3′ → 5′ exonucleolytic steps.

The study of mRNA stability in eukaryotic systems is receiving increasing attention because it is becoming evident that it is an important regulatory mechanism for gene expression (Cheadle *et al*., 2005; Mata *et al*., 2005; Pérez-Ortín *et al*., 2007; Sunnerhagen, 2007). There are several different methods of studying mRNA stability in yeast and other eukaryotes (Parker *et al*., 1991; Mata *et al*., 2005). All of them have some drawback (discussed in Parker *et al*., 1991; Sunnerhagen, 2007). The use of antibiotic drugs that inhibit RNA polymerase II is one of the preferred methods because of its simplicity and its application to all kinds of yeast strains. The most commonly used antibiotic is thiolutin (Jiménez *et al*.,1973), a metabolite produced by *Streptomyces luteoreticuli* (Celmer and Solomon, 1955) that inhibits all yeast RNA polymerases, mainly at the level of initiation (Tipper, 1973). However, it has also been reported to inhibit elongation in *E. coli* (Khachatourians and Tipper, 1974). It is commonly used at concentrations around 3 μg/ml but in some cases use of higher concentrations has been reported (Michan *et al*., 2005; Guan *et al*., 2006). The appearance of genomic techniques for the measurement of mRNA stabilities (Wang *et al*., 2002; Grigull *et al*., 2004) has incorporated the use of thiolutin at the genomic level. Despite its known effects as an inhibitor of translation (Jiménez *et al*., 1973; Tipper, 1973) and as an elicitor of stress responses (Adams and Gross, 1991; Grigull *et al*., 2004), the use of this antibiotic

is becoming more important in gene expression studies in yeast.

Here, we report that thiolutin has an inhibitory effect on mRNA degradation that becomes apparent at concentrations higher than 3 μg/ml. Because this effect involves the same process, mRNA stability, which is the goal of the studies that use thiolutin, its importance cannot be neglected. We have found that the apparent half-lives measured in individual or global mRNA stability studies are probably longer than the real ones, especially when using higher concentrations of thiolutin.

Materials and methods

Yeast strains, growth conditions, and sampling procedure for half-lives determination

The yeast strains BQS252 (*MAT***a**, *ura3-52*, derived from FY1679) and Z676 [*MAT***a**, *his3, leu2-3, ura3-52, rpb1::HIS3* transformed with plasmid *RY2522* (*rpb1-1, URA3, CEN, AMP*)] were used. Cells were grown in YPD (yeast extract 1%, peptone 2%, glucose 2%) with agitation at 28 °C and recovered by centrifugation at $OD_{600} = 0.5$. Cell samples were taken at different times after thiolutin addition and were frozen in liquid N_2. RNA samples were purified by phenol extraction as described (García-Martínez *et al*., 2004). For heat-shock experiments, pre-warmed (65 °C) YPD was added to the culture at 25 °C in order to quickly increase the temperature to 37 °C. Then samples were taken as previously described. Thiolutin was a gift from Pfizer. Several different batches were used.

Northern blot analysis

Electrophoresis was done in 1% agarose in 1× MOPS and 6.4% formaldehyde. The samples were transferred to a nylon membrane (Hybond N^+, Amersham) by capillarity with 6× SSC overnight and UV crosslinked. Then the filter was hybridized for 16 h at 42 °C in 50% formamide, 5× SSPE, 5% dextran sulphate, 0.5% SDS, 5× Denhardt's solution and salmon sperm DNA 200 μg/ml. DNA probes for *ACT1* and *RPL24* were obtained by PCR. The probes cover the entire ORF and were labelled by random primer using ^{33}P-dCTP and Ready-to-Go kit (Amersham). The filters were washed twice during 10 min at 42 °C (2× SSPE and 0.1% SDS) and once during 15 min at 65 °C (1× SSPE and 0.1% SDS) and exposed to an imaging plate (BAS-MP, Fujifilm).

Poly(A) RNA measurement

A dot–blot procedure to estimate the proportion of poly(A) mRNA in the total RNA was used. Three different dilutions of total RNA extracted from each time point were spotted, using a BioGrid robot (BioRobotics) on a nylon filter. The filter was hybridized with ^{32}P-5′-labelled poly(dT) as described previously (García-Martínez *et al*., 2004.) These data were used to calculate the proportion of poly(A) mRNA in each sample.

