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Genomic Run-On Evaluates Transcription Technique Rates for All Yeast Genes and Identifies Gene Regulatory Mechanisms

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Summary

Most studies of eukaryotic gene regulation have been done looking at mature mRNA levels. Nevertheless, the steady-state mRNA level is the result of two opposing factors: transcription rate (TR) and mRNA degradation. Both can be important points to regulate gene expression. Here we show a new method that combines the use of nylon macroarrays and in vivo radioactive labeling of nascent RNA to quantify TRs, mRNA levels, and mRNA stabilities for all the *S. cerevisiae* genes. We found that during the shift from glucose to galactose, most genes undergo drastic changes in TR and mRNA stability. However, changes in mRNA levels are less pronounced. Some genes, such as those encoding mitochondrial proteins, are coordinately regulated in mRNA stability behaving as decay regulons. These results indicate that, although TR is the main determinant of mRNA abundance in yeast, modulation of mRNA stability is a key factor for gene regulation.

Introduction

Eukaryotic gene expression is a complex process regulated at different levels, such as transcription rate (TR), mRNA processing, mRNA stability, and translation rate. Traditional gene expression methods focus on mRNA level (Sambrook and Russell, 2001). The absolute amount of an individual mRNA at a given time is the result of a balance between its TR and its stability. Whereas initiation of transcription has been well studied at the level of individual genes, the TRs have rarely been estimated (Iyer and Struhl, 1996).

The transcription run-on assay (TRO) has been used for a long time to directly quantify the density of elongating RNA polymerases. Assuming that RNA polymerases elongate at a constant rate, quantification of their density provides a measure of TR in the moment of RNA labeling (see Hirayoshi and Lis, 1999). The method, developed for higher eukaryotic nuclei, works very well with intact yeast cells (Birse et al., 1997). It consists of three steps: (1) cell permeabilization in a cold sarkosyl buffer that stops all physiological processes and disrupts most chromatin proteins but not elongating RNA polymerases, (2) run-on reaction at 30°C in the presence of radioactive UTP to label elongating RNA, and (3) hybridization of labeled run-on RNA to specific probes.

The ability to hybridize labeled run-on RNA to a single filter containing multiple gene probes allows for rigorous quantitative comparisons. This possibility has been used for some specific cases (Schuhmacher et al., 2001; Lilly et al., 2002). Fan et al. (2002) studied the cellular stress response of 1152 genes in H1299 cells at the level of TR. Legen et al. (2002) also studied run-on transcription of the 129 plastid genes of tobacco plants in wild-type and cyanobacterial RNA polymerase mutants. In both cases, the simultaneous measurement of mRNA amounts and run-on allowed them to determine the influence of TR and mRNA stability in the steady-state mRNA levels. The need for a nuclei or plastid isolation protocol introduces a potential source of nonphysiological effects. Moreover, the number of genes analyzed is relatively low and no conclusions were obtained on the absolute TR or on the relative TR between genes. This limitation was caused by the lack of comprehensive normalization methods and reference data in those systems. The yeast *Saccharomyces cerevisiae*, however, offers the possibility to overcome all these limitations due to (1) the existence of whole genome DNA chips that have provided exhaustive information about absolute mRNA levels (Holstege et al., 1998; Wang et al., 2002), (2) the possibility of using rigorous normalization methods, and (3) the ability to perform TRO assays on whole cells.

Here we present a method, called Genomic Run-On (GRO), which combines in vivo labeled RNA and nylon macroarrays to calculate TR for all yeast genes. This is the first time that a whole eukaryotic genome has been studied at the transcriptional level. The combined study of nascent transcription and steady-state levels of mature mRNA establishes the contributions of TR and mRNA stability to the total mRNA amount. We applied the GRO method to cells that were shifted from glucose to galactose. This shift has been demonstrated to cause large physiological changes (Jona et al., 2000). It has been previously shown that a change in carbon sources produces a rapid inhibition of translation but not a comparable decrease in the mRNA amount of certain genes (Ashe et al., 2000; Kuhn et al., 2001). We show that although global steady-state mRNA levels are little affected, TRs decrease to about 10% of the values observed in glucose, and they are much more variable along the time course of the experiment. This reveals an important contribution of mRNA stability as a regulatory mechanism. Clustering of the mRNA and TR profiles allows us to find novel functional relationships between genes and new regulatory pathways.

Results

GRO Protocol

We have adapted the well-known transcription run-on method to determine the transcription rates of all yeast genes (see Experimental Procedures and Figure 1).

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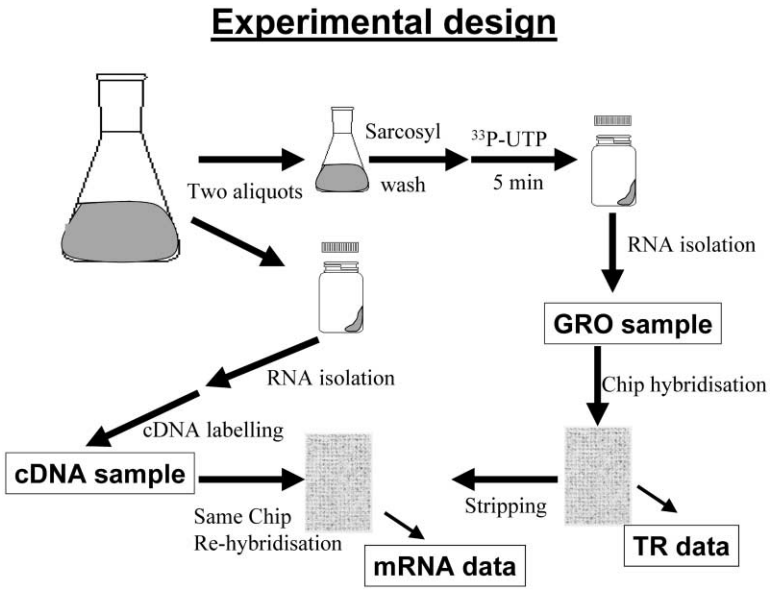

Figure 1. Experimental Design

Cells growing under the desired conditions are collected into two aliquots. The first aliquot is used for run-on analysis. We optimized the conditions of the labeling reaction for this purpose. Radioactivity was linearly incorporated up to 200 μCi of ^{33}P-UTP per sample, and 5 min was the optimum time for run-on (data not shown). Determination of the amount of radioactivity incorporated quantitatively reflects the amount of transcribing RNA polymerases in the precise moment of cell collection (Hirayoshi and Lis, 1999). By correcting the signal intensities by the number of uridines of every probe, the values obtained are proportional to the RNA pol II density on the probe region for every gene. The second aliquot is used for RNA isolation and then labeled by RT to cDNA. This sample is used for rehybridization of the same DNA macroarray used for GRO. Normalization between different hybridizations was done differently for mRNA and TR samples. When cells are subjected to external perturbations, it is not possible to assume that the amount of mRNA per cell is constant. We evaluated the total amount of poly(A) mRNA per cell and used this datum to normalize the different hybridizations of cDNA. For GRO hybridizations it is not possible to use a similar protocol because the variations in RNA pol II total transcription are not known. In this case we used the total RNA obtained after each run-on experiment to calculate the number of cells and to use this datum for normalization. The use of the same DNA chip for successive hybridization of cDNA and GRO samples improves the comparison between these two values for each gene (see Supplemental Data).

