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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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1 Introduction

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4 The process of eukaryotic gene expression involves
5 several regulated steps at the mRNA level. mRNA
6 is transcribed by RNA polymerase II and exported
7 to the cytoplasm. There, the turnover rate of mRNA
8 is regulated by different pathways. In yeast, two
9 main routes for mRNA degradation exist. In both
10 decay pathways, the degradation of mRNA is
11 initiated by the shortening of the poly(A) tail at the
12 3' end (reviewed in Parker and Song, 2004). Then,
13 either the cap structure of the 5' end is removed and
14 the transcript degraded by a 5' → 3' exonuclease
15 or, alternatively, the mRNA is degraded by a series
16 of 3' → 5' exonucleolytic steps.

17 The study of mRNA stability in eukaryotic sys-
18 tems is receiving increasing attention because it
19 is becoming evident that it is an important reg-
20 ulatory mechanism for gene expression (Cheadle
21 *et al.*, 2005; Mata *et al.*, 2005; Pérez-Ortín *et al.*,
22 2007; Sunnerhagen, 2007). There are several dif-
23 ferent methods of studying mRNA stability in yeast
24 and other eukaryotes (Parker *et al.*, 1991; Mata

et al., 2005). All of them have some drawback (dis-
cussed in Parker *et al.*, 1991; Sunnerhagen, 2007).
The use of antibiotic drugs that inhibit RNA poly-
merase II is one of the preferred methods because
of its simplicity and its application to all kinds
of yeast strains. The most commonly used antibi-
otic is thiolutin (Jiménez *et al.*, 1973), a metabo-
lite produced by *Streptomyces luteoreticuli* (Celmer
and Solomon, 1955) that inhibits all yeast RNA
polymerases, mainly at the level of initiation (Tip-
per, 1973). However, it has also been reported to
inhibit elongation in *E. coli* (Khachatourians and
Tipper, 1974). It is commonly used at concentra-
tions 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

1 is becoming more important in gene expression
2 studies in yeast.

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

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15 **Materials and methods**

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17 **Yeast strains, growth conditions, and sampling 18 procedure for half-lives determination**

19 The yeast strains BQS252 (*MATa*, *ura3-52*, derived
20 from FY1679) and Z676 [*MATa*, *his3*, *leu2-3*,
21 *ura3-52*, *rpb1::HIS3* transformed with plasmid
22 *RY2522* (*rpb1-1*, *URA3*, *CEN*, *AMP*)] were used.
23 Cells were grown in YPD (yeast extract 1%, pep-
24 tone 2%, glucose 2%) with agitation at 28 °C
25 and recovered by centrifugation at OD₆₀₀ = 0.5.
26 Cell samples were taken at different times after
27 thiolutin addition and were frozen in liquid N₂.
28 RNA samples were purified by phenol extraction as
29 described (García-Martínez *et al.*, 2004). For heat-
30 shock experiments, pre-warmed (65 °C) YPD was
31 added to the culture at 25 °C in order to quickly
32 increase the temperature to 37 °C. Then samples
33 were taken as previously described. Thiolutin was
34 a gift from Pfizer. Several different batches were
35 used.

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38 **Northern blot analysis**

39 Electrophoresis was done in 1% agarose in 1×
40 MOPS and 6.4% formaldehyde. The samples were
41 transferred to a nylon membrane (Hybond N⁺,
42 Amersham) by capillarity with 6× SSC overnight
43 and UV crosslinked. Then the filter was hybridized
44 for 16 h at 42 °C in 50% formamide, 5× SSPE,
45 5% dextran sulphate, 0.5% SDS, 5× Denhardt's
46 solution and salmon sperm DNA 200 µg/ml. DNA
47 probes for *ACT1* and *RPL24* were obtained by
48 PCR. The probes cover the entire ORF and were
49 labelled by random primer using ³³P-dCTP and
50 Ready-to-Go kit (Amersham). The filters were
51 washed twice during 10 min at 42 °C (2× SSPE

and 0.1% SDS) and once during 15 min at 65 °C 52
(1× SSPE and 0.1% SDS) and exposed to an 53
imaging plate (BAS-MP, Fujifilm). 54