Macroarray hybridization

cDNA labelling with a $(dT)_{15}VN$ primer, and hybridization on in-house prepared nylon membrane macroarrays were performed as described previously (Alberola *et al*., 2004). In order to measure the half-life, the intensities were normalized to the sum of all spot intensities and re-weighted using the poly(A) dot–blot data. Half-life data for yeast genes obtained in this work are available at: **http://scsie.uv.es/chipsdna/chipsdna-e.html#datos**. Original macroarray data were submitted to the GEO database, where they have been assigned Accession Nos GSE7261 and GSE8629.

Results

Apparent mRNA decay rates depend on the thiolutin concentration used

Thiolutin is commonly used as a transcriptional inhibitor for mRNA half-life determinations in yeast (Parker *et al*., 1991). Many researchers use 3 μg/ml as the working concentration (Herrick *et al*., 1990; Grigull *et al*., 2004) but the use of higher concentrations has also been reported (Michan *et al*., 2005; Guan *et al*., 2006). In order to test the working concentration for our experiments we tried several different ones in the range 1–30 μg/ml. We observed that addition of thiolutin at concentrations 3 μg/ml or higher stopped growth for several hours in an S288c background strain (not shown). Next, we used 3, 6, 10 and 18 μg/ml thiolutin for decay rate calculation (Figure 1). As shown in Figure 1, 3 μg/ml

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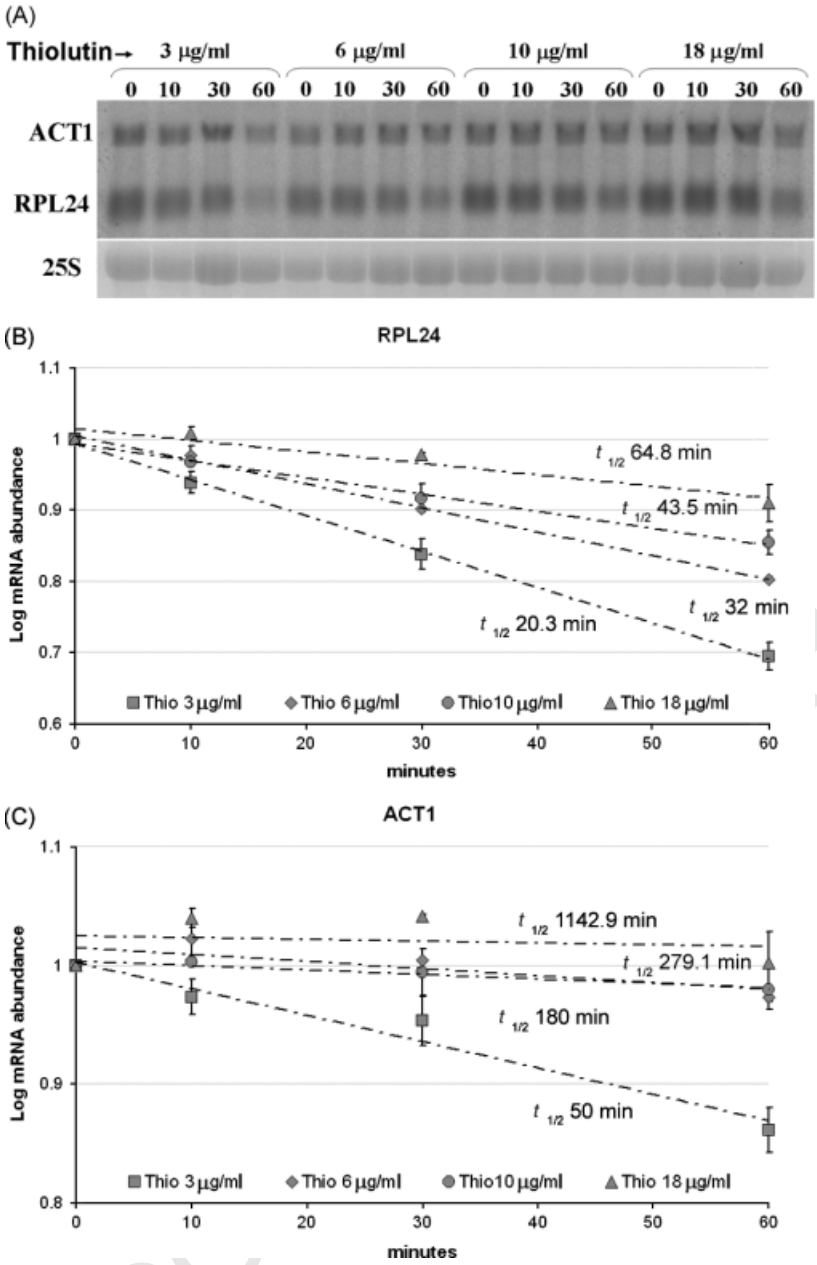

