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 (Celmér 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 (MATα, ura3-52, derived from FY1679) and Z676 [MATα, his3, leu2-3, ura3-52, rpbl::HIS3 transformed with plasmid RY2522 (rpbl-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 OD600 = 0.5. Cell samples were taken at different times after thiolutin addition and were frozen in liquid N2.

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 32P-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 32P-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)15VN primer, and hybridization on in-house prepared nylon membrane macroarrays were performed as described previously (Alberola et al., 1990). 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.

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 102
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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...
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 33P-dCTP by oligo-(dT)15VN priming and hybridized.

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
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 demon-
strates 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 distribu-
tions) 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 thi-
olutin (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/τ1/2)] 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 emerg-
ing 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 tran-
scription followed by determination of the rela-
tive 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 pur-
pose. 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 sta-

bility 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 concentra-
tion 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 pre-
viously noted by others (Brendolise et al., 2002).
Whereas for lower concentrations this result can
be easily explained because of incomplete inhibi-
tion of the transcriptional activity, the explana-
tion for the effect of higher concentrations is not

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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).

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 differ...
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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 deadenylation (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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