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Research Article

There is a steady-state transcriptome in exponentially growing yeast cells

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Abstract

The growth of yeast cells in batches in glucose-based media is a standard condition in most yeast laboratories. Most gene expression experiments are done by taking this condition as a reference. Presumably, cells are in a stable physiological condition that can be easily reproduced in other laboratories. With this assumption, however, it is necessary to consider that the average amount of the mRNAs per cell for most genes does not change during exponential growth. That is to say, there is a steady-state condition for the transcriptome. However, this has not been rigorously demonstrated to date. In this work we take several cell samples during the exponential phase growth to perform a kinetic study using the genomic run-on (GRO) technique, which allows simultaneous measurement of the amount of mRNA and transcription rate variation at the genomic level. We show here that the steady-state condition is fulfilled for almost all the genes during most exponential growth in yeast extract–peptone–dextrose medium (YPD) and, therefore, that simultaneous measures of the transcription rates and the amounts of mRNA can be used for indirect mRNA stability calculations. With this kinetic approach, we were also able to determine the relative influence of the transcription rate and the mRNA stability changes for the mRNA variation for those genes that deviate from the steady state. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: transcription rate; steady state; transcriptome; *Saccharomyces cerevisiae*; mRNA stability

Introduction

Evaluation of the amount of mRNA (RA) for a given gene by means of northern blot, qPCR or other techniques is a routine in many molecular biology experimental protocols. Comparisons between different physiological situations or between different cell types or mutants are habitually studied. As a significant number of mRNAs can vary their concentrations, even upon subtle changes in internal or external conditions, it is crucial to sample cells in identical situations in order to guarantee repetitiveness and comparativeness between experiments. This is especially true for batch cultures of free-living microorganisms where the growth rate and environment are continuously changing. This problem can be solved by using continuous culture conditions, but such conditions are more technically demanding. Therefore, most published experiments use batch conditions. For most batch culture analyses, researchers select exponential growth in a rich medium as the default state for comparison, as it is usually thought to be a physiologically constant condition in which most, or all, mRNAs remain unchanged in a steady-state condition. This can facilitate comparisons between experiments done in different laboratories. However, because the emergence of genomic techniques has raised the possibility of simultaneously quantifying most cellular mRNAs at the same time, the

need for a rigorous demonstration of the steady-state condition now extends to thousands of genes.

One of the most studied organisms in functional genomics is the yeast *Saccharomyces cerevisiae*. There are hundreds of experiments on this yeast using cells growing on YPD (yeast extract–peptone–dextrose) complete medium in the 'exponential growth condition'. It is known, however, that many genes vary during exponential growth when cells approach the diauxic shift (DeRisi *et al*., 1997) or change in a growth rate-dependent manner (Regenberg *et al*., 2006). As stated above, the use of chemostats has been described as a more reliable way to avoid such variations because they keep culture conditions stable (Daran-Lapujade *et al*., 2009; Hayes *et al*., 2002). However, most yeast researchers have used, and still use, the exponential growth condition in batch cultures for their experiments.

Although the majority of studies focus only on the determination of RA, it is becoming increasingly clear that gene expression should be studied as a kinetic process in which the amount of mRNA is controlled not only by transcription but also by the influence of mRNA stability (RS, also called mRNA half-life; Pérez-Ortín *et al*., 2007). In exponential growth a dynamic steady state can be assumed for RA, i.e. the transcription and degradation rates (TR and DR, respectively) are equal. TR follows a zero-order kinetics (does not depend on RA), whereas DR follows first-order kinetics:

$$DR = k_d\ RA$$

Therefore, in steady state:

$$TR = DR = k_d\ RA$$

where k_d is the degradation constant, which is inversely related to RS ($k_d = \ln 2/RS$). Thus, in this situation, *TR* can be calculated from experimentally determined RA and stability data (Holstege *et al*., 1998); alternatively, *DR* can be calculated from RA and TR data (García-Martínez *et al*., 2004). Both approaches are becoming increasingly popular for TR or DR calculations, although it has not yet been established whether the true steady-state condition for gene expression actually applies (Pérez-Ortín, 2007). A steady state defined for RA does not necessarily imply steady states for TR and DR. In fact, it would be theoretically possible for TR and DR to change simultaneously in parallel, keeping RA constant.

