Biochimica et Biophysica Acta 1849 (2015) 653–664

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

Impact of high pH stress on yeast gene expression: A comprehensive analysis of mRNA turnover during stress responses

David Canadell [a], José García-Martínez [b,d], Paula Alepuz [c,d], José E. Pérez-Ortín [c,d,*], Joaquín Ariño [a,**]

[a] Departament de Bioquímica i Biologia Molecular and Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona 08193, Spain
[b] Departamento de Genética, Universitat de València, Burjassot, València 46100, Spain
[c] Departamento de Bioquímica y Biología Molecular, Universitat de València, Burjassot, València 46100, Spain
[d] ERI Biotecmed, Universitat de València, Burjassot, València 46100, Spain

ARTICLE INFO

Article history:
Received 5 December 2014
Received in revised form 7 April 2015
Accepted 10 April 2015
Available online 18 April 2015

Keywords:
Alkaline stress
Environmental stress response
Genomic Run-On
mRNA stability
Saccharomyces cerevisiae

ABSTRACT

Environmental alkalinisation represents a stress condition for yeast *Saccharomyces cerevisiae*, to which this organism responds with extensive gene expression remodelling. We show here that alkaline pH causes an overall decrease in the transcription rate (TR) and a fast destabilisation of mRNAs, followed by a more prolonged stabilisation phase. In many cases, augmented mRNA levels occur without the TR increasing, which can be attributed to mRNA stabilisation. In contrast, the reduced amount of mRNAs is contributed by both a drop in the TR and mRNA stability. A comparative analysis with other forms of stress shows that, unlike high pH stress, heat-shock, osmotic and oxidative stresses present a common transient increase in the TR. An analysis of environmentally-responsive (ESR) genes for the four above stresses suggests that up-regulated genes are governed mostly by TR changes and complex transient bidirectional changes in mRNA stability, whereas the down-regulated ESR gene set is driven by mRNA destabilisation and a lowered TR. In all the studied forms of stress, mRNA stability plays an important role in ESR. Overall, changes in mRNA levels do not closely reflect the rapid changes in the TR and stability upon exposure to stress, which highlights the existence of compensatory mechanisms.

1. Introduction

Adaptation to environmental changes is an essential requirement for all living cells. Although the vegetative growth of yeast *Saccharomyces cerevisiae* occurs within a relatively wide range of external pHs, it is more vigorous at an acidic pH than at a neutral or alkaline one, and most laboratory strains cannot proliferate when the external pH exceeds 8.0–8.2 [1]. The electrochemical gradient of protons across the plasma membrane, required for most secondary active symporters and antiporters involved in the uptake of different nutrients, is created by the extrusion of protons mediated by plasma-membrane H^+-ATPase, encoded by the essential *PMA1* gene [2]. The coordinated regulation of Pma1 and vacuolar H^+-ATPase activity is also crucial for the maintenance of cytosolic pH homeostasis [3], and cytosolic pH has been proposed as a key player in the control of the cell growth rate according to the carbon source [4]. Therefore, it is evident that a brusque increase in the external pH, which leads to neutrality or moderate alkalinisation, represents a stress condition for budding yeast to which *S. cerevisiae* reacts by triggering an adaptive response.

The work done over the past 10 years has demonstrated that the adaptive response of *S. cerevisiae* to high pH stress involves extensive gene remodelling. Thus shifting cells from 5.5 to 8.0 induces changes in the mRNA levels of several hundreds of genes (see [1] for a review). These changes are the result of the activation of different signalling pathways, including Rim101–Nrg1 [5–8], Wsc1–Pkc1–Slt2 MAP kinase and calcium-activated calcineurin–Crz1 pathways [6,9,10], and the Snf1 pathway [11–13], as well as the down-modulation of the protein kinase A pathway [14]. In several cases, it has been possible to link the activation of a given pathway in response to a high pH with changes in the promoter activity of a set of genes as a result of the binding of specific positive or negative transcription factors. For instance, activation of calcineurin results in the dephosphorylation of the Crz1 transcription factor, which leads to its migration into the nucleus and its binding to specific sequences present in calcineurin-responsive genes [15–17]. In this way, activation of calcineurin is involved in the activation of around 10% of the genes induced by exposure to a high pH [9]. Similarly, the high pH-induced down-regulation of protein kinase A activity results in the activation and nuclear entry of Msn2 [14]. Msn2 and Msn4 are transcription factors that contribute to the general stress response of *S. cerevisiae* at the transcriptional level by binding to the 5′-CCCCT-3′ stress response element (STRE) [18].

* Correspondence to: J.E. Pérez-Ortín, Departamento de Bioquímica y Biología Molecular, Universitat de València, Burjassot, València 46100, Spain. Tel.: +34 96 3543467; fax: +34 96 3544635.
** Corresponding authors. Tel.: +34 93 5812182; fax: +34 93 5812011.
E-mail addresses: Jose.E.Perez@uv.es (J.E. Pérez-Ortín), Joaquin.Arino@uab.es (J. Ariño).

http://dx.doi.org/10.1016/j.bbagrm.2015.04.001
1874-9399/

The amount of mRNA at a given time, however, is not only determined by the transcription rate (TR), but also by the balance between the TR and the degradation rate (DR). In the last few years, with the advent of genomic technologies, it has been possible to evaluate the relative impact of transcription and degradation in the levels of most mRNAs [19,20]. For instance, the development of techniques such as the Genomic Run-On (GRO) method [21] and comparative Dynamic Transcriptome Analysis (cDTA) [22,23] has paved the way for such studies in yeast cells subjected to diverse forms of cellular stress. Thus in recent years, it has become evident that changes in mRNA levels in the yeast cells exposed to oxidative [24,25], osmotic [22,26,27], and heat-shock [28] stresses are not only due to changes in TR rates, but are also the result of variations in mRNA stability. Even more importantly, the emerging picture suggests that control of mRNA stability can play an important role in the overall response to stress. The impact of mRNA stability in stress responses differs, however, from the role of transcription. Whereas the TR usually determines the mRNA level during the response, mRNA stability is related more to the speed of the response. So as unstable mRNAs allow faster responses [20,29], a transitory decrease in mRNA stability (increase in DR) can be used to sharpen the response peaks of induced genes, although this non-homodirectional response implies a higher energy cost. This apparently contradictory behaviour is restricted to some up-regulated environmental stress response (ESR) genes [30]. There are, however, many cases in which the positive response is homodirectional: increase in mRNA levels by raising the TR and by increasing mRNA stability. For instance, in response to osmotic stress, the mRNA stability of induced genes increases at 5–6 min [26,27]. Likewise in that stress, the commonest behaviour for down-regulated genes is the homodirectional strategy, based on both a drop in the TR and in the mRNA stability, as observed for ribosomal protein (RP)-encoding genes [26,27]. In this way, energy consumption diminishes and the response speed accelerates. A non-homodirectional strategy in which both the TR and DR lower in response to changes in the environment has also been shown for some genes in yeast [25]. It has also been proposed that during response to stress, the majority of responsive genes show a single point change in mRNA stability, which suggests a single underlying regulatory event [31]. In some cases however, complex patterns of DR changes have been reported in both yeast and mammalian cells [20,24,28]. The existence of a variety of different behaviours and the need to clarify the respective roles of the TR and mRNA stability in stress responses prompted us to further explore the yeast alkaline stress response and to compare it with other previously studied stress responses by genome-wide techniques to evaluate both facets of the mRNA turnover equilibrium.

Our results show that the transcriptional response to high pH stress does not behave like a typical ESR since the influence of mRNA stabilisation is stronger for many up-regulated genes. Moreover, our comparative study of several stress responses also deciphers the relative weight of changes in the TR and mRNA stability to fine-tune gene expression profiles. In summary, our results demonstrate that the classical view of the ESR as a transcriptional response is a simplification, and highlight the central role that post-transcriptional mRNA stability regulation plays in the yeast environmental response.

2. Material and methods

2.1. Strains, growth conditions and experimental setup for the alkaline stress study

S. cerevisiae wild-type strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used in the GRO experiments. The MML830 strain [32], and its derivative MML874 (*tetO$_2$-CTT1*), in which the endogenous *CTT1* promoter was replaced with the *tetO$_2$* promoter as described in [33], were used for RT-PCR and specific mRNA half-life determinations. In these strains, *tetO$_2$*-controlled gene expression was up-regulated in the absence of doxycycline, and became repressed after the addition of 5 μg/mL of doxycycline.