Figure 1. Effect of different thiolutin concentrations in the estimation of apparent mRNA half-lives. (A) Northern blot hybridized with probes for *ACT1* and *RPL24* genes of samples taken at different times after the addition of increasing amounts of thiolutin. (B) Calculation of the half-life of *RPL24* mRNA for the different thiolutin concentrations shown in (A). (C) Calculation of the half-life of *ACT1* mRNA for the different thiolutin concentrations shown in (A). The experiments were done in duplicate and the error bars represent the standard deviation between replicates. All samples were normalized using ethidium bromide staining of the 25S rRNA

of thiolutin generates estimations of mRNA half-lives that are in agreement with those previously known (31–77 min for *ACT1* and 11–24 min for *RPL24*; Santiago *et al*., 1986; Herrick *et al*., 1990; Wang *et al*., 2002). However, the addition of higher concentrations of thiolutin leads to the estimation of apparently longer mRNA half-lives in a concentration-dependent manner. This effect

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depends on the strain used. For instance, W303 strain requires 8 μg/ml to obtain results equivalent to those observed using 3 μg/ml in the S288c background (L. Romero-Santacreu and P.M. Alepuz, personal communication). Because it seems unreasonable that transcription inhibition decreases with the increase in thiolutin concentration, we conjectured that another cellular process was affected. The simplest explanation would be that mRNA degradation was also inhibited by thiolutin.

Thiolutin inhibits mRNA degradation without affecting global poly(A) decay

To test the hypothesis that thiolutin inhibits mRNA degradation, we used the thermo-sensitive mutant of the large RNA pol II subunit *rpb1-1*, which is also commonly used for mRNA decay determinations (Parker *et al*., 1991; Wang *et al*., 2002; Grigull *et al*., 2004). As shown in Figure 2, the transcription stop due to a heat shock provokes a continuous decrease in the mRNA amount for *ACT1* and *RPL24* mRNAs that allows for the measurement of their half-lives. *RPL24* and *ACT1* apparent half-lives calculated from this experiment were 14 and 34 min in the absence of thiolutin. However, if thiolutin was added to 20 μg/ml at the same time as the heat-shock, the mRNA amounts remained stable, indicating that, although RNA pol II transcription was stopped by the RNA polymerase inactivation, the mRNA degradation pathways were affected by thiolutin.

In yeast, mRNA degradation pathways begin with the shortening of the 3′ poly(A) tail (Parker and Song, 2004). We wanted to determine whether the total amount of poly(A) tail was affected during the treatment. To address this point, we made a dot–blot analysis of the amount of total poly(A) in the different samples taken after thiolutin addition (Figure 3). We observed that the poly(A) amount decayed at all thiolutin concentrations used. In fact, the decay was somewhat faster for higher thiolutin concentrations. Therefore, we concluded that thiolutin-dependent block of mRNA degradation was not due to an overall inhibition of poly(A) shortening.

Thiolutin affects mRNA decay for all genes, but it differentiates between genes according to their function

In order to calculate the mRNA decay rates for all yeast genes, we used a similar method to that described by Wang *et al*. (2002) and Grigull *et al*. (2004). Samples from cells were taken at 0, 10, 20, 30 and 45 min after the addition of 3 μg/ml thiolutin. cDNA was labelled with ^{33}P-dCTP by oligo-$(dT)_{15}$VN priming and hybridized

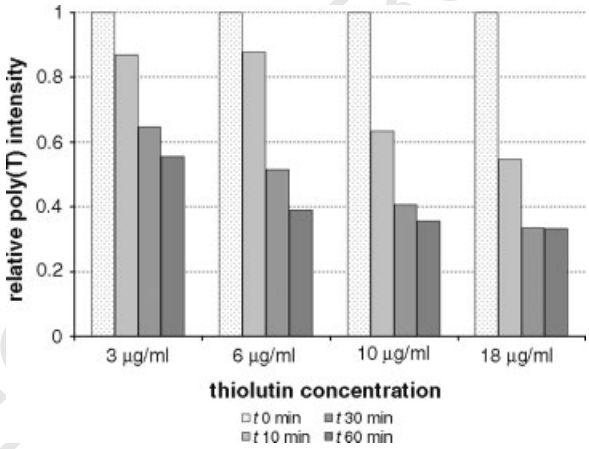

Figure 3. Total poly(A) quantisation after thiolutin treatments. Quantization of the spot intensities from a dot–blot made with total RNA from the samples used in Figure 1 hybridized with ^{32}P-labelled-poly(T). Intensity at time zero (before thiolutin addition) is arbitrarily taken as 1