In this paper we address the topic of assessing the steady-state condition for mRNA amounts in *S. cerevisiae* cells growing in YPD at the exponential phase. First we performed an experiment to determine whether the steady state applies for yeast in which we simultaneously measure TR and RA for all the genes. We also studied the kinetic behaviour of any gene that seems to deviate from it. In this way we have demonstrated that the steady-state condition for RA is true for most of the genes studied, but that there are small but significant changes in some groups of genes, which change either their TR or DR as the culture approaches the diauxic shift. Because TR also remains almost invariable, we can say that there is also a steady state for TR and DR. Finally, we reanalysed previously published data (Radonjic *et al*., 2005) to expand the time window of our study and to determine how long the steady-state condition is maintained. We found that the RA steady state is fulfilled over a long period within the exponential phase.

Materials and methods

Yeast strain and growth conditions

Yeast strain BQS252 (*MAT***a**, *ura3-52*; derived by sporulation from FY1679) was used. Cells were grown in YPD (yeast extract 1%, peptone 2%, and glucose 2%) with agitation (190 rpm) at 28 °C for the repeated sampling of exponentially growing yeast cells. Cell cultures were grown overnight until they reached the desired OD_{600}. Five time points were taken at 0, 10, 20, 30 and 40 min after the initial sample, which corresponded to $OD_{600} = 0.36$ (8×10^6 cells/ml), 0.38, 0.41, 0.44 and 0.47, respectively. Three biological replicates of the whole experiment were done.

Genomic run-on (GRO)

The GRO was done essentially as described in García-Martínez *et al*. (2004). Briefly, two aliquots of ca. 4×10^8 cells were harvested at each time point. One aliquot was used directly for the GRO protocol, in order to obtain TR data, while the other one was frozen for subsequent total RNA extraction. The cells of the GRO sample were

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permeabilized with 0.5% Sarkosyl and incubated for 20 min on ice. Then the cells were allowed to extend the nascent RNA in the presence of [α-^{33}P]-UTP. Finally, the radioactively labelled RNA was extracted and hybridized onto a nylon macroarray (Alberola *et al*., 2004). After TR determination, the total RNA sample was labelled by cDNA synthesis with random hexamers and hybridized in the same arrays in order to determine the RA.

Image analysis and data normalization

Images were quantified using Array Vision software, v. 7.0 (Imaging Research Inc.). The signal intensity for each spot was the background subtracted ARM (artifact removed median) density. Only values that were 1.35 times over the corresponding background were taken as valid measurements. The reproducibility of the replicates was checked using Array Stat software (Imaging Research Inc.). We considered the data to be independent and used a minimum number of two valid replicates in order to calculate the mean and standard deviation (SD) values for every gene. Normalization between conditions was done using the global median method.

Data analysis

We used the differential gene expression analysis program from the GEPAS suite (Montaner *et al*., 2006) to analyse whether there was any significantly different slope from 0 for the variation of RA during exponential growth (Radonjic *et al*., 2005) with regard to a continuous independent variable (time). Similar results were obtained when the culture OD_{600} was used as a continuous independent variable instead of the time (data not shown).

The detailed RA and TR data for exponential growth between OD_{600} 0.36 and 0.47 was calculated as previously described (García-Martínez *et al*., 2004; for the whole dataset, see Supporting information, Table S1). Briefly, the intensity of the mRNA or GRO hybridization was normalized using a genomic DNA hybridization signal and the U-richness (GRO) or C-richness (mRNA) correction. We computed the slope of RA and TR variations on a $\log_2$ scale with regard to time by assuming that the median TR and RA of the gene population remained constant during the experiment (see Supporting information, Table S2). Gene functional analyses were done by analysing either individual genes slopes (using FuncAssociate; Berriz *et al*., 2003) or gene sets (using Fatiscan from the BABELOMICS suite; Al-Shahrour *et al*., 2006). To analyse the changes in RS, we computed a theoretical RA variation dataset for all the genes, using the RA amount data obtained for the first time point and the experimentally obtained TR data for the whole experiment. Using these data, it is possible to calculate a theoretical RA for each point by assuming that the RS calculated the first time remains constant during the whole experiment (Pérez-Ortín *et al*., 2007). We compared the differences between this theoretically computed RA and the actual one, and assumed that the differences found would be due to stability changes during the experiment (see Figure 1A). To obtain a more robust RA variation, we performed a linear regression analysis of the actual and theoretical RA data and computed the predicted RA variation for each gene during our experiment, using all the time points. Finally, we computed the percentage of actual RA variation and the theoretical one (TR-dependent RA variation) for each gene. The difference between the actual and the theoretical RA directly gives the stability-dependent RA variation.