Cells were grown in YPD-rich medium (1% yeast extract, 2% peptone, 2% glucose) at 28 °C. For alkaline treatment, exponentially growing cells in YPD 50 mM TAPS, pH 5.5, were recovered by centrifugation and resuspended in YPD 50 mM TAPS adjusted to pH 8. Samples were taken immediately before the pH shift (time 0) and at 6, 13, 20, 30, 40 and 58 minutes afterwards.

2.2. Genomic Run-On (GRO)

The GRO analyses were done as in [21] with the modifications described in [24,28]. Briefly, at each sampling time, two different aliquots were taken, one for the TR and one for [mRNA] (RA) determinations. For the TR samples, the cells permeabilised using sarkosyl were mixed with a transcription buffer (containing AGC mix and [α-^{33}P]UTP) to perform an *in vivo* transcription reaction. The total RNA from the TR and RA samples was isolated as described in [28] and cDNA was synthesised from the RA sample as in [21]. Hybridisation, imaging and normalisation were done as in [21]. The reproducibility of three GRO (TR and RA) independent experiments was tested by the ArrayStat software (Imaging Research, Inc.) after considering the data to be independent and allowing the programme to take a minimum number of two valid replicates to calculate the normalised mean values for each gene. The decay rate constant (k_D) was calculated from the TR and RA data for all three independent GRO experiments using the method described in [24] and assuming steady-state conditions before the pH shift (t0) and non-steady state conditions after the onset of alkaline pH stress (t6–t58). The normalised values for both the TR and RA were used to evaluate the reproducibility of the calculated k_D. The total TR attributable to RNA polymerase II (Pol II) was defined as the median of the whole set of signals for all the Pol II transcribed genes. For sets of genes, the median TR, RA and k_D values of all the included genes were taken, and the average and standard deviation (SD) were calculated from the three replicates. For RA normalisation, the RNA amount per cell was estimated in parallel experiments using the same conditions and the same time points. For this purpose, total RNA was extracted according to the described protocol [21] and was quantified. poly(A) was estimated using the RNA samples dot-blotted on a membrane and hybridised with 5′-labelled oligo-d(T) as a probe [21]. The proportion of poly(A) mRNA per cell was calculated at each time point by measuring total RNA content and its poly(A) enrichment as described in [26].

Data for the other stresses (oxidative, osmotic and heat shock) were taken from the literature [24,26,28] as final processed data, which did not allow to include SD values. In any case, the statistical procedures applied in those sets of data are similar to those applied here and are described in the original papers.

2.3. Clustering procedures and TF dependence analysis

Changes in the TR, RA and k_D of all the yeast genes were evaluated by a cluster analysis of the normalised averaged values. For the cluster analysis, we used Cluster 3.0 and Java Treeview 1.1.6 [34,35]. To find the enrichment of Gene Ontology (GO) categories in the different groups generated in this study, we used FuncAssociate 2.0 (http://llama.mshri.on.ca/funcassociate) [36] and YeastMine (http://yeastmine.yeastgenome.org) [37]. An unsupervised analysis for transcription factor binding sites and the 3′ UTR motifs was carried out with the FIRE algorithm [38]. In a supervised analysis, the YEASTRACT database (http://www.yeastract.com) [39] and RSAT (http://rsat.ulb.ac.be/) [40] were used to search for the putative transcription factor consensus motifs in the targets genes.

Regarding their dependence for specific regulatory factors, genes were defined as Msn2,4 or Snf1-dependent in their transcriptional response to alkaline pH stress according to previously reported DNA microarray-based data [11,14]. For calcineurin dependence, a

list of genes was assembled based on several independent criteria: calcineurin-dependence in response to Ca^{+2} [41]; calcineurin-dependence in response to alkaline pH stress [9]; carbohydrate metabolism-related genes regulated by calcineurin under an alkaline pH [42]; transcriptional targets for Crz1 according to Saccharomyces Genome Database (SGD) criteria; and Crz1 genome-wide promoter recruitment under alkaline pH evaluated by Chip-seq (S. Petrezsélyová and J. Ariño, unpublished data). Genes were selected as calcineurin-dependent if classified as such by at least two of the five criteria. ESR genes were defined as in [43,44]. The genes classified as belonging to the RiBi regulon were extracted from [45].

2.4. RT-PCR assays

The samples of MML830 cells were treated for alkaline pH and collected at the same time points as in the GRO experiment. Total RNA was isolated with the Ribo Pure™-Yeast kit (Ambion) following the manufacturer's instructions. RNA quality was assessed by electrophoresis in a denaturing 1.2% agarose FlashGel RNA cassette (Lonza) and quantified by measuring the A_{260} in a BioPhotometer (Eppendorf). Then 60 ng of total RNA was used for semi-quantitative RT-PCR with the Ready-To-Go RT-PCR Beads kit (GE Healthcare) for 25 cycles. The *CTT1* (TCTTGACGAAGTAACAGAAG and CAAAGTATTGCGTAACTCTC), and *ACT1* (TGCTGTCTTCCCATCTATCG and ATTGAGCTTCATCACCAAC) specific pairs of oligonucleotides were used to determine the respective mRNA levels. Products were analysed by agarose (2%) gel electrophoresis.

2.5. Specific-gene mRNA half-life determinations

The MML874 (*tetO$_2$-CTT1*) strain was used in the experimental determination of the mRNA half-life ($t_{1/2}$) of the *tetO$_2$*-controlled genes. Cells were grown in YPD 50 mM TAPS, pH 5.5, until the exponential phase. One sample was taken for mRNA $t_{1/2}$ determination under standard growth conditions by adding doxycycline (final concentration of 5 μg/mL) and collecting cells every 5 min after doxycycline addition for 20 min. In parallel, the other cells were recovered by centrifugation and resuspended in YPD 50 mM TAPS, pH 8.0. For the mRNA $t_{1/2}$ determination at an alkaline pH, doxycycline was added to the samples (5 μg/mL) 10 or 40 min after the shift to pH 8. Once again, aliquots from each sample were taken every 5 min after doxycycline addition for 20 min. Total RNA was prepared as described in the RT-PCR assays section and 1 μg of RNA was used for cDNA synthesis by the iScript™ cDNA Synthesis Kit (Bio-Rad). cDNA was employed as a template in real-time quantitative PCR (qPCR) using SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) and specific primers for *CTT1* and *ACT1* (see above). Data from three biological replicates of the experiment were used to calculate the mean and the SD.

2.6. Accession numbers

The Gene Expression Omnibus (GEO) accession numbers are: GSE58051 for alkaline stress data; GSE24488 for heat-shock, GSE13100 for osmotic stress; and GSE9663/GSE9645 for oxidative stress.

3. Results

3.1. Genome-wide cell response to alkaline pH stress

In this work we studied the response of the yeast cells shifted from an external acidic pH (5.5), which is within the standard pH range for the normal proliferation of budding yeasts, to moderate alkalinisation (pH 8.0). This specific pH shift was selected because it has been well-characterised in our laboratory from both transcriptomic and physiological points of view [9,11,14,42,46]. It triggers a robust response at the mRNA level and, although it results in a transient delay in proliferation, it does not provoke a significant loss of cell viability. Whereas previous work was limited to study the changes of mRNA levels, here we aimed to extend these studies with the help of the GRO technique by interrogating cells for changes in both the TR and in mRNA stability in response to alkaline stress.

The overall transcriptional response to pH 8.0 is presented in Fig. 1A. The growth rate of cells transiently lowered and, as a result, a similar cell density was maintained during the 1-hour experiment. Although it has been shown that many cells transiently stop at the start checkpoint (discussed in [47]) for other stresses, this was not the case in the response to this level of alkaline stress. When the amount of mRNAs/cell was determined and plotted according to the time elapsed after stress, we observed a significant drop in the total mRNA level up to 15 min after the pH shift. mRNA levels did not further lower, but remained steadily at around 70% of the initial value. Overall Pol II TR was also calculated and plotted. As observed, the total TR abruptly lowered during the first 20 min after stress to slowly recover thereafter. However at the end of the experiment, the TR value was still clearly lower than that measured prior to alkalinisation. These results suggest that the overall tendency after high pH stress would be stabilisation of mRNAs. To evaluate this possibility, the overall decay rate constant (k_D) was calculated as previously reported [48]. We assumed steady-state conditions before the pH shift (t0) and non-steady state conditions after the onset of alkaline pH stress. As shown in Fig. 1B, a fast, transient and strong destabilisation of mRNAs occurred shortly after cells were exposed to stress, as indicated by the increase in the relative k_D values. However, this was followed by a more extended period (min 9 to 45, approximately) in which mRNAs were slightly more stable than under standard growth conditions. By the end of the experiment, the k_D value rose again to basal levels. These results are in agreement with an early work which postulated that changes in mRNA stability can be an adaptive mechanism [21,49] to confront a stress situation (see also the Introduction).