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56 **Poly(A) RNA measurement**

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58 A dot-blot procedure to estimate the proportion
59 of poly(A) mRNA in the total RNA was used.
60 Three different dilutions of total RNA extracted
61 from each time point were spotted, using a BioGrid
62 robot (BioRobotics) on a nylon filter. The fil-
63 ter was hybridized with ³²P-5'-labelled poly(dT)
64 as described previously (García-Martínez *et al.*,
65 2004.) These data were used to calculate the pro-
66 portion of poly(A) mRNA in each sample.

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69 **Macroarray hybridization**

70 cDNA labelling with a (dT)₁₅VN primer, and
71 hybridization on in-house prepared nylon mem-
72 brane macroarrays were performed as described
73 previously (Alberola *et al.*, 2004). In order to mea-
74 sure the half-life, the intensities were normal-
75 ized to the sum of all spot intensities and re-
76 weighted using the poly(A) dot-blot data. Half-
77 life data for yeast genes obtained in this work are
78 available at: [http://scsie.uv.es/chipsdna/chipsdna-](http://scsie.uv.es/chipsdna/chipsdna-e.html#datos)
79 [e.html#datos](http://scsie.uv.es/chipsdna/chipsdna-e.html#datos). Original macroarray data were sub-
80 mitted to the GEO database, where they have been
81 assigned Accession Nos GSE7261 and GSE8629.

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84 **Results**

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86 **Apparent mRNA decay rates depend on the 87 thiolutin concentration used**

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89 Thiolutin is commonly used as a transcriptional
90 inhibitor for mRNA half-life determinations in
91 yeast (Parker *et al.*, 1991). Many researchers use
92 3 µg/ml as the working concentration (Herrick
93 *et al.*, 1990; Grigull *et al.*, 2004) but the use
94 of higher concentrations has also been reported
95 (Michan *et al.*, 2005; Guan *et al.*, 2006). In order
96 to test the working concentration for our experi-
97 ments we tried several different ones in the range
98 1–30 µg/ml. We observed that addition of thio-
99 lutin at concentrations 3 µg/ml or higher stopped
100 growth for several hours in an S288c back-
101 ground strain (not shown). Next, we used 3, 6,
102 10 and 18 µg/ml thiolutin for decay rate calcu-
lation (Figure 1). As shown in Figure 1, 3 µg/ml