Since most probes in the chip are full-length ORFs (see Supplemental Data at http://www.molecule.org/cgi/content/full/15/2/303/DC1), we calculated the density of RNA polymerases along the entire gene. Transcribing RNA polymerases can be stalled at the 5′ (see Lis, 1998) or 3′ (Birse et al., 1997) ends of the gene. In any case, the values obtained in the GRO experiment are proportional to the average density of polymerases occupying a region somewhat longer than the probe (because the run-on elongation extends up to 300 nucleotides [nt]) (see Hirayoshi and Lis, 1999). Assuming a constant elongation rate for RNA pol II polymerases that have initiated the elongation mode (Lis, 1998), the polymerase density provides a measure of the TR at the moment of cell sampling.

We used another aliquot of the same cells to obtain a direct evaluation of the mRNA amounts using the same DNA chip (see Figure 1). Thus, both the TR and mRNA amount can be obtained for every gene. Moreover, because we normalized the signals obtained for TR and mRNA using a genomic DNA hybridization, values for individual genes are fully comparable. We obtained absolute mRNA copy number and corrected TR (mRNA transcribed per time unit per cell) using appropriate references (see below).

General Cell Responses to Carbon Source Shift

We applied the GRO method to study a large physiological change: the sudden depletion of glucose and its substitution by galactose. Other authors previously found that glucose removal or its change to galactose produces general effects such as inhibition of RNA pol II transcription, bulk mRNA stabilization (Jona et al., 2000), and inhibition of translation initiation (Ashe et al., 2000). We first analyzed the total amount of poly(A) mRNA and whole RNA pol II transcription (measured as the sum of individual spot intensities of a GRO experiment) per cell. Figure 2 shows the profile for both data sets. Whereas mRNA amount decreases only moderately during the time in which cells are arrested (t1–t3) the TR shows a sudden increase after carbon source shift (t1) and a large decrease, down to a 10% of the initial value, until cells resume active growth (t4). We can conclude, thus, that after changing from glucose to galactose cells, undergo a general downregulation in transcription rate but mRNA levels are maintained comparatively high because of a general stability increase. This increase is transient and begins to decline as cells resume active growth and start transcribing again.

Determination of Absolute mRNA Amount and TR for All Yeast Genes

Our protocol produces two independent data sets for every gene: mRNA amount and TR along the time course of the experiment. The first set is equivalent to those obtained with DNA chips in other experiments. The second one, however, is an entirely new kind of data set.

We determined the mRNA amount per cell for between 5753 and 5829 genes along the six sampling points (t0–t5). Comparable data have been previously reported only for t0, which corresponds to an exponentially growing culture in YPD (Velculescu et al., 1997; Holstege et al., 1998; Wang et al., 2002). We compared our results with those of Wang et al. (2002). By plotting the two data sets there is a reasonably good correlation ($r = 0.65$), taking into account the differences in the particular yeast strain, laboratory protocols, and DNA chip type (data not shown). We used the slope of that plot to convert our values into mRNA copies per cell. Then, because all data had been normalized with regard to t0,

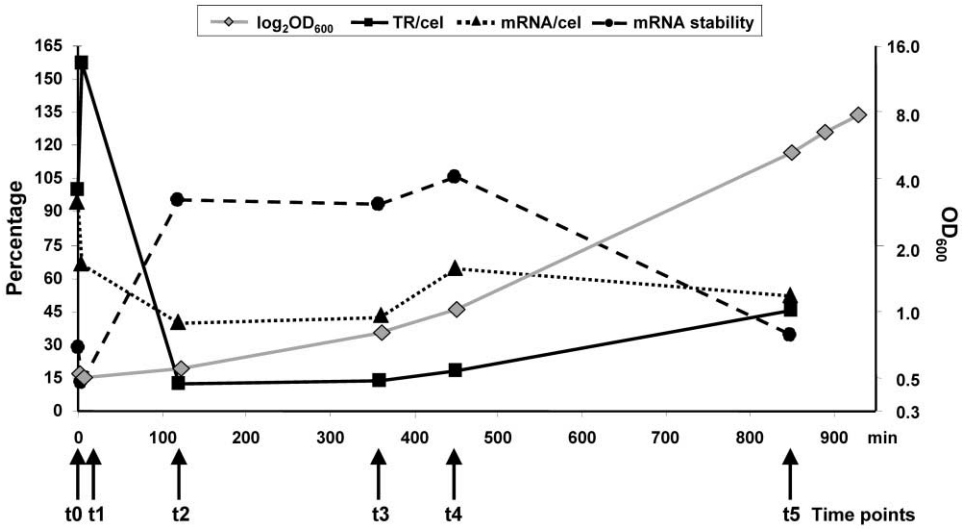

Figure 2. Time Course of the Glu→Gal Experiment

At time zero, exponentially growing cells (OD_{600} = 0.5) were collected and transferred to galactose medium as described in Experimental Procedures. A first pair of aliquots was taken and subjected to the protocol described in Figure 1 (t0 sample). Duplicated aliquots were taken at the indicated times (t1–t5). For each aliquot the total amount of mRNA/cell and RNA pol II TR/cell was calculated as described in Experimental Procedures. The mRNA stability was obtained as the ratio between mRNA/cell and TR/cell. Thus, it indicates the average stability for the bulk of cell mRNA. These three parameters were normalized to an arbitrary value of 100 at t0. Diamonds, log_2OD_{600}; squares, TR/cell; triangles, mRNA/cell; circles, mRNA stability.

we obtained mRNA copies/cell for all the genes analyzed in six different physiological conditions (t0–t5). The complete list of these results and the rest of the data obtained can be viewed at http://scsie.uv.es/chipsdna/#datos.

We determined RNA polymerase densities and, therefore, TRs for between 5593 and 5866 genes along the six sampling points (t0–t5) described. For the GRO experiment there is no reference data set. There is only an indirect estimation (calculated by the ratio of mRNA level and the mRNA decay rate) of transcriptional initiation for the *HIS3* gene in exponentially growing cells: one transcript per 140 s (Iyer and Struhl, 1996). This is equivalent to 0.43 mRNAs/min and to RNA pol II density of 0.28 molecules/kb, assuming an elongation rate of 25 nt/s (Edwards et al., 1991). Using this data as a reference, we converted arbitrary TR results into mRNAs/min per cell. The average values fluctuate between 0.005 (t3) and 0.084 (t1).