3.2. Gene-specific changes in TR and RA observed after alkaline stress

To gain an overall perception of the impact of alkaline stress on both the TR and RA for each specific gene, a set of genes with valid data (defined as having data from at least the 5 RA and 5 TR time points) was generated (5501 genes). In a first approach we selected genes that suffered statistically significant changes and classified them into three groups: those that showed an increase in the TR and/or RA; those that displayed a drop in the TR and/or in RA; and those that gave a biphasic response (they show a significant increase at certain time-points but decrease at others). As observed in Fig. S1, 235 genes underwent an increase in the RA or TR values (229 and 31, respectively). Twenty-five (10.6%) of them showed a simultaneous increase in RA and in TR (p-value $4.9E^{-30}$). The RA or TR values lowered for a large group of genes (2139). In this case the overlap (923 genes) represented 43.2% of all cases (p-value $< E^{-150}$). Remarkably, this percentage became even more dramatic when the genes that displayed a lower RA were examined because it rose to 96%. These results indicate that in most cases, an increase in the mRNA levels in response to high pH stress occurs with no substantial increase in the TR. Thus it is likely to result from the stabilisation of mRNA (see also Fig. 2). Conversely, a reduced RA correlates in most cases with a drop in the TR. This result, however, does not preclude that mRNA destabilisation might play a quantitatively relevant role in reduced mRNA (see later). Finally, 62 genes gave a biphasic response for the TR, whereas 55 showed such behaviour for RA. Only a relatively small percentage, although statistically significant (10 genes, 9.3% of the total number of 107 genes), displayed a biphasic response for both the TR and RA (p-value $2.9E^{-10}$).

In a second approach to analyse comparatively the genes having statistically and biologically relevant changes, the set of 5501 genes with valid data was filtered to recover those genes that underwent a

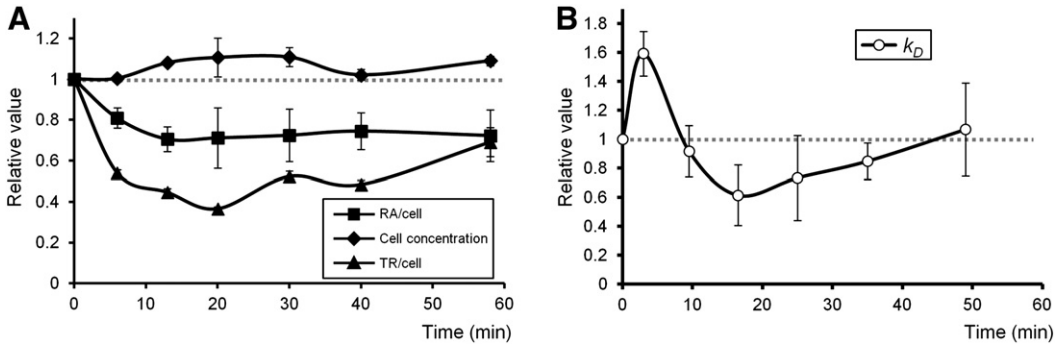


Fig. 1. Effect of alkaline pH stress on cell growth, overall mRNA content, TR and k_D. A) The cells growing exponentially in YPD 50 mM TAPS, pH 5.5, were shifted at time 0 to YPD 50 mM TAPS adjusted to pH 8.0. At the indicated times, aliquots were taken to measure cell concentration (diamonds), total mRNA per cell (RA, squares) and the Pol II-mediated transcription rate per cell (TR, triangles). See "Material and methods" for details. The parameters were referred to their respective time 0 values (defined as the unit). Bars represent the standard error of three independent experiments. B) Changes in the global k_D values in response to alkaline pH stress. The graph represents the average and standard deviation (SD) of three different experiments. Values are relative to the k_D value at time 0.

significant change for the TR (Z-test) for at least one time point (3319). A further filtering step was defined to recover those with at least six time points in which either the TR or the RA changed with a $\log_2$ value > |1.0|. This yielded 1744 genes. This set was subjected to a clustering analysis (presented in Fig. 2), which resulted in 20 major clusters. Clusters 1–6, 16 and 18 were composed mostly of up-regulated genes

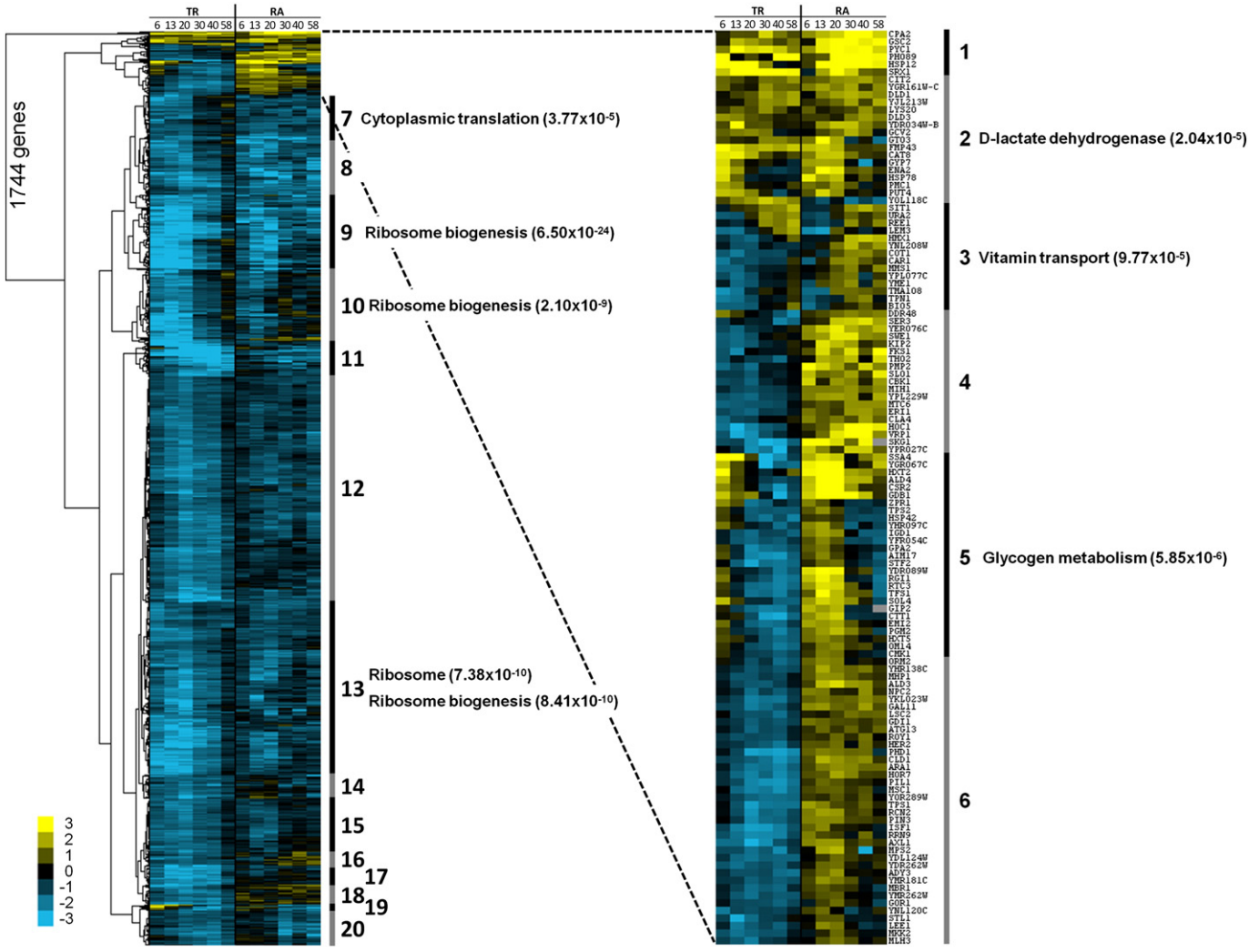


Fig. 2. Clustering of TR and RA data from alkaline pH stress. 1744 genes with statistically significant changes in the TR according to a Z-test and with at least six time points (considering both the TR and RA) showing a $\log_2$ value > |1| were selected and subjected to clustering (complete linkage, Euclidean distance). Shades of yellow denote values above 1 and shades of blue indicate values below −1. Twenty clusters were obtained and the most significant GO categories for each cluster (p value $\leq 10^{-4}$) are shown. Clusters 1 to 6 are expanded in the inset on the right.