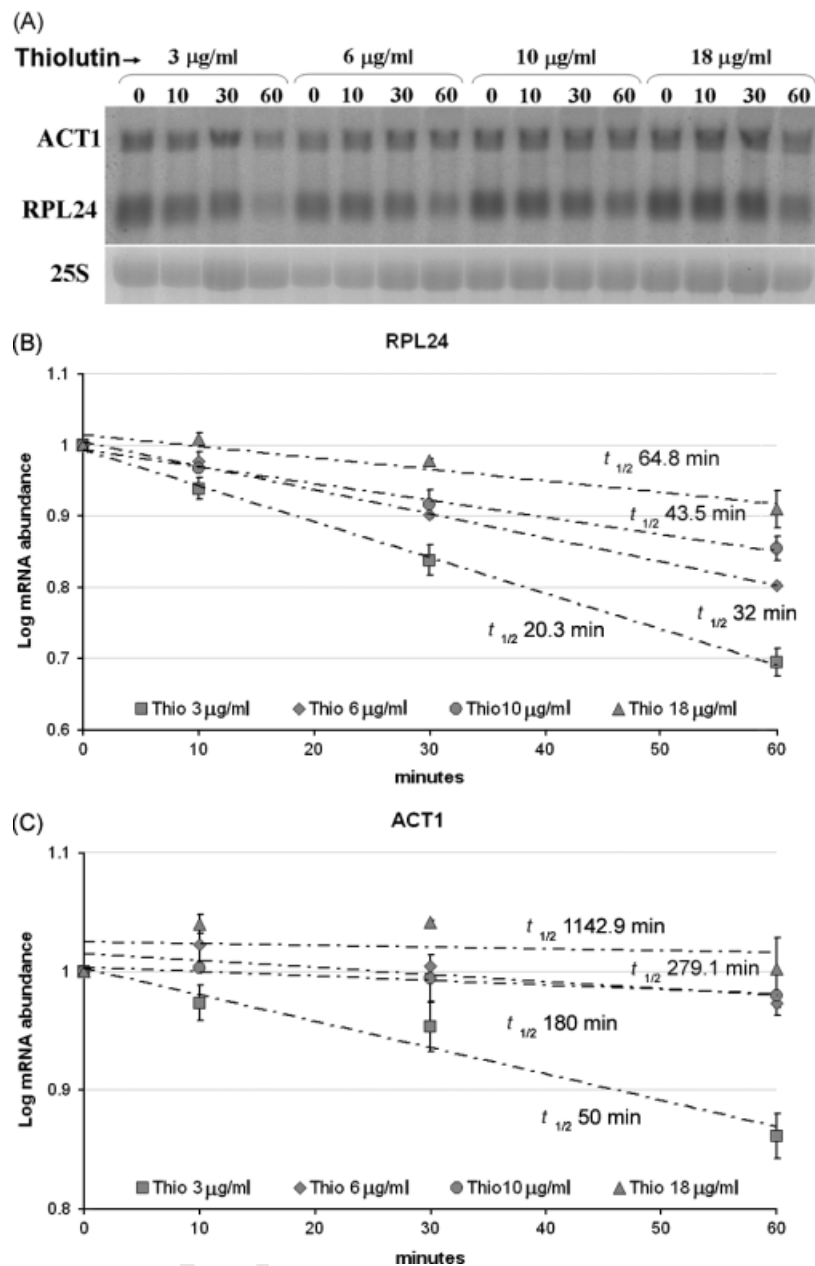


Figure 1. Effect of different thiolutin concentrations in the estimation of apparent mRNA half-lives. (A) Northern blot hybridized with probes for *ACT1* and *RPL24* genes of samples taken at different times after the addition of increasing amounts of thiolutin. (B) Calculation of the half-life of *RPL24* 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

1 of thiolutin generates estimations of mRNA half-
 2 lives that are in agreement with those previously
 3 known (31–77 min for *ACT1* and 11–24 min
 4 for *RPL24*; Santiago *et al.*, 1986; Herrick *et al.*,

1990; Wang *et al.*, 2002). However, the addition
 5 of higher concentrations of thiolutin leads to the
 6 estimation of apparently longer mRNA half-lives
 7 in a concentration-dependent manner. This effect
 8

1 depends on the strain used. For instance, W303
2 strain requires 8 $\mu\text{g/ml}$ to obtain results equivalent
3 to those observed using 3 $\mu\text{g/ml}$ in the S288c back-
4 ground (L. Romero-Santacreu and P.M. Alepuz,
5 personal communication). Because it seems unre-
6asonable that transcription inhibition decreases with
7 the increase in thiolutin concentration, we con-
8 jectured that another cellular process was affected.
9 The simplest explanation would be that mRNA
10 degradation was also inhibited by thiolutin.