Gene Profile Clustering for TR and mRNA

Apart from the interest of absolute mRNA or TR quantity, the relative changes in both values along the time course reflect the gene behavior in transcription and in mRNA level. In order to find functional relationships between genes, we clustered mRNA (Figure 3) and TR (Figure 4) data sets. A total of 3046 genes gave nonflat mRNA patterns. Because 99% of the genes showing flat patterns have signal intensities 10-fold higher than the background, we rule out that they correspond to poorly detected mRNAs. The nonflat pattern genes were organized in 27 clusters: 1c–27c. Clusters 16c–27c group together. They are significantly enriched in genes with gene ontology (GO) categories related with energy pathways. *GAL* structural genes are included in cluster 18c. Clusters 1c–7c are characterized by a smooth decrease in mRNA amount from t0 to t5. Finally, clusters 8c–15c contain most of the genes analyzed and are characterized by a valley in t2. Among them, clusters from 9c to 13c, that have the sharper valley, are enriched in GOs related with macromolecular biosynthesis.

Clustering of the TR profiles is shown in Figure 4. In this case 5749 genes gave nonflat patterns. Most of the genes and clusters show profiles similar to the overall TR profile shown in Figure 2. We conclude that the overall profile is not a consequence of a group of highly transcribed genes but a general feature of the RNA pol II transcription. It is interesting to note that the only cluster that shows an increase from t0, 19g, contains *GAL* structural genes and two other hexose metabolism-related genes, *PCK1* and *ADR1*. Almost all the rest show a profound valley in t2–t3 points. This means that RNA pol II transcription is reduced to a minimum during the arrested growth. Clusters 16g–18g and 21g are characterized by a significant enrichment in macromolecular biosynthesis GO categories. Clusters 5g–14g contain most of the genes and they are very similar to the overall profile of Figure 2. They are enriched in GOs related with energy pathways but with a low statistical significance (except for 7g).

Estimation of mRNA Stability

Having determined the number of mRNA copies and TR per cell, we were able to calculate mRNA stability for every gene. This calculation is based on the hypothesis of steady-state mRNA levels. We divided the mRNA amount by the TR for every gene and time point. In this way an estimation of the mRNA stability in time units is obtained (see http://scsie.uv.es/chipsdna/#datos). Because time points t0, t2, t3, and t5 represent quite "sta-

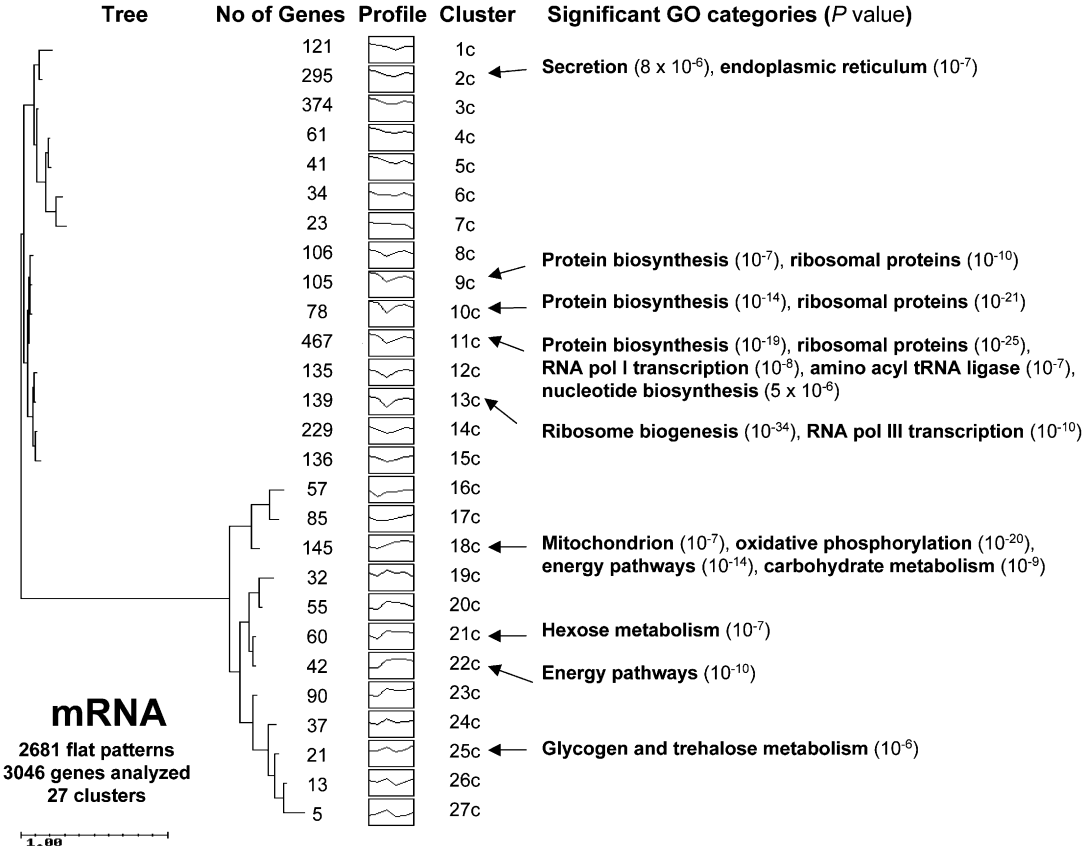

Figure 3. Clustering of mRNA Data

The tree obtained after clustering is shown on the left. For each cluster, the number of genes contained, its profile, and name are indicated. Only the most significant GO categories ($<8 \times 10^{-6}$) are shown.

ble" physiological conditions (either exponential or arrested growth), we think that it is reasonable to assume that steady-state conditions are observed for most of the mRNAs. We compared the values obtained here for 26 genes at t0 with the published data using the *rpb1-1*^{ts} allele of RNA pol II shifted to 37°C (Herrick et al., 1990; Braus, 1991; Moore et al., 1991; Li et al., 1999; Albig and Decker, 2001). The correlation is reasonably good, Pearson coefficient r = 0.72 (Supplemental Figure S1).

We assume, however, that because the mRNA stability determination is indirect it may be affected by the errors in TR and mRNA amount determinations, especially for genes with very low values in any of them. We obtained an SD value for every datum based on reproducibility in the three repeats of the experiment (see http://scsie.uv.es/chipsdna/#datos). Another potential drawback of the method is that for some transcripts with very rapid decay there is no a perfect relationship between steady-state levels and decay rates for a given TR, e.g., *CTF13* (see discussion in Cao and Parker, 2003).