(classified according to their RA response). Cluster 19 contained both up-regulated and down-regulated genes. Finally clusters 7–15, 17 and 20 included mostly down-regulated genes. Clusters 1 and 2 contained the genes that showed an increased TR and RA typically throughout the experiment. Cluster 2 was enriched in the lactate dehydrogenase gene ontology (GO) category (Fig. 2, right panel). Clusters 4 and 6 showed increased mRNA, but no increased TR, which suggests post-transcriptional regulation. Cluster 5 contained many genes related to the carbohydrate metabolism (mainly glycogen, but also trehalose metabolism, as well as glucose transport). These genes exhibited a common trait: a highly transient increase in the TR, followed by a delayed increase in RA (Fig. 2, right). In spite of this, and as deduced from Fig. 2 (left), the majority of the response involved lowered TR and RA values (88.9% of the clustered genes), according to the general tendency of the global TR and RA values illustrated in Figs. 1A and S1. Clusters 7, 9, 10 and 13 showed strong enrichment in the genes related to translation and ribosome biogenesis (RiBi). Plotting the TR, RA and k_D values for the RP and RiBi genes (Fig. S2) exhibited a drop in RA, which was the result of a sharp rapid decrease in both TR and mRNA stability (increase in k_D). RiBi genes sharply decreased in the TR and RA more than RP genes, but were affected less by mRNA destabilisation. These groups are analysed in depth in the following sections.

We then examined each cluster presented in Fig. 2 for the specific decay rate to extract possible common patterns. As shown in Fig. 3, most clusters were grouped in three major profiles. Group A included clusters 1, 4–6 and 16 (114 genes, 6.5%), dominated by strong rapid mRNA stabilisation, the opposite behaviour to the general one shown in Fig. 1B. Group B was by far the most numerous (1455 genes, 83.4%) and contained clusters 3, 8–15, 17 and 18. These mRNAs were immediately destabilised, then stabilised, and returned approximately to the initial values by the end of the experiment. Noteworthy, clusters 3 and 18 corresponded to the genes with increased RA (in cluster 3, some simultaneously displayed an increase in the TR), whereas the rest corresponded to the down-regulated ones (see Fig. 2). This scenario reveals that opposite transcriptional responses can share similarly shaped mRNA stability profiles upon alkaline stress. Group C contained clusters that did not share a common pattern (2, 7, 19 and 20). Cluster 7 was enriched in the genes involved in translation. However, its pattern differed from that of the genes in RP and RiBi regulons; in this case, mRNAs were destabilised throughout the experiment (compare with clusters 9, 10 and 13 in Fig. 3B).

We then subjected the set of 1744 genes to a search for known transcriptional regulatory elements using the FIRE algorithm [38]. Clusters 5 and 14 were enriched in STRE motifs (Msn2/Msn4 binding sequences, p-value $4.73E^{-10}$ and $1.80E^{-7}$, respectively). This was not surprising because it has been recently shown that the induction of a significant fraction of genes whose mRNA shortly increased upon high pH stress was dependent on Msn2/Msn4, which migrated to the nucleus in response to the inhibition of PKA caused by alkaline stress [14]. In addition to this PKA-mediated effect, activation of calcineurin phosphatase and the Snf1 kinase is the basis of mRNA accumulation for many genes upon high pH stress [6,9,11]. In order to evaluate the relative input of

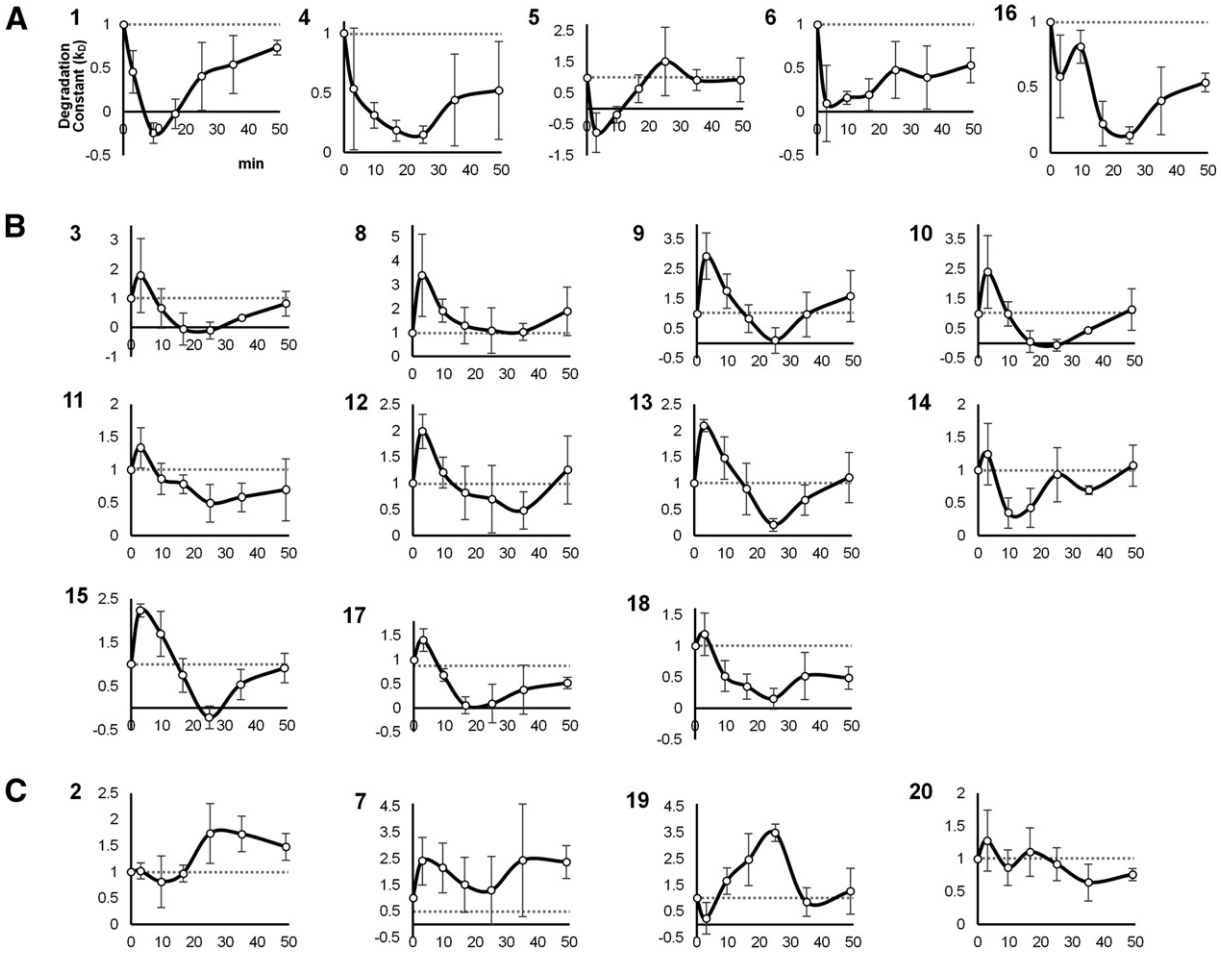


Fig. 3. mRNA stability after alkaline pH stress. The median of the k_D values corresponding to all the genes for each cluster depicted in Fig. 2 was calculated and related to the median k_D value at time 0 (set to the unit). Data are represented as the average ± SD of three experiments. The 20 clusters were manually classified into three groups (panels A to C) according to their general profile. Negative k_D values are due to the mathematical artefacts caused by experimental noise in the TR and RA measurements. Labels for axes are shown only in the first graph for simplicity.

PKA-Msn2/4, calcineurin and Snf1 in the subset of genes that showed increased RA (194), we resorted to previously published transcriptional data sets (see Material and methods). As shown in Supp. Fig. 3, we see that cluster 1 was composed basically of Crz1-dependent genes, sometimes with the additional contribution of Snf1-mediated regulation. Cluster 2 also included the Crz1 and Snf1-regulated genes, together with genes under the regulation of Snf1 or Msn2/4 only. In contrast, clusters 5 and 6 were enriched in Msn2/4-dependent genes, with the additional contribution of Snf1 and, to a lesser extent, of Crz1. Finally in cluster 19, most genes were sensitive to Snf1, either alone or in combination with Msn2/4 and/or Crz1.