11 Thiolutin inhibits mRNA degradation without 12 affecting global poly(A) decay

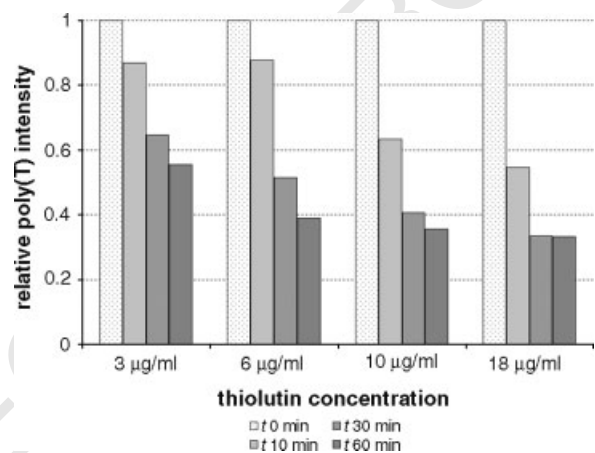
13 To test the hypothesis that thiolutin inhibits mRNA
14 degradation, we used the thermo-sensitive mutant
15 of the large RNA pol II subunit *rpb1-1*, which
16 is also commonly used for mRNA decay deter-
17 minations (Parker *et al.*, 1991; Wang *et al.*, 2002;
18 Grigull *et al.*, 2004). As shown in Figure 2, the
19 transcription stop due to a heat shock provokes
20 a continuous decrease in the mRNA amount for
21 *ACT1* and *RPL24* mRNAs that allows for the
22 measurement of their half-lives. *RPL24* and *ACT1*
23 apparent half-lives calculated from this experiment
24 were 14 and 34 min in the absence of thiolutin.
25 However, if thiolutin was added to 20 $\mu\text{g/ml}$ at
26 the same time as the heat-shock, the mRNA amounts
27 remained stable, indicating that, although RNA pol
28 II transcription was stopped by the RNA poly-
29 merase inactivation, the mRNA degradation path-
30 ways were affected by thiolutin.

31 In yeast, mRNA degradation pathways begin
32 with the shortening of the 3' poly(A) tail (Parker
33 and Song, 2004). We wanted to determine whether
34 the total amount of poly(A) tail was affected dur-
35 ing the treatment. To address this point, we made a
36 dot-blot analysis of the amount of total poly(A) in
37 the different samples taken after thiolutin addition
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(Figure 3). We observed that the poly(A) amount
40 decayed at all thiolutin concentrations used. In fact,
41 the decay was somewhat faster for higher thio-
42 lutin concentrations. Therefore, we concluded that
43 thiolutin-dependent block of mRNA degradation
44 was not due to an overall inhibition of poly(A)
45 shortening.

46 Thiolutin affects mRNA decay for all genes, but 47 it differentiates between genes according to 48 their function

49 In order to calculate the mRNA decay rates for
50 all yeast genes, we used a similar method to
51 that described by Wang *et al.* (2002) and Grigull
52 *et al.* (2004). Samples from cells were taken at
53 0, 10, 20, 30 and 45 min after the addition of
54 3 $\mu\text{g/ml}$ thiolutin. cDNA was labelled with ^{33}P -
55 dCTP by oligo-(dT)₁₅VN priming and hybridized
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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

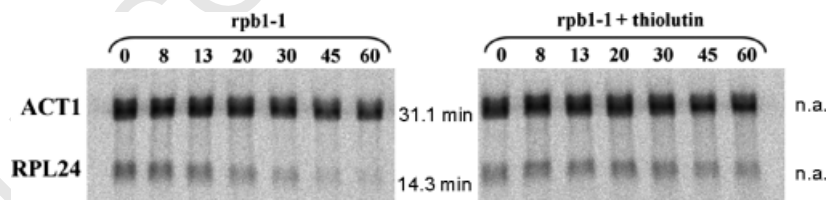


Figure 2. Inhibition of mRNA degradation by thiolutin. Northern blot of samples taken at different times after heat-shock at 37 °C of the *rpb1-1* strain with or without addition of thiolutin to 20 $\mu\text{g/ml}$. The calculated half-lives are shown on the right of each series. n.a. indicates that 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

1 to nylon macroarrays as described (Alberola *et al.*,
 2 2004). The use of such an oligonucleotide, which
 3 only primes at the beginning of the poly(A) tail,
 4 guarantees that the labelling is only dependent
 5 on the number of poly(A) molecules and not on
 6 their length. Normalization of the array signals
 7 was done using global poly(A) decay. The decay
 8 rates data obtained were reasonably similar to those
 9 obtained by Grigull *et al.* (2004) using thiolutin.
 10 The correlation coefficient between the degradation
 11 constants (k) is 0.40, which is similar to the
 12 correlation observed between the *rpb1-1* data of
 13 Grigull *et al.* (2004) and Wang *et al.* (2002), viz.
 14 0.42.