Accession numbers

The genomic data are stored in the Valencia Yeast (VYdBase; **http://vydbase.uv.es/**) and GEO databases. The GEO Accession No. for the set of different hybridizations is GSE11521.

Results

Verification of the steady state for mRNA on yeast cells using genomic run-on (GRO) analyses

To determine whether the yeast transcriptome was in a steady-state condition during the exponential phase on the YPD medium, we measured the variation of mRNA at five time points during exponential growth. As most of the published experiments start with a very low OD_{600} after inoculation and reach a middle exponential phase of 0.3–0.6 at OD_{600}, we decided to monitor the mRNA variation under these conditions. We also used our GRO

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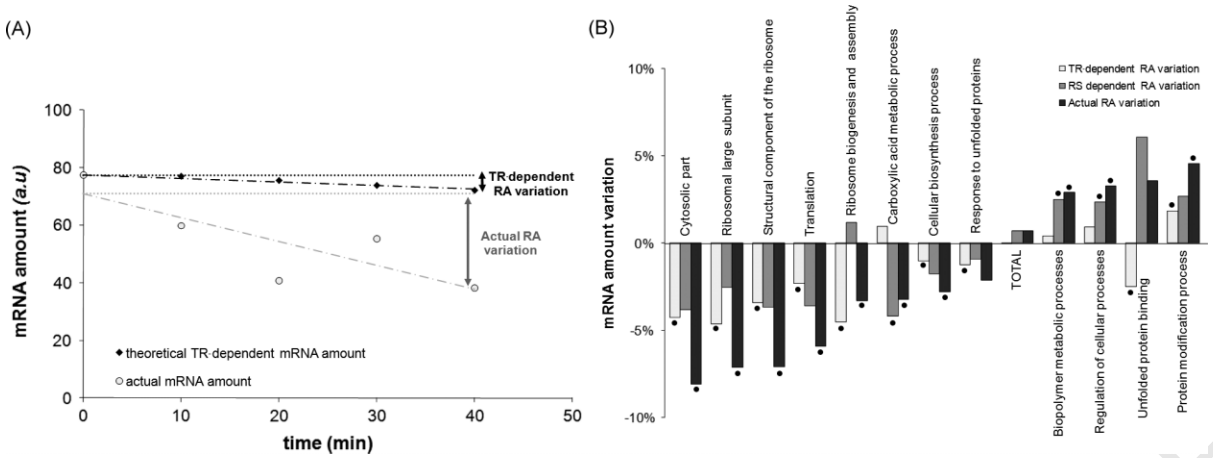

Figure 1. Relative contributions of the transcription rate (TR) and mRNA stability (RS) to the mRNA amount (RA) change. (A) Scheme of the protocol used to compute the mRNA variation, which is dependent in either TR or RS. The theoretical RA values in the experiment were computed for each gene by taking in account only the TR variations and by assuming a constant RS (in black). When comparing this theoretical TR-dependent RA change with the actual one (experimentally measured, in grey), it is possible to compute the percentage of RA variation for each gene, which is due to changes in either TR or RS. The data shown correspond to a sample gene (*YAL004W*) that has been chosen to show an important contribution of RS to RA variation. It is shown in natural scale to make the differences more evident. However its RA slope does not reach the significance cut-off for being statistically different from 0 (in either natural or $\log_2$ scale; FDR p value >0.8). (B) Relative contribution of TR (light grey) or RS (dark grey) to the total RA variation (black) for selected gene groups shown in Table 1. Bars represent the median value for the variation for all the genes in each group. The groups with significant differences from 0 (as shown in Tables 1 and S3) are marked with black dots

protocol, which allows the simultaneous determination of TR and RA data. In this way, if the steady-state condition was verified, we could also calculate the mRNA stabilities from the RA and TR data (García-Martínez *et al*., 2004).