3.3. Comparative analysis of transcriptional responses to different stress conditions

Work carried out in the last few years by GRO technology has provided the specific profile of the transcriptional response of yeast cells subjected to diverse stresses for periods of time similar to those used in this work. For instance, Molina-Navarro et al. [24] reported the response of yeast cells to oxidative stress (*t*-butyl hydroperoxide) as a time course of over 60 min. Similarly, the response to heat shock (37 °C) has been investigated for 40 min [28], whereas the impact of mild osmotic shock (0.4 M NaCl) has also been reported [26], although only the first 15 min were documented in this case. Therefore, we decided to integrate existing data with those described in this work in an attempt to extract a comprehensive overview of the yeast response to stress. The effect of osmotic stress on the mRNA synthesis rate and stability has also been investigated by Miller et al. [22] using Dynamic Transcriptome Analysis (DTA) technology, in this case over 30 min after stress (0.8 M NaCl). Unfortunately, the nature of the data generated in this approach does not allow a straightforward comparison to be made and has not, therefore, been included here.

In Fig. 4 we show a comparison of the time-dependent variation of three key parameters, global TR, overall mRNA levels and overall k_D values, determined in yeast cells subjected to high pH, heat-shock, osmotic and oxidative stresses, obtained from the present work and the above-mentioned ones. As deduced from this figure, a drop in mRNA levels was observed in the cells exposed to oxidative stress and, at least for the first few minutes, to osmotic stress. This behaviour is similar to that described above for alkaline stress. In contrast, the cells subjected to heat-shock presented an increased amount of mRNAs during the first 15 min, after which time the mRNA levels were maintained. A comparison of TR profiles for the different stress kinds revealed a striking difference. Whereas heat-shock, osmotic and oxidative stresses presented a common pattern, that is, an almost immediate increase in TR followed by a tendency of returning to the baseline TR values, the cells exposed to high pH underwent, as indicated above, a marked decrease in the TR shortly after stress initiation, followed by a very slow return to the baseline values. The comparison of mRNA stability patterns shortly after exposure to diverse stresses indicated that they had a similar k_D profile: a tendency to lower the stability of mRNAs. In all cases, with the only exception of the oxidative stress response, the k_D values showed a return to the baseline stability levels, or even increased stability (as deduced from the k_D values lower than 1).

In order to find specific strategies in the yeast transcriptional stress response, in addition to the overall comparison described above, we decided to perform genome-wide comparisons for the individual TR and RA gene data. We firstly performed a clustering analysis by integrating the RA and TR data for all four stress types. Only the genes for which at least 80% of the data was available were selected (4009 genes). As shown in Fig. 5A, the heat map shows a strong impact on high pH stress on TR reduction when compared with other stress types. Our clustering analysis grouped the genes into 27 clusters. Each cluster was processed by the FIRE algorithm to search for common signatures. It is worth noting that the genes located in clusters 1, 2, 12 and 25 were significantly enriched in their 5′ regions in the Msn2/Msn4 binding sequences (Table 1). The 3′ regions of genes in cluster 27, which is highly enriched in the genes involved in ribosome biogenesis and ribosomal proteins, showed a predominance of the consensus sequences for Puf4. Puf4 is a member of the PUF protein family, defined by the presence of Pumilio homology domains that confer RNA binding activity [50], and is known to be quite specific for RiBi and RP mRNAs [51].

3.4. Gene expression strategies for ESR genes

The environmental stress response (ESR) has been described by [44] to be composed of two parts: the up-regulated part (about 1/3 of the total), regulated by mainly the Msn2/Msn4 transcription factors, and a down-regulated part, which encompasses mainly ribosomal protein-encoding genes and many genes involved in ribosomal biogenesis and translation. We thought it would be interesting to analyse if both parts of the ESR were controlled only by transcriptional regulation, as

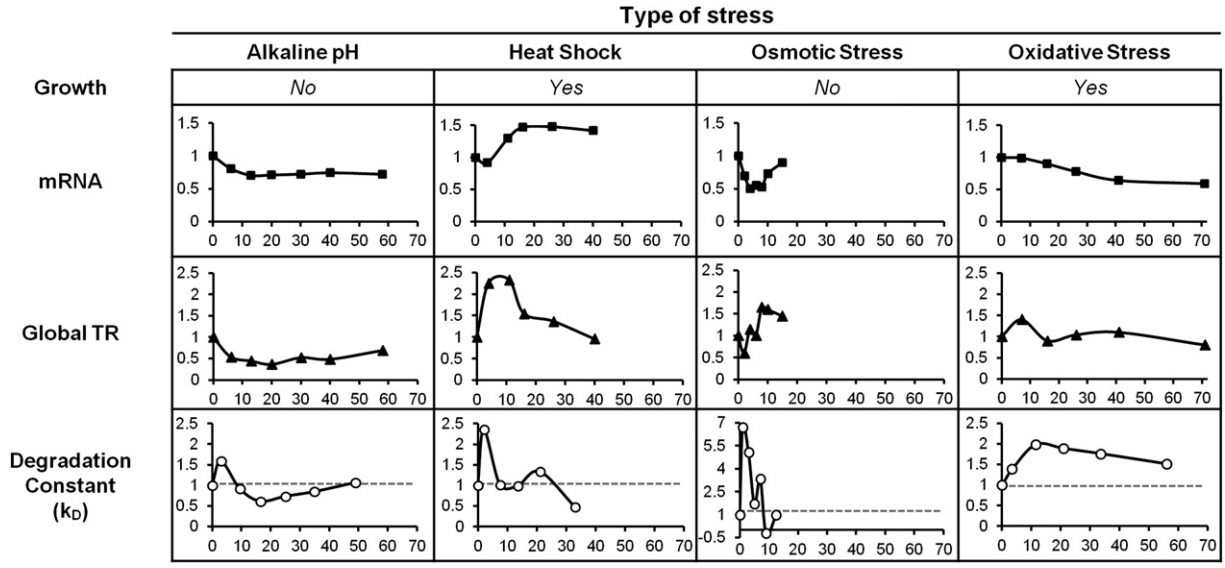


Fig. 4. Comparison of the overall mRNA levels, transcription rates and degradation constants for high pH, heat shock, osmotic and oxidative stress. The time course values of the TR, RA and k_D were extracted from the original reports based on GRO experiments, and represented as in Fig. 1. Oxidative stress data were taken from [24], for osmotic stress from [26], and for heat shock from [28]. The row denoted as "Growth" indicates whether cells proliferated (Yes) or not (No) during the time span of each experiment.

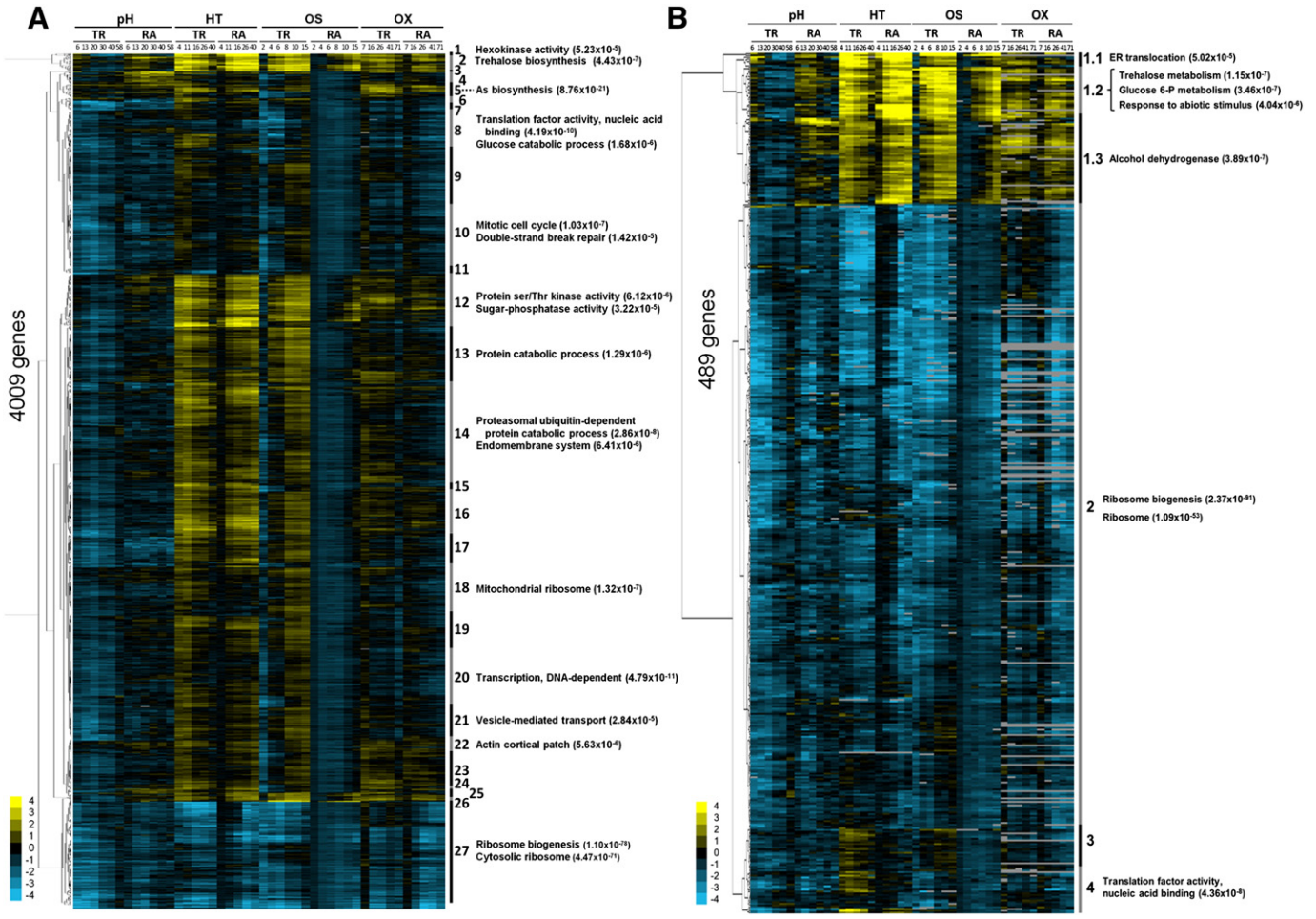
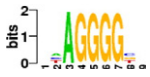
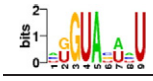