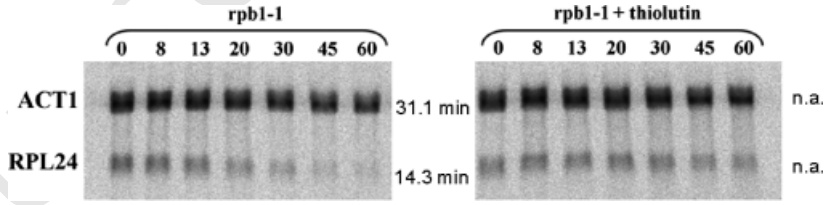

Figure 2. Inhibition of mRNA degradation by thiolutin. Northern blot of samples taken at different times after heat-shock at 37 °C of the *rpb1-1* strain with or without addition of thiolutin to 20 μg/ml. The calculated half-lives are shown on the right of each series. n.a. indicates that the amount of mRNA increases during the experiment and, therefore, half-life calculation is not applicable. All samples were normalized using ethidium bromide staining of the 25S rRNA

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to nylon macroarrays as described (Alberola *et al*., 2004). The use of such an oligonucleotide, which only primes at the beginning of the poly(A) tail, guarantees that the labelling is only dependent on the number of poly(A) molecules and not on their length. Normalization of the array signals was done using global poly(A) decay. The decay rates data obtained were reasonably similar to those obtained by Grigull *et al*. (2004) using thiolutin. The correlation coefficient between the degradation constants (k) is 0.40, which is similar to the correlation observed between the *rpb1-1* data of Grigull *et al*. (2004) and Wang *et al*. (2002), viz. 0.42.

In order to ensure that increases in thiolutin concentration affected the apparent half-life of all genes, we treated another yeast culture with 10 μg/ml thiolutin and calculated the apparent half-lives. A comparison of the results obtained using the two thiolutin concentrations is shown in Figure 4. The general trend of yeast mRNAs is to increase their apparent half-life, which demonstrates that the thiolutin-dependent effect of mRNA degradation pathways is general. We used the northern blot data from *ACT1* (which remains in the same relative position in the two distributions) in order to normalize the two sets of data. However, when analysing the bias of functionally-related groups of genes, it was evident that some groups were more affected (apparent half-lives more increased) that the average, e.g. ribosomal proteins and RiBi regulon (Jorgensen *et al*., 2004). On the other hand, some groups were less affected than the average, e.g. sulphur amino acid transport, siderophore transport, sulphate assimilation and iron transport. The functional categories present in the second class suggested that the transcription of those genes may be induced in response to the weak stress induced by low concentrations of thiolutin (see Grigull *et al*., 2004), but that at higher drug concentrations the transcriptional block was too fast to allow gene induction.

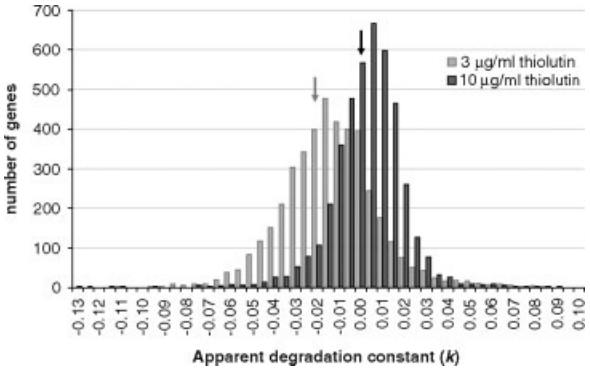

Figure 4. Genomic analysis of thiolutin effect on mRNA decay. Histogram of the apparent degradation constants [$k = (-1/t_{1/2})$] using different thiolutin concentrations. *ACT1* half-life calculated from Northern blot data (Figure 1) was used to normalize the results. This gene remained in the same relative position (marked with an arrow) to the distribution in both cases. The experiments were done in duplicate being the Pearson correlation coefficients between replicates of 0.9 or greater