Profiles of the mRNA stability data show tendencies for each gene along the experiment. We obtained 24 clusters (1s–24s) for 5603 genes, which gave nonflat patterns (Figure 5). We observed three groups of clusters. The first one is composed of clusters 1s–7s that comprise 62% of the genes and are similar to the overall profile shown in Figure 2: stabilization during the arrested growth followed by a decrease when cells resume growth in galactose. This group is enriched in energy-related genes. The behavior of the other two groups (8s–24s) is somewhat different, but the stabilization at intermediate times and a decrease at the final time point are general features as well. Therefore, as shown for TR profiles, there is a common behavior for most yeast mRNAs. Clusters 8s–13s comprise 26% of the genes. They show some GOs with low significance. The third group, clusters 14s–24s includes mostly translation-related genes, although it accounts only for 12% of the total genes. This result suggests that this class of genes is especially regulated at the level of mRNA stability.

Cross-Correlation between the Three Types of Gene Profiles

We analyzed the respective contribution of the TR and the mRNA stability to the specific mRNA level profile of each gene. For this objective, we made a correlation plot for each pair of TR versus mRNA or stability versus mRNA values for the six time points for every gene.

As shown in Figure 6, most of the genes have high positive correlations between their TR and mRNA data. The histogram shows that a majority of the genes have r Pearson coefficients above +0.5. The distribution has a median of +0.60 and a peak at +0.82. This means

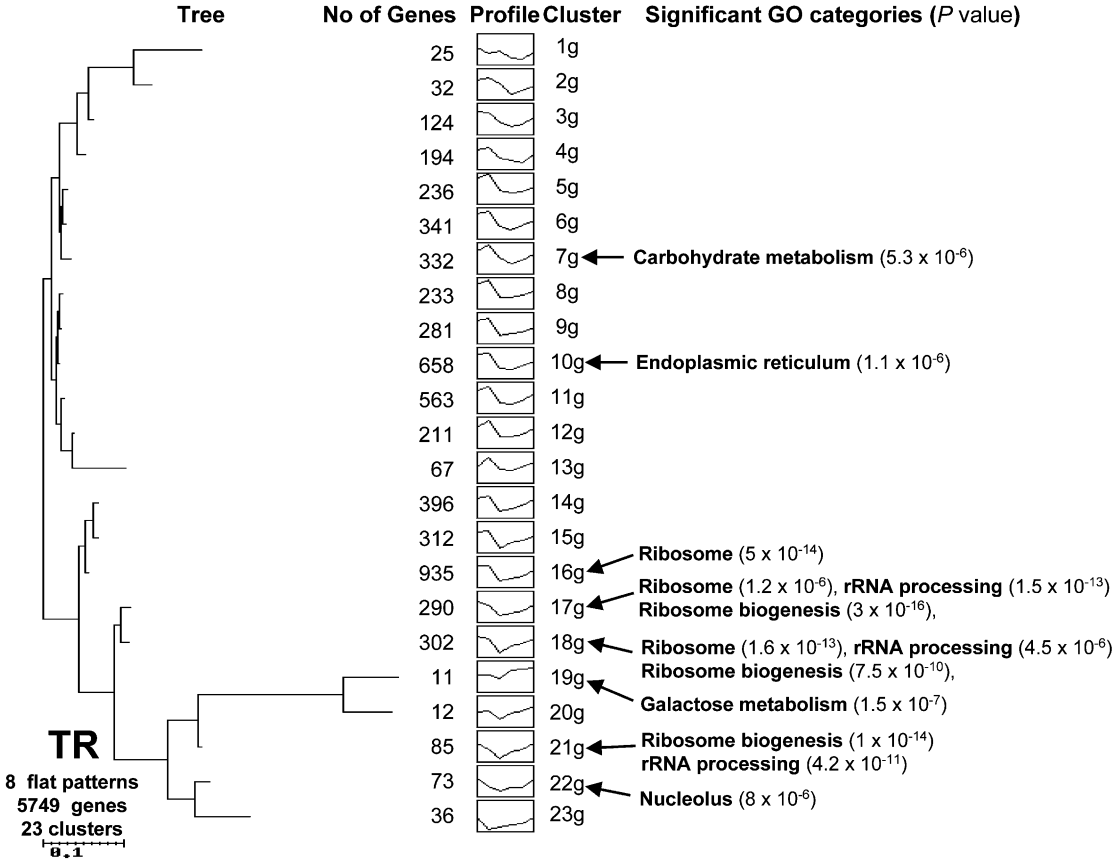

Figure 4. Clustering of TR Data

Description as in Figure 3.

that TR is the main cause of mRNA level gene profiling in this experiment in yeast. However, a significant amount of genes (41%) have correlations below $+0.5$ and there is even a small group (8%) that has highly negative correlations. We analyzed the presence of significant GO categories in the groups of $+0.5 > r > -0.5$ and $r < -0.5$. Within the first group, the GO categories "transcription factors" ($p = 7 \times 10^{-7}$) and "mitochondrial ribosomal protein" (8×10^{-6}) were significant. Within the second group, several GO categories related with mitochondria appeared: "energy pathways" (10^{-17}), "oxidative phosphorylation" (4×10^{-17}), "mitochondrial ribosomal protein" (10^{-7}), and "mitochondrion" (10^{-27}) (see Supplemental Table S2). In fact, many mitochondrial genes (260 genes out of 450) have negative r giving a very low median for this class ($+0.12$). Thus, for mitochondrial proteins, the mRNA level seems to be very poorly correlated with TR.

When comparing the correlations of mRNA level and mRNA stability data for the whole set of genes (Figure 6), it is evident that there is a high tendency to have an inverse correlation (median $= -0.24$). This means that, in our experiment, the mRNAs stability follows completely different rules than TR. It has less influence than TR on the mRNA level but, because, in general, it is opposite to TR profiles (see Supplemental Figure S7), it has an important influence in the final mRNA level and, consequently, in gene expression.

Discussion

In vivo labeled RNA obtained by run-on has been successfully used to probe nylon filters containing all *S. cerevisiae* ORFs. This technique, named Genomic Run-On (GRO), allows for the genome-wide analysis of yeast transcription. The measurement of TR should be very useful for the study of gene regulation because this is the step in which transcription factors act. Theoretically, genes regulated by a common transcription factor (a regulon) should have similar TR profiles. GRO analysis combined with regular steady-state analysis (transcriptome) provides an accurate measurement of the expression of every gene at two different levels. It also, indirectly, reveals the stability of each yeast mRNA during the biological process studied.