We performed an experiment in triplicate for the five time points at OD_{600} 0.36–0.47 (spanning 40 min, at 10 min intervals), using cultures inoculated 16 h beforehand. Under these conditions, the diauxic shift started several hours later at $OD_{600} \approx 2$ (V. Pelechano, unpublished observation). All 15 samples were subjected to the GRO protocol for calculating RA and TR. The repetitiveness of the samples was very good (see Supporting information, Figure S1). Pearson's correlation was, on average, 0.934 for the TR data and 0.936 for the RA data between the biological replicates. The correlation for the data points for a single growth curve was even better: 0.965 for TR and 0.964 for RA. This suggests that there is more biological variation between growth curves in different experiments than within the time points of a single exponential phase curve. However, as we fused the different replicates, only the time-dependent variations (common for all three experiments) were taken into account.

We reasoned that the slight changes occurring during growth in the culture composition would affect the cells' physiology, leading to a continuous change in RA or TR, which would be visible as positive or negative slopes in their respective plots. In this way, artifactual or random changes would not be accounted for. However, when we analysed these data using the GEPAS suite (a differential expression analysis tool; Montaner *et al*., 2006), no individual slope of any gene differed significantly from zero (FDR >0.6). This result confirms that the steady-state condition applies for all (at least most of) the yeast genes. Therefore, no important error is associated when taking samples for mRNA quantification from cells growing in the early exponential phase. Thus, either TR or RS can be calculated in these growth conditions from the RA experimental data and either from the respective RS data, measured by conventional methods (Grigull *et al*., 2004), or from the TR data, measured by GRO by means of mathematical calculations (Pérez-Ortín, 2007).

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However, the behaviour of genes can be too subtle to be discovered from studying single genes tendencies only. Therefore, in order to analyse this result in more depth, we used a tool from the BABELOMICS suite (Fatiscan algorithm; Al-Shahrour *et al*., 2006), which scans the whole set of gene data to discover any common trends in the GO categories. Specifically, this algorithm does a segmentation test that checks whether there are any significant asymmetries in the distribution of each GO. In this way, minor changes that are often too minor to be significant for individual genes can be statistically stressed by the common GO group behaviour. We scanned all the GO categories at all the possible levels. With this analysis, we found that some groups significantly increased or decreased during the experiment (Table 1A). Moreover, as we simultaneously measured RA and TR, we were able to distinguish between the different regulatory strategies.

Some group changes were detected in both TR and RA, but others were observed in only one of them. For instance, the translation-related categories were seen to lower RA and TR, which is in agreement with the decrease noted in the cell duplication rate that the culture would undergo several hours afterwards. Moreover, the differences between RA and TR behaviours illustrate the possibility of detecting the regulatory strategies followed for the mRNA changes: are the changes in RA due to changes in transcription or to changes in mRNA stability? If we assume that the steady-state condition applied for this experiment, then we could calculate the respective influence of TR and RS on the RA change (for a detailed explanation, see Figure 1A and Materials and methods). With this protocol, we were able to calculate their relative influence on the changes noted in each GO category, as seen in Table 1 and Figure 1B. We analysed the slope of the RA variation that could be explained by RS changes using Fatiscan. Then we selected the groups with either a significant positive contribution (meaning increased stability) or a significant negative one (meaning decreased stability) (Table 1B). The fact that the number of statistical significant groups found for the RS changes is smaller than the ones found in the direct measures could be explained mainly by two factors; because it only takes into account the part of the variation for the RA, due to stability

Table 1. Functional groups with a significant deviation from the steady state during exponential growth