15 In order to ensure that increases in thiolutin
 16 concentration affected the apparent half-life of
 17 all genes, we treated another yeast culture with
 18 10 $\mu\text{g/ml}$ thiolutin and calculated the apparent
 19 half-lives. A comparison of the results obtained
 20 using the two thiolutin concentrations is shown in
 21 Figure 4. The general trend of yeast mRNAs is
 22 to increase their apparent half-life, which demon-
 23 strates that the thiolutin-dependent effect of mRNA
 24 degradation pathways is general. We used the
 25 northern blot data from *ACT1* (which remains
 26 in the same relative position in the two distribu-
 27 tions) in order to normalize the two sets of data.
 28 However, when analysing the bias of functionally-
 29 related groups of genes, it was evident that some
 30 groups were more affected (apparent half-lives
 31 more increased) than the average, e.g. ribosomal
 32 proteins and RiBi regulon (Jorgensen *et al.*, 2004).
 33 On the other hand, some groups were less affected
 34 than the average, e.g. sulphur amino acid transport,
 35 siderophore transport, sulphate assimilation and
 36 iron transport. The functional categories present in
 37 the second class suggested that the transcription
 38 of those genes may be induced in response to the
 39 weak stress induced by low concentrations of thi-
 40 olutin (see Grigull *et al.*, 2004), but that at higher
 41 drug concentrations the transcriptional block was
 42 too fast to allow gene induction.

45 Discussion

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 47 The determination of mRNA stability is of increas-
 48 ing relevance because mRNA turnover is emerg-
 49 ing as an important factor in the regulation of
 50 gene expression in eukaryotic cells (Mata *et al.*,
 51 2005; Pérez-Ortín *et al.*, 2007). In yeast, the use

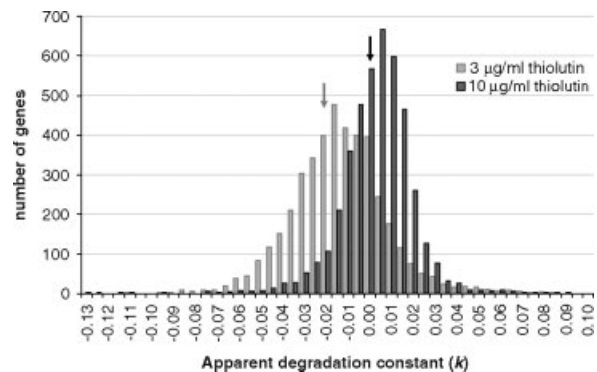


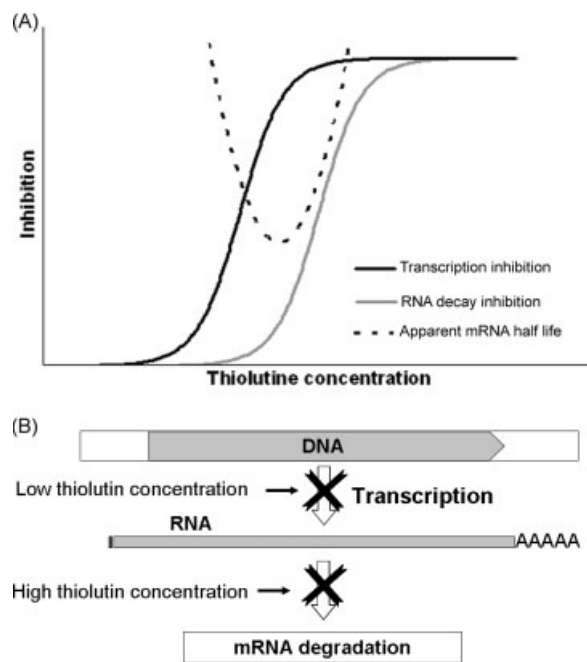
Figure 4. Genomic analysis of thiolutin effect on mRNA decay. Histogram of the apparent degradation constants [$k = (-1/t_{1/2})$] using different thiolutin concentrations. *ACT1* half-life calculated from Northern blot data (Figure 1) was used to normalize the results. This gene remained in the same 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