We studied, with this new tool, the transition from glucose to galactose of an exponentially growing yeast culture. Cell growth suffers a quick arrest when the carbon source changes. There is a drop in the global transcriptional activity. However, a much less severe decrease in the steady-state RNA is detected. That event suggests a general stabilization of mRNAs during the phase of adjustment to the new metabolic situation. Other researchers found that some glucose-repressible mRNAs were stabilized (Cereghino and Scheffler, 1996; Yin et al., 2000) or destabilized (*HYP2*) (Vasudevan and Peltz, 2001) when comparing exponential growth in glu-

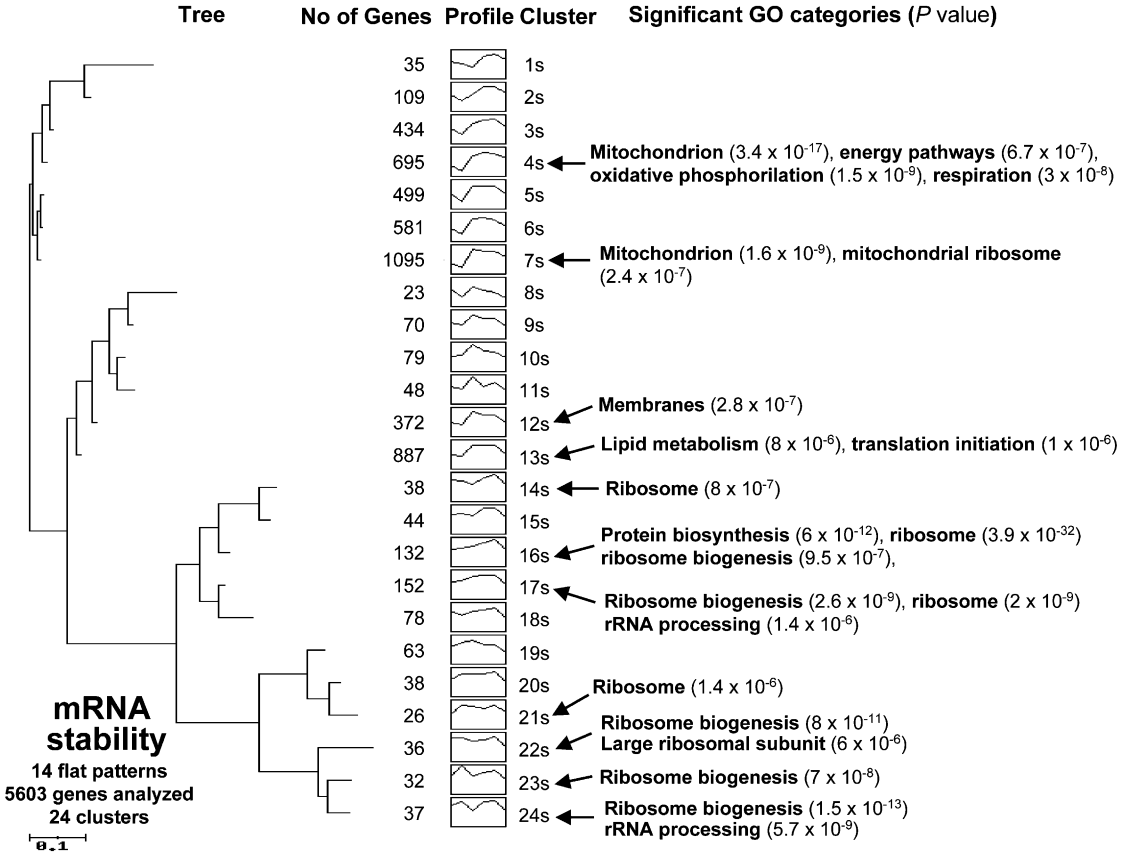

Figure 5. Clustering of mRNA Stability Data

Description as in Figure 3.

cose to other carbon sources. In our case, when comparing t0 to t5 data (growth in glucose or galactose, respectively), we observed the same effects except for *PDC1* that behaves oppositely (it is destabilized in galactose).

The effect of glucose deprivation has been shown to stabilize mRNAs encoding ribosomal proteins (Yin et al., 2003) and some other mRNAs for some hours after the shift (Jona et al., 2000). The overall curve (Figure 2) but, especially, the mRNA stability profiles (Figure 5), indicates that the stabilization is not a specific signature of certain genes but is the general rule. The sudden depletion of glucose has been shown to cause other consequences as well. Martínez-Pastor and Estruch (1996) showed that some mRNAs are present, but they are not translated. Ashe et al. (2000) extended this observation to other mRNAs and showed that this effect is general and that it is due to an inhibition of translation initiation that spans for longer than 2 hr. Thus, it seems that when cells are depleted of glucose, transcription and translation are almost abolished and a general increase of mRNA stability maintains most individual mRNA levels. This coincides with a considerable loss of ribosome association to mRNAs (Ashe et al., 2000; Kuhn et al., 2001). The molecular mechanisms that govern the striking phenomenon of mRNA stabilization are not known. Ashe et al. (2000) demonstrated that cellular responses after depletion of glucose are not dependent on the TOR pathway, but on the glucose-repression pathways, especially on cAMP protein kinase, that restores, in the midterm, the translation competence of the mRNAs. Yin et al. (2000) demonstrated that the mRNA destabilization of some glucose-regulated genes is controlled by glucose-repression pathways. We suggest that the overall mRNA stabilization after glucose removal and its return to similar stability levels when growing in YPGal may be governed by similar regulatory pathways.

The overall profile observed for TR is striking. Jona et al. (2000) observed that an inhibition of overall transcription happens after the glucose removal. In their study they only analyzed incorporation of labeled uridine into RNA, which accounts for the sum of transcription of all three RNA polymerases. We obtained similar results with that protocol (Supplemental Figure S3). However, since RNA pol I accounts for more than 60% of the total transcription and it is shut off in circumstances of arrested growth (reviewed in Warner et al., 2001), the behavior of RNA pol II is not seen in their results. The quantification of RNA pol II TR made by macroarray analysis reveals a sharp increase at 5 min, specific for this polymerase. Because the overall transcription has no increase (Supplemental Figure S3) the conclusion is that RNA pol I+III start their decrease in TR since the very beginning but RNA pol II decreases a few minutes later, after a short pulse. What causes the specific increase in RNA pol II is not known. We think that it is not