Fig. 5. Clustering of the TR and RA profiles for high pH, heat shock, osmotic and oxidative stress. Panel A. A total of 4009 genes with valid data (TR and RA) for high pH (pH), heat-shock (HT), osmotic (OS) and oxidative (OX), stresses were clustered (complete linkage, Euclidean distance). A GO analysis was performed for the 27 clusters obtained and the most significant GO categories (p value $\leq 10^{-4}$) are shown. Panel B. Four hundred eighty-nine genes, defined as belonging to the ESR class (see the main text for details), were clustered as in Panel A. The most statistically significant GO categories in each cluster are shown.

commonly assumed, or if mRNA stability control could also play a role in the ESR. To this end, from the list of 719 genes described as ESR by Gasch and coworkers [44], we selected those with valid TR data in our analysis whose mRNA level changed by at least 2-fold in at least three of the four stress conditions under study. By doing so, we obtained a final common list of 489 genes (Suppl. Fig. S4). An analysis of individual gene responses for this subset (Fig. 5B) showed a large component (355 genes, 72.6%) of commonly repressed genes (cluster 2) composed mostly of RP and RiBi genes (224 genes). At first sight, and except for the first time point in oxidative stress, an overall TR and RA decrease was observed in this cluster. This could be interpreted as if, for these genes, the changes in RA would be driven by TR. However, when using our previously described method for k_D calculation [48] it was clearly deduced (Fig. S5) that, at short times, mRNA destabilisation greatly contributed to the RA decrease. It can be concluded that the repressive component of the ESR in *S. cerevisiae* results from rapid decreases in both the TR (Fig. 5) and mRNA stability (Fig. S5). Although both parameters exhibited particular kinetics for each stress response, the analysis of k_D in this gene set showed that, for all four stress responses analysed, on the whole, these genes underwent strong and continuous mRNA destabilisation (Fig. S5).

Clusters 3 and 4 contained genes which behaved differentially in osmotic and, especially, heat-shock stresses. Cluster 4 contained part of the genes corresponding to translation activity. This cluster displayed decreased RA at most time points (as expected from previous studies, see [44]). Interestingly it also showed a slight, but evident, increase in the TR (especially after the heat-shock response). Therefore, these genes, which have been previously classified as down-regulated on the basis of the RA profiles, in fact underwent an increase in the TR.

Table 1
Over-represented motifs in clustered genes (Fig. 5A). PAC, polymerase A and C motif; RRPE, rRNA-processing element.

Motif	Name	Position	Over-represented	
			Cluster	*p-Value*
	Msn2,4	5′ DNA	1	7.08×10^{-7}
			2	3.45×10^{-12}
			12	1.55×10^{-9}
			25	3.58×10^{-9}
	Gcn4, Bas1	5′ DNA	5	2.83×10^{-11}
	PAC	5′ DNA	27	2.26×10^{-39}
	RRPE	5′ DNA	27	3.02×10^{-16}
	Puf4	3′ UTR	27	1.92×10^{-11}

Accordingly, and in parallel with RP and RiBi genes (Fig. S5), their k_D profiles (Fig. S6) exhibited strong mRNA destabilisation in heat-shock-, oxidative- and osmotically-stressed cells. An increase in the TR associated with mRNA destabilisation would lead to an apparently futile increase in mRNA turnover with no clear purpose. It is noteworthy that this behaviour is not shared by RP and RiBi genes, which showed no increase in the TR.

Cluster 1 accounts for 17.6% of the analysed ESR genes, corresponds to the inductive part of the ESR (85 genes) and can be divided into three subclusters (Fig. 5B). Cluster 1.1 included the majority of genes, and exhibited a fast transient TR increase after alkaline stress, which extended longer in time than that observed for the other genes induced by this stress (clusters 1.2 and 1.3). In contrast for the rest of the stress responses, the three subclusters showed a marked increase in the TR, followed by RA increase (note that the shorter time span analysed for osmotic stress only allowed the RA increase to be viewed at the end of the time course). As expected, for all the stresses, TR peaks preceded RA peaks [52], but the particular profiles were also influenced by the stabilities of mRNAs [24,26,28,52]. These results suggest that, except for alkaline stress, the inductive ESR is governed mainly by TR changes.

3.5. Relative influences of TR and mRNA stability in the ESR

All previous data have indicated that, in many cases, mRNA stability also influences the level of mRNAs during yeast stress responses. Thus we decided to further examine the relative contributions of TR and k_D changes during the ESR. Of the 489 genes in the data set shown in Fig. 5B, 85 genes were up-regulated and 404 genes were down-regulated in response to all stresses when RA was considered. Each subset was subsequently classified depending on the TR profile (Fig. S4). Fifty-two genes could be classified as being up-regulated according to both the RA and TR profiles, whereas 318 genes could be considered down-regulated in accordance with both the RA and TR values (the TR-ESR genes in Fig. 6). All the other genes that did not fit this classification (Fig. S4) were denoted as non-TR-ESR genes. Therefore, we conclude that during the ESR, approximately 61% of the cases showing mRNA accumulation and 79% of those resulting in an mRNA decrease were likely to be significantly affected at the transcription level. However, the contribution of changes in mRNA stability cannot be discarded.

Therefore, in an attempt to inspect the influence of k_D changes during the ESR, we plotted (Fig. 6) the median k_D for the TR-ESR and non-TR-ESR genes, as defined above. In the up-regulated group (Fig. 6, A–C), the RA plots showed no considerable differences between the two classes of genes, except for the oxidative stress response (Fig. 6A). The differences seen in the TR (Fig. 6B) between TR-ESR and non-TR-ESR were only quantitative and were compensated by differences in k_D (Fig. 6C). Thus it can be concluded that the up-regulated genes, irrespectively of their classification as TR-ESR or non-TR-ESR, did not differ qualitatively, but only quantitatively, in their response. The k_D profiles were particular for each stress type, but shared a common stabilisation phase (between 5 and 20 min) that was preceded and followed by two destabilisation phases. It is important to remark that the ESR up-regulated genes (RA) in the alkaline stress response were in fact down-regulated at the TR level during most of the response (Fig. 6B, see also Fig. 2). So for these genes, the contribution of k_D changes to the actual RA change was crucial.

The RA profiles for the down-regulated genes were more divergent between the TR-ESR and non-TR-ESR groups in all the stresses (Fig. 6D), which were accompanied by large differences in the TR (Fig. 6E) and k_D (Fig. 6F). The most striking case was represented by the heat-shock response, in which the non-TR-ESR genes did not undergo any changes in the TR, whereas the TR of the TR-ESR genes were clearly down-regulated and there was no difference in k_D profiles. Apart from heat-shock, the other stresses showed less variation between the non TR and TR selected classes. Hence for the down-regulated genes, apart from the TR decrease, destabilisation was a predominant trend, as concluded by the fact that most k_D time points were above value 1 (Fig. 6F), although each stress had a particular k_D shape.