(A) ***Direct measures***			
Groups in which Transcription Rate (TR) increases		**Groups in which mRNA amount (RA) increases**	
Adjusted *p* value	**Functional group**	**Adjusted *p* value**	**Functional group**
$6.35 \cdot 10^{-3}$	Protein modification	$3.36 \cdot 10^{-4}$	Biopolymer metabolic process
$7.26 \cdot 10^{-3}$	Post-translational protein modification	$5.71 \cdot 10^{-4}$	Regulation of cellular process
$1.66 \cdot 10^{-2}$	Cation transporter activity	$1.27 \cdot 10^{-2}$	Regulation of transcription
$2.53 \cdot 10^{-2}$	Meiosis I		
$3.39 \cdot 10^{-2}$	Oxidoreductase activity, oxidizing metal ions		
Groups in which transcription rate (TR) decreases		**Groups in which mRNA amount (RA) decreases**	
Adjusted *p* value	**Functional group**	**Adjusted *p* value**	**Functional group**
$2.82 \cdot 10^{-10}$	Cytosolic part	$2.52 \cdot 10^{-8}$	Structural constituent of ribosome
$1.33 \cdot 10^{-6}$	Ribosome	$2.78 \cdot 10^{-7}$	Ribosome
$8.91 \cdot 10^{-5}$	Ribosome biogenesis and assembly	$4.00 \cdot 10^{-6}$	Cytosolic part
$2.32 \cdot 10^{-4}$	Structural constituent of ribosome	$7.23 \cdot 10^{-5}$	Large ribosomal subunit
$6.35 \cdot 10^{-3}$	Translation	$1.09 \cdot 10^{-4}$	Translation
$6.35 \cdot 10^{-3}$	Cellular biosynthetic process	$5.29 \cdot 10^{-3}$	Ribosome biogenesis and assembly
$1.18 \cdot 10^{-2}$	Cytoplasmic exosome (RNase complex)	$4.16 \cdot 10^{-2}$	Monocarboxylic acid metabolic process
$2.15 \cdot 10^{-2}$	Histidine biosynthetic process		
$2.15 \cdot 10^{-2}$	Riboflavin metabolic process		
$3.39 \cdot 10^{-2}$	Response to unfolded protein		
$3.39 \cdot 10^{-2}$	Response to protein stimulus		

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Table 1. Continued

(B) *Indirect measures*			
Groups in which mRNA stability (RS) increases		**Groups in which mRNA stability (RS) decreases**	
Adjusted *p* value	**Functional group**	**Adjusted *p* value**	**Functional group**
$1.07 \cdot 10^{-5}$	Biopolymer metabolic process	$2.06 \cdot 10^{-3}$	Organic acid metabolic process
$2.06 \cdot 10^{-3}$	Regulation of biological process	$2.06 \cdot 10^{-3}$	Carboxylic acid metabolic process
$2.86 \cdot 10^{-3}$	Regulation of cellular process		

(A) Functional groups with significant slopes for experimentally determined (direct measures) TR or RA along the five time points analysed during early exponential growth. (B) Functional groups with a significantly high contribution of RS to the RA increase or decrease (mRNA stabilization or destabilization, respectively). The RS-dependent RA slope was mathematically computed (indirect measures), as explained in Figure 1A, and used for the functional analysis. Only some representative GO are shown (for the complete list, see Supporting information, Table S3). All the analyses were done using the Fatiscan algorithm from BABELOMICS (Al Shahrour *et al*., 2006); the FDR-adjusted *p* values are shown.

changes, and because it is an indirect measure. The fact of being an indirect measure depending on the TR and RA data makes it more noisy (in fact the SD for the RS-dependent RA slopes is larger (0.0074) than that from TR or RA direct measures (0.0058 and 0.0057, respectively). It can be seen that both TR and RS cooperate to either lower or increase RA for most categories; that is to say, homodirectional changes occurred, although in variable proportions. In some groups, however, the changes observed operated in opposite senses. RiBi genes had a decreased RA, due to a large effect on TR that compensated slight mRNA stabilization, whereas unfolded protein response mRNAs increased because of strong mRNA stabilization, despite a certain decrease in TR. It is important to note that all these changes were very subtle, and could be taken in account only for those GOs in which significant changes for the kinetic parameters had been previously detected. These results illustrate the variable methods used by the yeast cell to change the mRNA level (Pérez-Ortín *et al*., 2007).

Determination of the time window when assuming the steady state for mRNAs in yeast cells growing in the exponential phase

As our experiment covered only a relatively small time window of exponential phase growth in yeast ($OD_{600} = 0.3–0.5$), we decided to assess whether this steady state for the transcriptome is maintained in later phases of exponential growth. To determine this, we reanalysed the data from the comprehensive study of Radonjic *et al*. (2005). In their experiment, the authors analysed the transcriptome of wild-type yeast (S288c background) growing in a batch culture in YPD since the inoculation on fresh medium to the late stationary phase. They use $OD_{600} = 0.5$ as the starting condition, which precludes a direct comparison with our conditions. The growth curve included nine experimental points in the exponential phase in the range 3.9–9 h after inoculation (see Figure 1A from Radonjic *et al*., 2005). This study measured only the RA changes, but this is enough to check the steady-state condition. First, we analysed this data using the GEPAS suite (a differential expression analysis tool; Montaner *et al*., 2006). Probably because the analysed time window in this experiment is longer, covering more physiological variations, the number of genes with a slope different from zero (FDR <0.01) was higher. We obtained 304 genes showing a positive slope, and 271 showing a negative slope when their $\log_2$ RA data were plotted against the time course (Figure 2A). They were enriched in some GO categories. Specifically, the mRNA levels of those categories related with respiration and protein catabolism increased, unlike the mRNA levels of those categories related to ribosome biogenesis, which decreased (Figure 2B). Both results fitted the expected changes in cell metabolism, which would take place when entering the diauxic shift some hours later (DeRisi *et al*., 1997; Radonjic *et al*., 2005), and they are in agreement with the slight changes detected in our previous experiment. A close inspection of the results, however, showed that most of the changes noted in relation to the initial exponential phase at 3.9 h in those genes took place at the last two time points analysed,