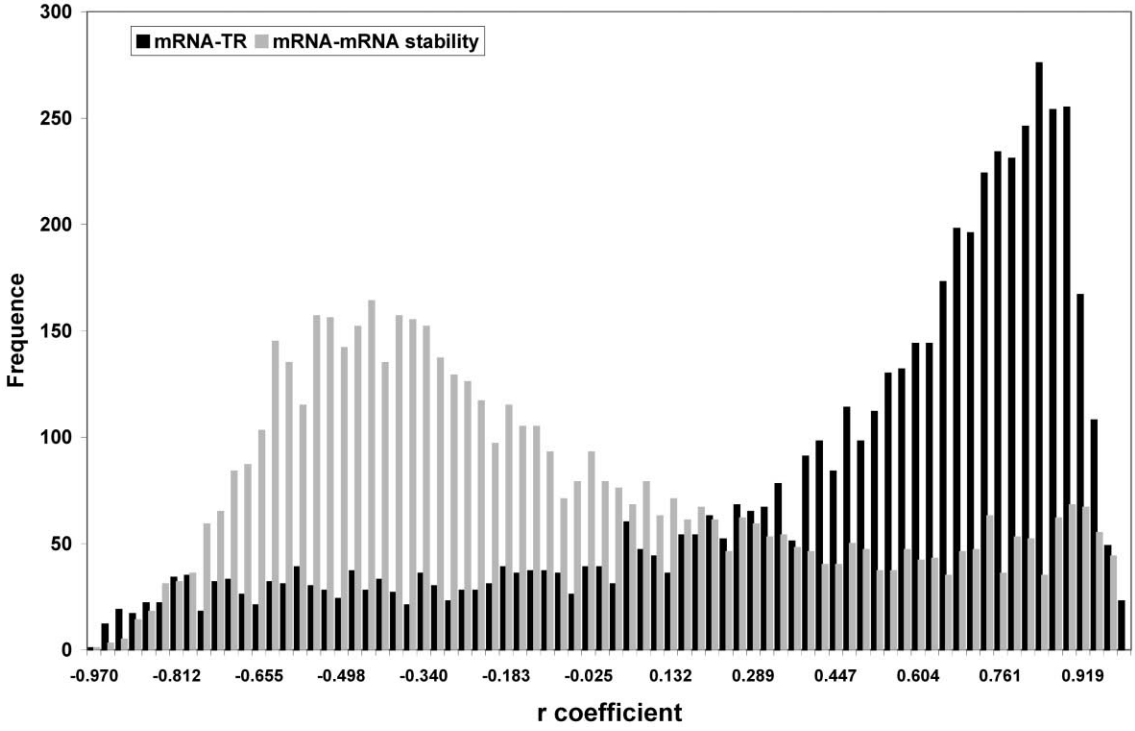

Figure 6. Dependence of the mRNA Amount on the TR and the mRNA Stability

Histograms of the Pearson coefficient (r) for correlations of individual values of TR versus mRNA data (black columns) and mRNA stability versus mRNA (gray columns).

an artifact because 33% of the genes (clusters 1g–3g and 16g–23g) show TR profiles different from the overall pattern (Figure 2) with no increase at t1 (see Figure 4). Thus, the shock suffered by cells when changing carbon source does not impact on all the RNA pol molecules in the same way: RNA pol I (and, perhaps, RNA pol III) and 30% of the genes transcribed by RNA pol II, including most of those involved protein biosynthesis, experiment an immediate decrease in TR. This coincides with the general shut off the macromolecular synthesis machinery that has been shown to be controlled by the TOR pathway (Powers and Walter, 1999). The rest of the genes, 70% of the RNA pol II ones, show a transient rise in TR.

The comparison of our individual TR data with the published TR value for *HIS3,* indirectly obtained by Iyer and Struhl (1996), allowed us to obtain TR in mRNA molecules/min for a yeast cell. Due to the use of a single reference instead the average of multiple genes, the absolute TR values are less accurate than the mRNA level data. They provide for the first time, however, a complete picture of the cell transcription process. For YPD exponential growth (t0) the mean and the median TR are, respectively, 0.055 and 0.018 molecules/min. It is interesting to note that histones, and *COX* and *MFA* genes, are between the most transcribed, about 1.5–2 molecules/min. This corresponds to 1–1.3 RNA pol II molecules/kb, far from the density observed for RNA pol I in active rRNA genes in yeast, 7.6 molecules/kb (French et al., 2003). It is interesting to note that the ribosomal protein genes, considered to be heavily transcribed (Warner et al., 2001; Lieb et al., 2001), have only 0.260 and 0.143 molecules/min (mean and median), much higher than the average but also much lower than the most heavily transcribed genes.

TR data provide a new proof for real genes. For instance, in our experiment GRO analysis reveals transcription, in some of the six time points, for 164 genes (96 of unknown function) that showed no detectable mRNA level in any condition. This may be caused by low steady-state mRNA levels for those genes. (The complete list of these genes can be viewed in the Supplemental Table S1).

Another source of new information comes from the clustering of TR profiles (Figure 4). It is evident that TRs are much more altered during the experiment than mRNA levels. The low number of flat patterns and the profiles' shapes suggest that genes undergo much more important changes in RNA pol II occupation during physiological changes than in final mRNA level. Moreover, the different TR and mRNA profiles indicate that important additional mechanisms act to control the steady-state mRNA level (see below).

The combined analysis of mRNA and TR clusters also provides interesting new information. Some groups show a high tendency to cluster together in both analyses. One example are the amino acyl tRNA ligase genes. Twelve genes (out of seventeen analyzed) cluster identically in TR and mRNA. The conclusion is that, for these genes, the regulatory mechanisms should be mostly at the level of TR, given that profile clustering in TR and mRNA coincides. Posttranscriptional mechanisms do

not specifically alter the steady-state mRNA level of individual genes. A similar case is seen in the cytosolic ribosomal proteins. This group has been shown in previous studies to have an homogeneous behavior in many stressful physiological situations (Gasch et al., 2000; Warner et al., 2001). We observed a similar conduct: decrease in mRNA levels after carbon source shift and recovery coordinated with yeast growth (clusters 9c–11c). There is a coordinated regulation at the level of TR: they cluster together (16g and the closely related 18g) and follow the same profile of the macromolecular synthesis machinery (see above). Logically, this provokes a tight clustering in mRNA stability as well (clusters 14s, 16s, 17s, and 21s). Ribosomal proteins are, therefore, different in all their features from the majority of the yeast genes. This result suggests that specific regulatory mechanisms exist for ribosomal protein genes not only for transcription initiation (see references in Planta et al., 1995; Warner et al., 2001) but also for mRNA stability. In fact, it has been previously noted that mRNAs encoding ribosomal proteins have comparatively short half-lives (Herrick et al., 1990; Yin et al., 2003) and that they are transiently stabilized (for up to 3 hr) after glucose addition (Yin et al., 2003). Kuhn et al. (2001) also showed that ribosomal protein mRNAs behave differentially, displaying a more pronounced reduction in polysomal fraction after carbon source shift. It can be concluded that glucose addition or removal provokes an immediate mRNA stabilization for these proteins. This may be a protective mechanism that avoids the waste of these extremely abundant mRNAs (30% of the total mRNA) (Warner et al., 2001) during metabolism remodeling circumstances.

A third group that behaves coordinately is the structural *GAL* genes that show a unique profile in TR (cluster 19g). They are the only genes that show no decrease in TR in t2/t3. Therefore, they are the only genes for which transcription is activated in order to remodel the metabolism. Their typical mRNA stability profile (4s) demonstrates that they are not subjected to specific posttranscriptional regulation. Curiously, the mRNA profile (18c) is common to other carbohydrate metabolism and energy pathway-related genes. This means that posttranscriptional mechanisms should act on genes, *GAL*, and others, such that, despite their different TR programs, they converge to similar mRNA profiles.