3.6. Specific analysis of the genes induced by reactive oxygen species (ROS)

It is known that high pH stress results in ROS generation, which becomes evident after only 30 min of stress, and is shown by a marked increase in the mRNA levels of the genes known to transcriptionally respond to oxidative stress [9]. We found many of these genes among the few hundred that displayed increased RA in our alkaline stress experiment. Although those included in the clustering shown in Fig. 2 did not accumulate in specific clusters, we considered the possibility that these genes could still display some common traits as to their transcription rate and/or mRNA stability patterns. It is known that heat-shock also triggers a situation of oxidative stress [53,54]. We then wondered whether or not these genes would share a common profile when cells are subjected to direct oxidative stress (*t*-butyl hydroperoxide), an alkaline pH or heat-shock stress (which also results in an increased mRNA accumulation for these genes). To investigate this possibility, we selected a group of 28 genes that are known to transcriptionally respond to oxidative stress (Supplementary Table 1). Then we calculated the average TR and RA values, which are plotted in Fig. 7A. As observed, the RA values peaked between 10 and 30 min for high pH and oxidative stress, and then declined almost to the initial levels (this decline cannot be observed for heat-shock stress, perhaps due to the shorter time course). As deduced from Fig. 7A, both oxidative and heat shock stress trigger a fast increase in the TR, followed by mRNA accumulation. In alkaline stress, however, the increase in RA was not paralleled by the increased TR, which implies that mRNA stabilisation would be the main cause of the accumulation of these specific mRNAs. This was reflected in the profile of the calculated k_D (Fig. 7B), which sharply declined during the first 10–20 min to later return to almost the baseline values.

We considered it interesting to experimentally confirm by an alternative method the presumed stabilisation of mRNAs upon high pH stress. To this end we selected *CTT1*, which encodes cytosolic catalase, whose involvement in the oxidative stress response has been known for many years [55]. Fig. 7C shows that the RA and TR profiles for *CTT1* extracted from our high pH GRO experimental data were similar to those of the overall group (Fig. 7A). The mRNA accumulation for *CTT1* after high pH stress (8.0) was followed by semi-quantitative RT-PCR. In this case, a different genetic background (W303-1A) to that employed for the GRO experiments (BY4741) was used. As shown in Fig. 7D, fast mRNA accumulation, followed by a return to initial levels, was observed, which is in keeping with previous data. We then experimentally evaluated the mRNA stability kinetics for *CTT1* in the cells subjected to high pH stress. For this purpose, the original gene promoter was substituted for the doxycycline-regulatable $tetO_2$ promoter. The mRNA half-life was determined in this strain by measuring the amount of mRNA by qRT-PCR prior to exposing cells to pH 8.0 and at two different times after onset of stress (10 and 40 min), which were selected based on the decay kinetics predicted for the gene (Fig. 7C). As deduced from Fig. 7E, the half-life of *CTT1* significantly increased after high pH stress (from 14.1 to 18.0 min), which likely explains the transitory increase in *CTT1* mRNA with no substantial contribution of transcriptional activity. These results confirm the predicted stabilisation of *CTT1* mRNA upon high pH stress and further support the notion that the mRNA accumulation mechanism of the genes defined as being responsive to oxidative stress differs according to the nature of the stress.

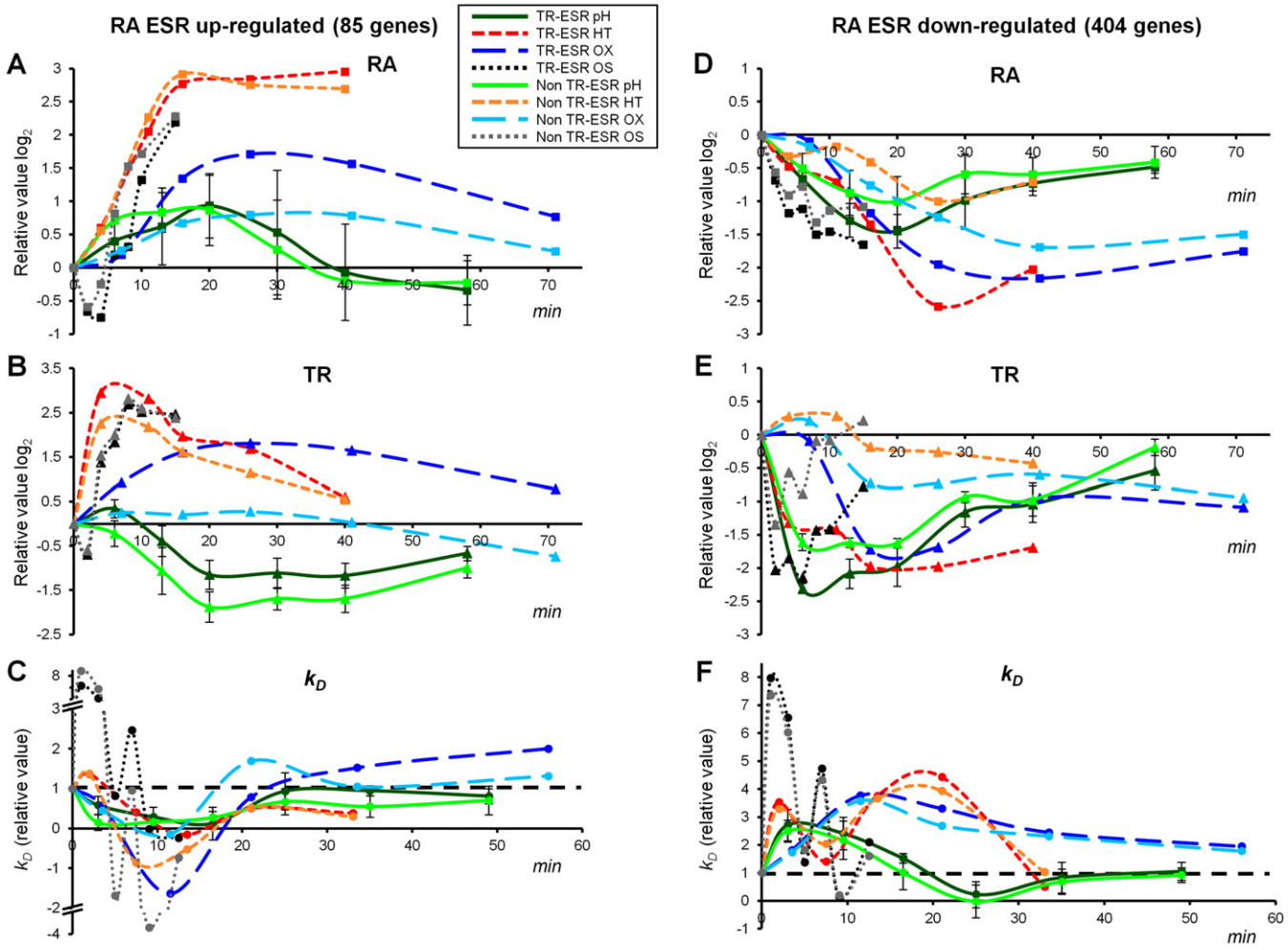


Fig. 6. Relative influences of TR and mRNA stability in the ESR. The genes classified as the ESR were divided into two categories: TR-ESR when the TR values followed the RA pattern and non-TR-ESR when this did not occur (see Fig. S4). The medians during the stress period for RA, TR and k_D for the RA up-regulated (panels A–C) or down-regulated genes (panels D–F) are shown for each stress type (see inset for key symbols). Note that in RA and TR plots the Y axis is in $\log_2$ scale, whereas k_D is shown on a natural scale as in previous figures. The final data (without SD) for stresses OX, OS and HT were taken from references [24,26,28]. See also the explanation for the negative k_D values in the legend of Fig. 3.