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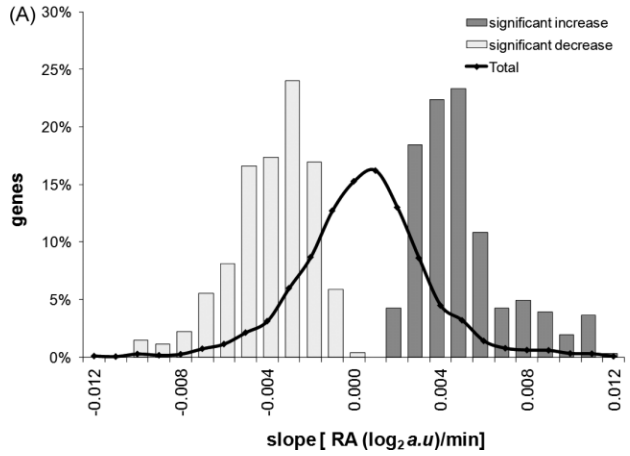

(B) **Gene groups with a significant positive RA slope for 3.9-9 h**

p-value	*p*-adj	GO Attribute
$3.65 \cdot 10^{-15}$	<0.001	vacuolar protein catabolic process
$5.73 \cdot 10^{-11}$	<0.001	response to abiotic stimulus
$1.08 \cdot 10^{-10}$	<0.001	cytoplasmic part
$8.30 \cdot 10^{-10}$	<0.001	mitochondrial part
$1.17 \cdot 10^{-07}$	<0.001	oxidation reduction
$3.75 \cdot 10^{-07}$	<0.001	actin cortical patch
$1.03 \cdot 10^{-06}$	<0.001	oxidoreductase activity

Gene groups with a significant negative RA slope for 3.9-9 h

p-value	*p*-adj	GO Attribute
$3.15 \cdot 10^{-41}$	<0.001	nucleolus
$4.12 \cdot 10^{-32}$	<0.001	ribosome biogenesis
$9.20 \cdot 10^{-31}$	<0.001	rRNA processing
$3.91 \cdot 10^{-13}$	<0.001	ribosomal large subunit biogenesis
$4.53 \cdot 10^{-09}$	<0.001	nucleus

Figure 2. mRNA amount variation during exponential growth. (A) Histogram of slopes for the mRNA amount (RA) variation (in $\log_2$ scale for arbitrary units) as a function of time (min) for the 3.9–9 h interval, according to Radonjic *et al.* (2005). The distribution of all the gene slopes is shown as a black line. Bars represent the genes with slopes that significantly differ (positively in dark grey and negatively in light grey) from 0 (FDR <0.01). The *y* axis represents the percentage of genes, with regard to the total number analysed, belonging to each class. (B) The gene groups that are significantly enriched in genes that increase or decrease RA. Significance was calculated using FuncAssociate (Berriz *et al.*, 2003) Only the most representative GO categories are shown