The analysis of the gene profiles with regard to specific gene features is interesting. TR and mRNA stability profiles do not appear to be dependent on the expression level. However, the mRNA amount changes oppositely from sampling time t1 to t2: genes with high mRNA level decrease their level whereas those with very low level show an increase (Supplemental Figure S4). On the other hand, the TR decreases exponentially with the length of the ORF (see Supplemental Figure S5). This can reflect an increasing probability of the RNA polymerase to come off the gene as the transcription proceeds.

A final conclusion obtained from the mRNA stability profiles is that the existence of clusters of functionally related genes supports the hypothesis of mRNA decay regulons. This hypothesis has been proposed based only on the observation of similar transcript decay rates for components of multiprotein complexes (Wang et al., 2002; Khodursky and Bernstein, 2003). Here we show that, as expected for a regulon, some genes behave coordinately along a variety of circumstances. Moreover, we show that not only multiprotein complexes but other noninteracting, but functionally related proteins, such as those of mitochondrial energy pathways, are part of decay regulons. As can be seen in Figure 5, decay regulons are especially clear for two groups of genes: those coding for ribosomal and mitochondrial proteins. Mitochondrion GO category genes show profiles similar to the general one, but exacerbated for the case of those involved in energy pathways (Supplemental Table S2 and Figure S6). The ribosome GO category, however, shows a particular profile that can be split into two other ones. One of them (see Supplemental Figure S6B) is very different from the rest, and it is particularly enriched in proteins of the large subunit of the ribosome.

The discover of gene groups with, or without, coordinated regulation at TR and mRNA stability raises the question of how important the respective contribution of transcription and mRNA stability mechanisms is for the steady-state profile of a given mRNA. The application of Pearson coefficient to the plot of individual values of TR and mRNA level, or mRNA stability and mRNA level, is a simple way to quantify these contributions. Our results show that, as expected, TR is the main step for regulation, but they also show that the contribution of posttranscriptional mechanisms is very important. As pointed out by other authors, regulation of transcript stability may be a more important step in gene regulation than it is currently assumed (Wang et al., 2002). Regulation at this level is faster than the regulation at transcription initiation (Yang et al., 2003). Moreover, it is cost effective because already synthesized mRNAs are preserved for future use when the metabolism has been remodeled for galactose utilization and the cell is ready to resume growth.

Some genes seem to increase their mRNA level even having a decrease in TR. As shown in Figure 6, some genes have negative correlations in TR versus mRNA and positive in stability versus mRNA plots. This set of genes is highly enriched in mitochondrial proteins, especially those related with energy production. For instance, 10 out of 15 genes for TCA cycle enzymes have $r < -0.5$ in TR versus mRNA plot. This group has an average r coefficient of $+0.89$ in stability versus mRNA plot. Other GO subcategories such as "oxidative phosphorylation," "cytochrome oxidase complex," and "metabolism" are especially enriched within the group of mitochondrial proteins with a highly negative correlation. Looking at the profiles, it seems that the mRNAs of proteins involved in energy generation, that should be active during the arrested growth, are subjected to a exacerbated stabilization program (Supplemental Figure S6) that produces an increase in their mRNA levels (18c, 22c) while the cell has a general decrease in TR that otherwise would decrease them.

We believe that the GRO method will be useful to generate a more comprehensive analysis of transcription and gene regulation in yeast because it is suitable to be used in almost all physiological situations, providing "instant pictures" of the transcription state of the cell. When combined with standard mRNA analysis, it provides a more complete picture of gene expression. Moreover, GRO could be easily adapted to other eukary-

otes, provided that run-on methodologies are feasible in a given experimental system.

Experimental Procedures

Yeast Strain, Growth Conditions, and Sampling Procedure

We used the *S. cerevisiae* yeast strain BQS252 (*Mat*a, *ura3-52*, *GAL2*) that was obtained by esporulation of FY1679 (Thierry and Dujon, 1992). Cells were grown overnight at 28°C in 800 ml of YPD medium (2% glucose, 2% peptone, 1% yeast extract) in 2 liter flasks at 120 rpm up to exponential growth phase ($OD_{600} = 0.5$). Then cells were recovered by centrifugation, YPD medium was removed, and they were resuspended in YPGal medium (2% galactose instead of glucose). Cells were allowed to grow in the same conditions for 14–15 hr after the shift.

Cell samples were taken at different times: t0 (exponential growth in YPD medium, just before the shift to galactose medium), t1, t2, t3, t4, and t5, corresponding to 5, 120, 360, 450, and 850 min, respectively, after the carbon source shift. The t5 sampling time corresponds to exponential growth in galactose medium (see Figure 2).

At every sampling time, two different aliquots from the cell culture were taken. One of them was immediately processed to measure transcription rates, according to the run-on protocol (next section). Cells from the second aliquot were recovered, washed with cold distilled water, and kept frozen to proceed later according to mRNA measurement protocol (see corresponding section). The experiment was repeated three times.

Genomic Run-On Protocol

Transcription run-on (TRO) protocol (Birse et al., 1997; Hirayoshi and Lis, 1999) was scaled up for a large amount of cells. Around 6×10^8 yeast cells were used to perform in vivo transcription. After spinning down cells, they were washed in cold water and the cell pellet was resuspended in 900 μl of sterile cold water (final volume 950 μl). Then, the cell suspension was transferred to a fresh microcentrifuge tube, 50 μl of 10% N-lauryl sarcosine sodium sulfate (sarkosyl) were added, and cells were incubated for 20 min on ice. After the permeabilization step, cells were recovered by low-speed centrifugation and the supernatant was removed. In vivo transcription was performed by resuspending cells in 120 μl of 2.5× transcription buffer (50 mM Tris-HCl [pH 7.7], 500 mM KCl, 80 mM $MgCl_2$), 16 μl of AGC mix (10 mM each of CTP, ATP, and GTP), 6 μl of DTT (0.1 M), and 13 μl of [α-^{33}P]UTP (3000 Ci/mmol, 10 μCi/μl). Cells were maintained on ice at all times. The final volume was adjusted to 300 μl with distilled water and the mix was incubated for 5 min at 30°C to allow transcription elongation. The reaction was stopped by adding 1 ml of cold distilled water to the mix. Cells were recovered by centrifugation to remove the nonincorporated radioactive nucleotide.

Total RNA was isolated using the Fast-Prep (Bio101, Inc.) device by resuspending the cells in 500 μl of LETS buffer (0.1 M LiCl, 10 mM EDTA, 0.2% SDS, 10 mM Tris-HCl [pH 7.4]), 200 μl glass beads, and 500 of water-saturated acid phenol. Contaminants were removed by extraction successively with phenol-chloroform and chloroform. Labeled RNA was precipitated by adding 0.1 vol of 5 M LiCl and 2.5 vol of cold ethanol overnight at −20°C. After centrifugation at maximum speed for 15 min, labeled RNA was washed once with 70% ethanol and dried. Total extracted RNA was spectrophotometrically quantified. An aliquot was used for specific radioactivity determination in a scintillation counter. All of the in vivo labeled RNA was used for hybridization ($0.35–3.5 \times 10^7$ dpm).