Discussion

The determination of mRNA stability is of increasing relevance because mRNA turnover is emerging as an important factor in the regulation of gene expression in eukaryotic cells (Mata *et al*., 2005; Pérez-Ortín *et al*., 2007). In yeast, the use of antibiotics to inhibit RNA polymerase II transcription followed by determination of the relative abundance of the mRNAs is a useful method to evaluate half-lives because it is applicable to any strain. The antifungal thiolutin (Jiménez *et al*., 1973) is the most common drug used for this purpose. It is thought that thiolutin inhibits all nuclear RNA polymerases by directly interacting with the enzymes and does not inhibit elongating RNA polymerases (Tipper, 1973).

The thiolutin concentration used for mRNA stability experiments in yeast varies in the range 3–25 μg/ml (Adams and Gross, 1991; Grigull *et al*., 2004; Guan *et al*., 2006; Minvielle-Sebastia *et al*., 1991; Herrick *et al*., 1990; Parker *et al*., 1991; Michan *et al*., 2005). In general, it is assumed that any concentration above 3 μg/ml has, essentially, the same effect (Herrick *et al*., 1991). However, in the course of our work we have noted that the apparent half-lives of several mRNAs vary depending the particular concentration used (Figure 1). Concentrations below (not shown) and above 3 μg/ml lead to mRNA half-life estimates that are longer than expected. Problems with mRNA half-life determination have been previously noted by others (Brendolise *et al*., 2002). Whereas for lower concentrations this result can be easily explained because of incomplete inhibition of the transcriptional activity, the explanation for the effect of higher concentrations is not

straightforward. We hypothesized that this effect could be the consequence of an additional inhibition of mRNA degradation at higher concentrations (Figure 5). In fact, thiolutin is known to inhibit other biological processes, such as translation and rRNA processing, when used at 20 μg/ml (Jiménez *et al*., 1971). The overlapping effect of increasing transcriptional inhibition from low thiolutin concentrations and the increasing mRNA degradation from higher concentrations would produce a curve with a minimum for half-life determination (Figure 5). According to our data and those from other groups (Guan *et al*., 2006), the result depends on the particular strain background used.

We have provided experimental evidence for our hypothesis by using a conditional RNA polymerase II mutant strain (Figure 2). As transcription is blocked by temperature inactivation of Rpb1p in this strain, the addition of thiolutin cannot have additional effects on transcriptional shut-off if the inactivation is complete. Alternatively, if Rpb1p inactivation were not complete, the shut-off caused by thiolutin would reduce the apparent half-life. Because the relatively high concentration of thiolutin used (20 μg/ml) produces an increase of the mRNA half-lives, the simpler explanation is that concentration affects the mRNA degradation pathways. It is interesting to mention that a similar experiment was done by Guan *et al*. (2006) but using 10 μg/ml of thiolutin. They found, however, that thiolutin did not change the apparent half-life measured. Because the addition of 10 μg/ml had an effect on the mRNA half-life determination (Figure 1) in our hands, we think that the discrepancy could be due to variations in the activity of different thiolutin batches. In fact, Guan *et al*. (2006) found that the W303 background requires 2.5 times (25 μg/ml) higher concentration of thiolutin than for S288c (10 μg/ml) to completely inhibit transcription. This result is in accordance with our observation that the W303 strain required 8 instead of 3 μg/ml (2.67 times higher) to obtain the minimum apparent half-life. We also have observed that different thiolutin batches have varying biological activities. These differences are likely due to the fact that thiolutin preparations contain important amounts of contaminants — up to 20% in some batches, as determined by mass spectrometry (not shown). This would explain the use of higher thiolutin concentrations by other authors (Guan *et al*., 2006; Michan *et al*., 2005).