Table 2. Change in the mRNA amount slope during exponential growth

Functional group	3.9–4.75 h	4–5.25 h	4.25–5.75 h	4.75–6.5 h	5.25–7.25 h	5.75–9 h
Cytoplasm	↑ **$2.29 \cdot 10^{-8}$**	↑ **$9.53 \cdot 10^{-8}$**	↑ **$2.76 \cdot 10^{-5}$**	↑ **$2.05 \cdot 10^{-7}$**	↑ **$3.35 \cdot 10^{-16}$**	↑ **$1.14 \cdot 10^{-6}$**
Mitochondrial part	↑ **$3.42 \cdot 10^{-4}$**	—	—	↑ **$3.78 \cdot 10^{-10}$**	↑ **$2.88 \cdot 10^{-16}$**	↑ **$5.54 \cdot 10^{-12}$**
Oxidoreductase activity	↑ **$2.31 \cdot 10^{-6}$**	—	—	—	↑ **$4.72 \cdot 10^{-4}$**	↑ **$2.03 \cdot 10^{-7}$**
Alcohol metabolic process	↑ **$6.73 \cdot 10^{-3}$**	—	—	—	—	↑ **$5.55 \cdot 10^{-3}$**
Response to unfolded protein	↑ **$4.67 \cdot 10^{-3}$**	—	—	—	↑ **$3.07 \cdot 10^{-9}$**	↑ **$4.12 \cdot 10^{-3}$**
Response to stress	—	—	—	—	↑ **$1.56 \cdot 10^{-3}$**	↑ **$5.18 \cdot 10^{-3}$**
Integral to membrane	↑ **$1.34 \cdot 10^{-5}$**	↑ **$1.00 \cdot 10^{-7}$**	↑ **$6.59 \cdot 10^{-4}$**	↓ $4.44 \cdot 10^{-4}$	—	—
Ribosome biogenesis and assembly	↓ $7.85 \cdot 10^{-12}$	↓ $4.75 \cdot 10^{-6}$	—	↓ $1.21 \cdot 10^{-3}$	↓ $5.56 \cdot 10^{-24}$	↓ $1.05 \cdot 10^{-28}$
Structural constituent of ribosome	↓ $6.20 \cdot 10^{-3}$	—	—	—	↓ $1.17 \cdot 10^{-17}$	↓ $8.83 \cdot 10^{-3}$
Translation	↓ $6.09 \cdot 10^{-4}$	—	—	—	↓ $1.59 \cdot 10^{-12}$	↓ $1.97 \cdot 10^{-6}$
Nucleus	↓ $8.04 \cdot 10^{-14}$	↓ $2.04 \cdot 10^{-6}$	—	—	↓ $3.13 \cdot 10^{-8}$	↓ $1.83 \cdot 10^{-6}$

Gene-set enrichment analysis using Fatiscan (Al Shahrour *et al.*, 2006) according to the RA slopes at different intervals during exponential growth (Radonjic *et al.*, 2005). The categories which increase RA (↑, enriched in the positive slopes) are shown in bold type, and the categories which decrease RA (↓, enriched in the negative slopes) are depicted in normal type. All the slopes were computed using four time points. Only some representative functional groups are shown.

i.e. at 7.25 and 9 h after inoculation. When we considered only the first seven time points, from 3.9 to 6.5 h, no gene's slope significantly differed from zero when we used the same criteria. This result confirms that the time window in which a steady state for the transcriptome can be widely assumed is extended to up a couple of hours before the diauxic shift.

In order to confirm the small deviations from the steady state that we were able to calculate in our previous experiment, we used the same gene set enrichment analysis (Fatiscan algorithm; Al-Shahrour *et al.*, 2006) to detect the groups of the related genes showing slight but significant changes in their RA in the Radonjic *et al.* (2005) data. Table 2 shows how the RA of some GO

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categories tended to increase (↑, bold numbers) or decrease (↓) along the growth curve. As expected, the respiration and stress response categories were significantly represented in the RA increase, while the translation categories were significantly represented in the RA decrease.

Therefore, although we conducted a kinetically detailed study of the transcriptome only during mid-exponential growth, these results confirm that our conclusions can be extrapolated to most of the exponential growth phase.

Discussion

It is commonly assumed that mRNAs are approximately in a steady state during exponential growth in a free-living microorganism. For instance, we have used this assumption in order to calculate the mRNA stabilities in the yeast *S. cerevisiae* by means of GRO experiments (García-Martínez *et al*., 2004). However, an experimental demonstration is currently lacking.