of antibiotics to inhibit RNA polymerase II trans-
 52 cription followed by determination of the rela-
 53 tive abundance of the mRNAs is a useful method
 54 to evaluate half-lives because it is applicable to
 55 any strain. The antifungal thiolutin (Jiménez *et al.*,
 56 1973) is the most common drug used for this pur-
 57 pose. It is thought that thiolutin inhibits all nuclear
 58 RNA polymerases by directly interacting with the
 59 enzymes and does not inhibit elongating RNA
 60 polymerases (Tipper, 1973).
 61

The thiolutin concentration used for mRNA sta-
 62 bility experiments in yeast varies in the range
 63 3–25 $\mu\text{g/ml}$ (Adams and Gross, 1991; Grigull
 64 *et al.*, 2004; Guan *et al.*, 2006; Minvielle-Sebastia
 65 *et al.*, 1991; Herrick *et al.*, 1990; Parker *et al.*,
 66 1991; Michan *et al.*, 2005). In general, it is
 67 assumed that any concentration above 3 $\mu\text{g/ml}$
 68 has, essentially, the same effect (Herrick *et al.*,
 69 1991). However, in the course of our work we
 70 have noted that the apparent half-lives of several
 71 mRNAs vary depending the particular concentra-
 72 tion used (Figure 1). Concentrations below (not
 73 shown) and above 3 $\mu\text{g/ml}$ lead to mRNA half-life
 74 estimates that are longer than expected. Problems
 75 with mRNA half-life determination have been pre-
 76 viously noted by others (Brendolise *et al.*, 2002).
 77 Whereas for lower concentrations this result can
 78 be easily explained because of incomplete inhi-
 79 bition of the transcriptional activity, the explana-
 80 tion for the effect of higher concentrations is not
 81

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

15 We have provided experimental evidence for
 16 our hypothesis by using a conditional RNA poly-
 17 merase II mutant strain (Figure 2). As transcription
 18 is blocked by temperature inactivation of Rpb1p in
 19 this strain, the addition of thiolutin cannot have
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44 **Figure 5.** A model for thiolutin actions in yeast cells.
 45 (A) Thiolutin inhibition of transcription (black) and of
 46 mRNA degradation (grey) provokes a complex response for
 47 the measured mRNA half-life. The solid curve represents the
 48 approximate effect of transcription and mRNA degradation
 49 and the dotted line represents for the predicted sum of
 50 effects on apparent half-life. (B) Blocking of transcription
 51 occurs at the level of initiation (Tipper, 1973) at any
 concentration whereas blocking of mRNA degradation
 occurs only at high concentration (this work)

52 additional effects on transcriptional shut-off if the
 53 inactivation is complete. Alternatively, if Rpb1p
 54 inactivation were not complete, the shut-off caused
 55 by thiolutin would reduce the apparent half-life.
 56 Because the relatively high concentration of thio-
 57 lutin used (20 µg/ml) produces an increase of the
 58 mRNA half-lives, the simpler explanation is that
 59 concentration affects the mRNA degradation path-
 60 ways. It is interesting to mention that a similar
 61 experiment was done by Guan *et al.* (2006) but
 62 using 10 µg/ml of thiolutin. They found, however,
 63 that thiolutin did not change the apparent half-
 64 life measured. Because the addition of 10 µg/ml
 65 had an effect on the mRNA half-life determina-
 66 tion (Figure 1) in our hands, we think that the
 67 discrepancy could be due to variations in the activ-
 68 ity of different thiolutin batches. In fact, Guan
 69 *et al.* (2006) found that the W303 background
 70 requires 2.5 times (25 µg/ml) higher concentration
 71 of thiolutin than for S288c (10 µg/ml) to com-
 72 pletely inhibit transcription. This result is in accord-
 73 ance with our observation that the W303 strain
 74 required 8 instead of 3 µg/ml (2.67 times higher)
 75 to obtain the minimum apparent half-life. We also
 76 have observed that different thiolutin batches have
 77 varying biological activities. These differences are
 78 likely due to the fact that thiolutin preparations con-
 79 tain important amounts of contaminants — up to
 80 20% in some batches, as determined by mass spec-
 81 trometry (not shown). This would explain the use
 82 of higher thiolutin concentrations by other authors
 83 (Guan *et al.*, 2006; Michan *et al.*, 2005).