Nylon filters were made using PCR-amplified whole ORF sequences as probes (see Supplemental Data). After prehybridizing nylon membranes for 1 hr in 5× SSC, 5× Denhart's, 0.5% SDS, hybridizations were performed using 5 ml of the same solution containing labeled RNA. Hybridizations were conducted during 16–18 hr in a roller oven at 65°C. After hybridization, filters were washed once in 2× SSC, 0.1% SDS for 30 min, and twice in 0.2× SSC, 0.1% SDS for 30 min. Filters were exposed for 7 days to a imaging plate (BAS-MP, FujiFilm) which was read at 50 μm resolution in a phosphorimager scanner (FLA-3000, FujiFilm).

Measurement of mRNA Levels

As mentioned before, a second cell aliquot was taken at each sampling time and was kept frozen at −20°C. After thawing the samples on ice, total RNA was isolated following the procedure described for TRO samples. Again, RNA yield was measured spectrophotometrically. About 30–40 μg of total RNA were reverse transcribed into cDNA by adding 200 U of RT polymerase SuperScript II (Invitrogen), 3 μl of 10× random decamers (Ambion, Inc.), 1 μl RNaseOUT (Invitrogen), 6 μl 5× First Strand Buffer (Invitrogen), 1.5 μl dNTP mix (16 mM dATP, dTTP, dGTP, and 100 μM dCTP), and 5 μl [α-^{33}P]dCTP (3000 Ci/mmol, 10 μCi/μl) in a final reaction volume of 30 μl. The labeling reaction was incubated for 1 hr at 43°C. 1 μl of EDTA 0.5 M was added to stop the reaction. The labeled cDNAs were purified by a S300-HR MicroSpin column (Amersham BioSciences).

Hybridization was done in the same conditions as described for GRO experiment except that labeled cDNA was at 3.5×10^6 dpm/ml and that filters were exposed for 24 hr to an imaging plate. Note that a total of six different nylon filters (one for each sampling time) were used in that experiment. Both GRO and cDNA labeled samples belonging to the same sampling time were successively hybridized against the same filter. For the three repeats of the experiment, different nylon filters were used for the same time point. Therefore, each time point determination is obtained from the averaged results of three independent filters (see Supplemental Data).

Estimation of Total and Messenger RNA

In order to facilitate further normalizations, we estimated the RNA amount obtained from a fixed amount of cells along the experiment. Thus, five different cell aliquots were taken at each of the six sampling times (t0–t5) from a mock experiment. Total RNA was extracted according to the protocol described above and an average value for RNA amount was calculated. Standard deviation was below 2%. Knowing the RNA amount obtained from a fixed number of cells, we transformed the RNA amount obtained in the real experiment (the one used for GRO hybridizations and for cDNA synthesis) to number of cells. Thus, we calculate values for transcription rates and mRNA molecules per cell.

In addition, we used a dot-blot procedure to estimate the proportion of poly(A) mRNA in the total RNA. Three different dilutions of total RNA extracted from each time point of the three repeated experiments were spotted, using a BioGrid robot (BioRobotics) on a nylon filter. The filter was hybridized with 5′-labeled poly(dT). About 1 μg of poly(dT) (Sigma-Aldrich) was incubated at 37°C for 30 min with 5 μl of 10× PNK buffer, 2 μl of polynucleotide kinase (PNK, 10 U/μl), and 5 μl of [γ-^{32}P]ATP (3000 Ci/mmol, 10 μCi/μL), in a final volume of 50 μl. The enzyme was inactivated by incubating the mix at 70°C for 10 min. Filter hybridizations were performed at 40°C using the same protocol and solutions described above. Using these data we calculated the proportion of poly(A) mRNA per microgram of total RNA and, thus, per cell in each of the time points (t0–t5).

Quantification of Hybridization Signals and Normalization Procedures

Both GRO and cDNA experiments were performed in triplicate, swapping the filters in each replicate among the different sampling times. Images were quantified by using ArrayVision 7.0 software (Imaging Research, Inc.), taking the sARM density (with the corresponding subtracted background) as signal. Filters were also hybridized with total yeast genomic DNA labeled by random priming. Genomic hybridization signals were used to normalize both GRO and cDNA signals in each respective filter (see Supplemental Data).

cDNA hybridizations were normalized within each experiment replicate by the global mean procedure. Correction factor to normalize between experiments was calculated from global mean values for the t0 sampling times. Reproducibility of the replicates was tested by the ArrayStat software (Imaging Research, Inc.), considering the data as independent and allowing the program to take a minimum number of valid replicates of 2 in order to calculate the mean values for every gene (only one of the three replicates is allowed to be a removable outlier). Average cDNA values for each gene were finally corrected by percentage of guanines in each probe-coding strand. Normalization between sampling points was made using the amount of mRNA/cell to give values of mRNA copies/cell for each gene

in every time point. These values were used for cluster analysis and comparisons.

Since in GRO hybridizations the total amount of extracted labeled RNA is used for hybridizing the filters, we estimate the total transcription per cell by correcting the values by the amount of RNA extracted and by the amount of total RNA per cell along the experiment. Thus, the correction is specific for each sampling time and for each experiment. Normalization between experiments, similarly to cDNA results, was done by calculating the correction factor from t0 values and applying it to the rest of the sampling times. Statistical validation of replicates was performed as in cDNA values. After that, average TR values for each gene were finally corrected by percentage of uridines present in each probe-coding strand. Again, the corrected average values were used for gene cluster analysis and other calculations. In this instance normalization between time points cannot be done by using internal or external references. We assumed that the total TR for RNA pol II, calculated by summing up the whole set of signals in every hybridization, from the same number of cells, is reflected in the total filter hybridization signal. Variations due to differences in in vivo label incorporation, RNA extraction, and hybridization protocol are reduced by averaging the three independent experiments. However, the reproducibility was quite good given that only between 0.8% and 5% of the genes analyzed were discarded by the ArrayStat software because of their inconsistency. This indicates that the results reflect actual changes in TR for every gene.

Clustering Procedures

Changes in TR and mRNA amount, as well mRNA stability estimates for all yeast genes, were evaluated by cluster analysis of normalized averaged values.

For cluster analysis of the results we used the WEB server of CNIO Bioinformatic Unit (http://bioinformatica.cnio.es) included in the Gene Expression Pattern Analysis Suite v 1.0 (GEPAS). First, log-transformed data were preprocessed with the preprocessor tool (Herrero et al., 2003) in order to remove genes with more than 80% missing values. KNNimpute option was used to impute missing values. Flat patterns were filtered according their standard deviation using a threshold of 0.5.

Preprocessed data were used for cluster analysis by transferring it to SOTA tree server (Herrero et al., 2001) using linear correlation coefficient as distance between genes. The tree was allowed to growth up using a variability threshold of 40% as training conditions.

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