Here we report a detailed study of the gene expression in yeast during exponential growth. In this experiment we simultaneously checked both RAs and TRs. The general conclusion is that a steady state for the transcriptome and transcription rates can be assumed, and that small deviations from it can be detected, but only when looking at all the genes from a given GO at the same time. This last analysis procedure is much more sensitive because it detects common tendencies for a group of related genes. However, when we analysed them individually, each yeast gene was within the steady-state condition for its mRNA. In addition, our kinetically orientated approach enables us to determine the respective contributions of TR and RS to the putative changes in RA. For most cases, both changes work in the same direction. However, certain exceptions indicate how the cell uses mRNA stability as an additional controller of the gene expression. Moreover, the demonstration of steady state for TR allows the conclusion that the cells keep the RA values constant by also maintaining constant turnover, not by coordinately changing TR and DR — a more complicated option but theoretically possible.

To extend our results and to determine how long the steady state for the transcriptome can be assumed during exponential growth, we used data from a comprehensive microarray study by Radonjic *et al*. (2005), which is representative for many others. Using these data, we verified that the steady-state condition is met by a sample series over a long period (for at least 3 h and up to 2 h before the diauxic shift). There is nevertheless a significant tendency of the mRNAs related with the growth rate (translation) to decrease and the genes related to the diauxic shift (mitochondria) to increase. This probably reflects the cell's early response to the forthcoming changes to take place in metabolism, and corroborates the suggestion that yeast cells use a feed-forward strategy, anticipating growth changes with gene expression changes (Levy and Barkai, 2009).

Having established that the steady-state condition during exponential growth had been fulfilled, we can say that our protocol to calculate mRNA half-lives indirectly, as well as that used by other authors to calculate TR from the RA and RS data, have been verified. The correlations for direct and indirect computed TRs are about 0.5 (Pérez-Ortín, 2007). This is a significant correlation but is not as high as expected. There may be several reasons for this: first, the mathematical error associated with indirect calculations; second, the error associated with the direct measurement of the mRNA half-life (see below). Both problems are unavoidable and will mostly introduce random noise into the indirect data and, therefore, decreased correlation. Another reason for the low correlation obtained could be that indirect TR measures the appearance of mature mRNA in the cytoplasm, whereas GRO (or other methods to estimate the TR, such as RPCC (Pelechano *et al*., 2009), measures the density of RNA polymerases. Density can only be converted into TR by assuming a constant RNA *pol II* speed (Hirayoshi and Lis, 1999). Direct methods measure 'nascent TR', which can differ from 'mature TR' (increase of mature, cytoplasmic mRNAs over time) if the proportion of productive transcription (finished mRNAs) is not the same for all the genes, or if the transport of mRNAs is not equally efficient for them all. This discrepancy, however, is potentially interesting because it opens a way to determine the differences in transcription elongation or mRNA processing between different groups of genes.

The calculation of the RS data indirectly from the RA and TR data can also be compared with those calculated by direct methods. In this case,

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no correlation exists (see Pérez-Ortín, 2007). This result is surprising and suggests potential confusing influences when mathematically operating with variables that have already been correlated. For instance, TR and RA correlate positively, and both correlate negatively with RS (García-Martínez *et al*., 2007). Thus, when computing TR with the RA and RS data (TR α_λ RA/RS), we obtain a new dataset by dividing one dataset that correlates positively with the TR by one that is inversely correlated. The result is, therefore, a new dataset that should mathematically correlate positively with the TR, as is in fact the case. However, when we attempted to compute RS using the TR and RA data (RS α_λ RA/TR), we divided two datasets that negatively correlated to RS. Thus, this negative correlation in the new dataset decreased, due to a confusing effect of the different variables. Another source of discrepancy is the dilution effect caused by the continuous growth of the culture when calculating indirect RS that contributes to mRNA concentration reduction besides the mRNA degradation itself (Alon, 2006), whereas it does not affect indirect methods because the growth of the culture is stopped due to the transcription stop. Additional limitations in the calculations are related to the use of nascent TR (see above) and the well-known problems brought about by the stressing situation caused to cells because of the transcription stop that they require (Grigull *et al*., 2004; Pérez-Ortín *et al*., 2007).

Finally, the confirmation of a transcriptional steady state during the exponential phase means that the functional analyses of gene expression done in yeast to date are reliable, as this steady state confirms that the different time points within that phase can be considered as identical with regard to all mRNA levels.

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Supporting information on the internet

The following suporting information may be found in the online version of this article:

Table S1. TR and RA values for the experiment

Table S2. Relative contribution of TR to RA changes

Table S3. Complete lists of functional groups with a significant deviation from the steady state during exponential growth

Figure S1. Correlation between the different time points for the amount of mRNA (RA) and the transcription rate (TR)

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