84 In yeast, mRNA degradation is usually started by
 85 poly(A) tail shortening (Parker and Song, 2004).
 86 We have found that, whereas northern analy-
 87 sis shows that individual mRNAs are stabilized by
 88 high thiolutin concentrations (Figures 1, 2) the
 89 global poly(A) amount for yeast mRNAs decays
 90 at those concentrations (Figure 3). Our results are
 91 in agreement with the previous results reported by
 92 Minvielle-Sebastia *et al.* (1991) and Herrich *et al.*
 93 (1990), who showed that blocking transcription
 94 either by temperature shift of an *rpb1-1* mutant or
 95 by addition of thiolutin in a wt strain did not block
 96 poly(A) shortening. Northern blots and macroar-
 97 ray experiments detect a slower mRNA degrada-
 98 tion in the presence of high thiolutin concentration;
 99 hence, they are not sensitive to the change in pro-
 100 portion between polyadenylated and deadenylated
 101 pools of the mRNAs. Although we cannot discard
 102 the suggestion that individual mRNA species can

1 have different behaviours, we suggest that inhibi-
 2 tion of mRNA degradation occurs after deadenylation.
 3 It has been shown that thiolutin induces
 4 some stress in yeast cells (Grigull *et al.*, 2004) and
 5 that stress induces mRNA stabilization (reviewed
 6 in Bond, 2006). However, the stress stabilization
 7 of mRNA occurs by inhibition of deadenylation
 8 (Hilgers *et al.*, 2006), hence the effect of thiolutin
 9 cannot be an indirect consequence of the stress.
 10 Thiolutin also has an inhibitory effect on translation
 11 at high concentrations (Jiménez *et al.*, 1973)
 12 and it is known that inhibition of translation blocks
 13 mRNA degradation at the level of decapping, without
 14 inhibiting deadenylation (Beelman and Parker,
 15 1994). Thus, it is possible that the inhibition of
 16 mRNA degradation observed by addition of high
 17 thiolutin concentrations is due to its effect on translation.
 18

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

36 The use of thiolutin, and other drugs, to stop
 37 transcription has some drawbacks. It was already
 38 known that these drugs can cause stress responses
 39 in yeast which precludes their use for mRNA stability
 40 determinations for stress-responsive genes
 41 (Grigull *et al.*, 2004). Moreover, we have shown
 42 here that thiolutin has another unavoidable drawback.
 43 The sum of its inhibitory effects on RNA
 44 polymerase II and mRNA degradation led to poor
 45 estimates of mRNA half-lives (Figure 5). If the
 46 concentration used is too low (<2–3 µg/ml for
 47 the S288c background), transcription is not fully
 48 stopped. If the concentration used is too high
 49 (>3 µg/ml), the inhibitory effects of mRNA degradation
 50 are seen. In any case, a longer half-life
 51 than the actual one will be measured. This effect

was probably the reason why mRNA half-lives 52
 were slightly longer when measured by thiolutin 53
 treatment than by other techniques (Herrick *et al.*, 54
 1990). We recommend to check which is the right 55
 concentration of thiolutin for each strain to avoid 56
 secondary effects. 57

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