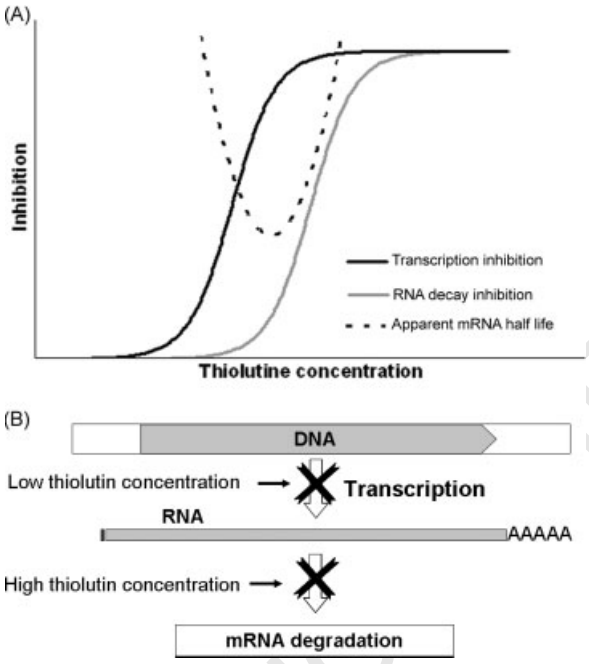

Figure 5. A model for thiolutin actions in yeast cells. (A) Thiolutin inhibition of transcription (black) and of mRNA degradation (grey) provokes a complex response for the measured mRNA half-life. The solid curve represents the approximate effect of transcription and mRNA degradation and the dotted line represents for the predicted sum of effects on apparent half-life. (B) Blocking of transcription occurs at the level of initiation (Tipper, 1973) at any concentration whereas blocking of mRNA degradation occurs only at high concentration (this work)

In yeast, mRNA degradation is usually started by poly(A) tail shortening (Parker and Song, 2004). We have found that, whereas northern analysis shows that individual mRNAs are stabilized by high thiolutin concentrations (Figures 1, 2) the global poly(A) amount for yeast mRNAs decays at those concentrations (Figure 3). Our results are in agreement with the previous results reported by Minvielle-Sebastia *et al*. (1991) and Herrich *et al*. (1990), who showed that blocking transcription either by temperature shift of an *rpb1-1* mutant or by addition of thiolutin in a wt strain did not block poly(A) shortening. Northern blots and macroarray experiments detect a slower mRNA degradation in the presence of high thiolutin concentration; hence, they are not sensitive to the change in proportion between polyadenylated and deadenylated pools of the mRNAs. Although we cannot discard the suggestion that individual mRNA species can

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have different behaviours, we suggest that inhibition of mRNA degradation occurs after deadenylation. It has been shown that thiolutin induces some stress in yeast cells (Grigull *et al*., 2004) and that stress induces mRNA stabilization (reviewed in Bond, 2006). However, the stress stabilization of mRNA occurs by inhibition of deadenylation (Hilgers *et al*., 2006), hence the effect of thiolutin cannot be an indirect consequence of the stress. Thiolutin also has an inhibitory effect on translation at high concentrations (Jiménez *et al*., 1973) and it is known that inhibition of translation blocks mRNA degradation at the level of decapping, without inhibiting deadenlylation (Beelman and Parker, 1994). Thus, it is possible that the inhibition of mRNA degradation observed by addition of high thiolutin concentrations is due to its effect on translation.

Our study shows that the effect of thiolutin on mRNA degradation is at the genomic level. We found that 3533 genes of the 4222 analysed had an increase in their apparent mRNA half-life (Figure 4). This effect was, however, not identical for all of them. For some groups related to sulphur, sulphate and iron metabolism, the deviation from the average behaviour came, probably, from the physiological stress caused by this drug (Grigull *et al*., 2004). The apparent half-lives of the mRNAs in other groups, mainly those related to translation, are more affected by thiolutin. Because it is known that different mRNA degradation pathways act in parallel (Parker and Song, 2004), this would indicate that, for those mRNAs, the pathway(s) inhibited by thiolutin have an increased importance with regard to the average of yeast genes.

The use of thiolutin, and other drugs, to stop transcription has some drawbacks. It was already known that these drugs can cause stress responses in yeast which precludes their use for mRNA stability determinations for stress-responsive genes (Grigull *et al*., 2004). Moreover, we have shown here that thiolutin has another unavoidable drawback. The sum of its inhibitory effects on RNA polymerase II and mRNA degradation led to poor estimates of mRNA half-lives (Figure 5). If the concentration used is too low (<2–3 μg/ml for the S288c background), transcription is not fully stopped. If the concentration used is too high (>3 μg/ml), the inhibitory effects of mRNA degradation are seen. In any case, a longer half-life than the actual one will be measured. This effect was probably the reason why mRNA half-lives were slightly longer when measured by thiolutin treatment than by other techniques (Herrick *et al*., 1990). We recommend to check which is the right concentration of thiolutin for each strain to avoid secondary effects.

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