4. Discussion

In this report we provide data on the TR and mRNA stability at the genome-wide level in the yeast cells exposed to sudden alkalinisation in their environment. This new data set, together with other previously reported data sets that explore the same transcriptional parameters in response to other stressful situations, such as oxidative stress, heat-shock or osmotic shock, allowed us to carry out the first comprehensive analysis of the respective influences of the transcription and degradation rates during stress responses on eukaryotes. The general comparison of the four stress responses showed that alkaline stress displayed some differential traits when compared with other stresses. During alkaline stress, the global TR immediately lowered, and did not recover until at least 1 h after the shock. This fast effect was reminiscent of the very transient effect provoked by the addition of Na^+ to cells during osmotic stress (at the 2 min time point; see Fig. 4). Interestingly for salt stress, fast destabilisation of protein–DNA complexes has been reported to occur, and it has been assigned to the washing effect of salt on chromatin-bound transcription factors [56]. In both cases, cells underwent changes in an attempt to restore physiological cation concentrations. For mild osmotic stress (0.4 M NaCl), the change was very fast and the initial TR levels were reached only 4 min later. Therefore, it could be hypothesised that H^+ depletion in the yeast nucleus caused by sudden extracellular alkalinisation could similarly trigger destabilisation of protein–DNA complexes to thus lead to a drop in the overall TR. It would also appear that an alkaline pH did not trigger a regular ESR, but a limited version of it. This could be related to the differential effect of this stress on the yeast cell cycle. Although the yeast cells arrested in the cell cycle by α-factor have shown delayed cell cycle entry in an alkaline environment (Clotet, J. personal communication), the asynchronic cultures shifted to pH 8 did not arrest at START, which occurs in most other stresses. O'Duibhir et al. recently showed that an ESR-induced gene expression pattern was strikingly similar to what these authors called a "slow growth signature" [47]. In fact slow-growing cells behave as if they were under mild stress [57]. This signature (and the ESR itself) was due to an increased cell accumulation at the late G1, before the START checking point [47]. So the fact that cells did not accumulate in G1 after alkaline stress would explain their differential expression pattern. We should also consider that, unlike other stress types, such as osmotic and oxidative stress, in which only a few pathways are initially activated, exposure to environmental alkalinisation triggers a fast response, which involves multiple signalling mechanisms, including activation of calcineurin, Snf1, the *PHO*-pathway, the cell wall integrity Slt2-mediated pathway, and so on (see [1] for a review). Alkaline stress has been reported to not trigger an osmotic shock response, but resulted in ROS formation and oxidative stress [9,10]. The complexity of the mechanisms elicited by exposure to high pH stress is likely to be the basis of its characteristic response pattern.

Our comparative analyses of four different stress responses also allowed us to find interesting new clues to the mechanisms that the

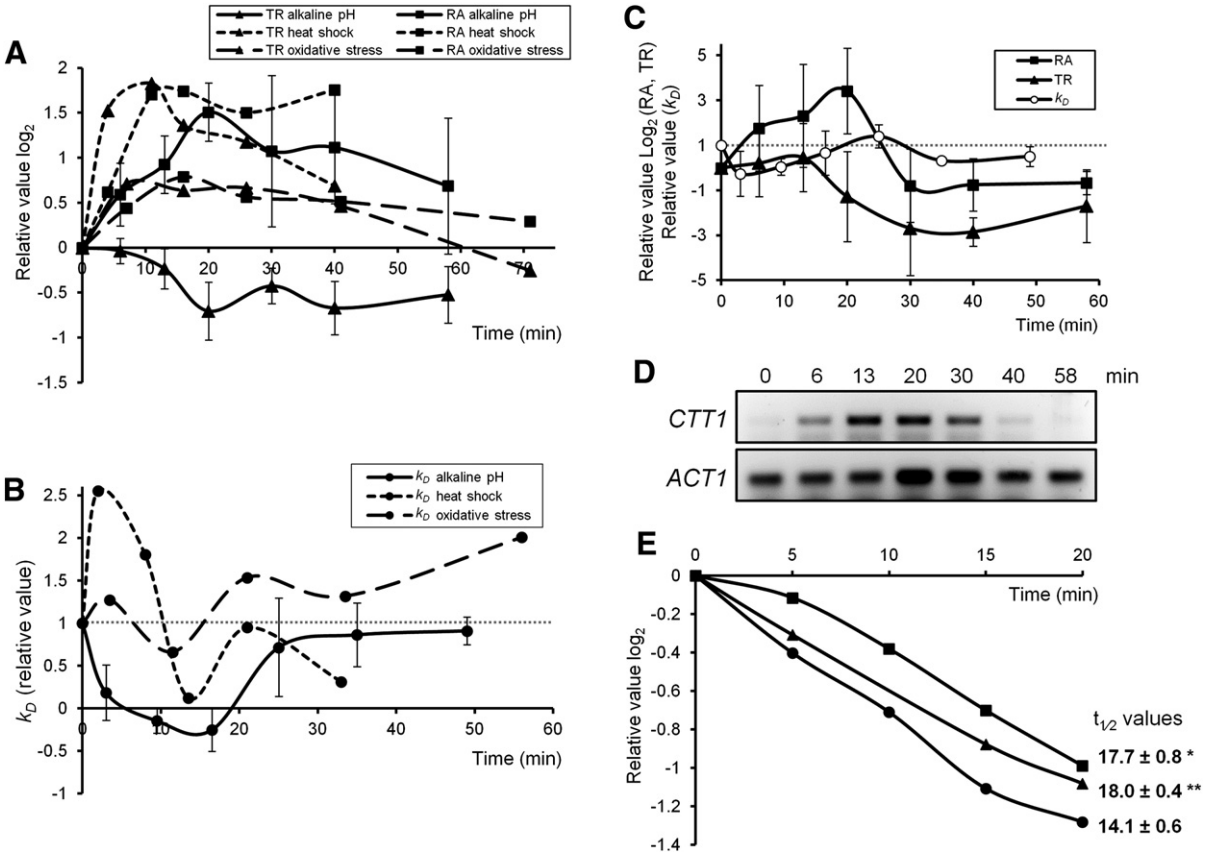


Fig. 7. Analysis of oxidative stress-responsive genes after alkaline pH stress. A) The genes known to be responsive to oxidative stress, whose mRNA was induced upon alkaline stress (this work, see Supplemental Table 1), were selected and their TR and RA data were compared with those previously reported for heat shock [28] and oxidative stress [24]. Log_2 values are used in this graph to allow a better comparison among data sets. B) Representation of the medians of the k_D values corresponding to the set of genes represented in A. The k_D value at time 0 was set as the unit. The SD of three replicates is shown for the alkaline pH data. For the other stresses, see the legend of Fig. 6. C) Plot of the TR, RA, and k_D data for the *CTT1* gene under alkaline stress derived from our GRO experiment. D) A semi-quantitative reverse transcriptase-PCR analysis of *CTT1* mRNA abundance. The MML830 cells treated for alkaline pH, as in the GRO experiment, were collected using the same GRO time points. Sixty nanograms of total RNA, prepared as described in "Material and methods", was used. The *ACT1* gene was included for comparison purposes. E) The exponentially growing cultures of strain MML874 (*tetO2-CTT1*) were prepared, 5 μg/mL doxycycline was added at time 0 (circles), or at 10 (squares) and 40 (triangles) min after the shift to YPD, adjusted at pH 8.0. In each case, aliquots were taken every 5 min after adding doxycycline for 20 min. The RNA was extracted and retrotranscribed and cDNA levels were determined by qPCR (see "Material and methods"). The relative (*CTT1*/*ACT1*) mRNA ratio on the log_2 scale is represented according to time for a representative experiment. The linear regression of the experimental data, including only points for which linearity was maintained, was used for $t_{1/2}$ determination. The mean of $t_{1/2} \pm$ SD for all three independent experiments is indicated. Asterisks denote statistically significant differences, as defined by the Student's *t*-test (* $p < 0.05$, ** $p < 0.01$).

yeast cell uses to cope with alterations provoked by changes in the environment. On the one hand, in general we saw that stress conditions provoked rapid changes in the TR and k_D (Figs. 1 and 4), probably due to a direct effect on the enzymes (e.g. Pol II, decaysome) involved in mRNA metabolism. However, these changes were much less marked in the RA profiles, which reflects the existence of compensatory effects for total RA homeostasis, and reinforce and extend the results obtained along the same lines in non-stressed cells [20,23,58].

On the other hand, using data sets of independently measured individual TR and RA helps to determine the respective contributions of both TR and k_D to the ESR. The general behaviour indicates that although the ESRs were governed by the transcriptional (TR) level, mRNA stability (k_D) also had a strong influence. Most ESR gene profiles showed both decreased and increased mRNA stability during stress responses. Quantitatively, it would seem that an effect of mRNA destabilisation was especially relevant for the down-regulated genes (Figs. 6 and S3, S5 and S6). The most relevant functional categories in this group, the RiBi and RP genes, were particularly regulated at the mRNA stability level. Indeed, these mRNAs have been shown to be regulated as a post-transcriptional regulon [49,51]. Post-transcriptional regulons, consisting of RNA-binding proteins (RBPs) and their target genes, are essential for the coordination of gene expression [49,59]. In the particular case of the RiBi and RP genes, the RBP is the Puf4 protein [49,51], whose consensus sequences appear to be highly enriched at the 3′-UTR of these genes (Table 1). Therefore, it would be reasonable to propose that Puf4p is the effector of the mRNA destabilisation for the RP and RiBi genes during the ESR. In fact, based on computational studies of several transcriptomic data sets, Foat et al. [49] already suggested that Puf4 is a de-stabiliser and is activated by the stress caused by transcriptional arrest. Our results confirmed that the down-regulated part of the ESR was, in a large portion, driven by the destabilisation of cytosolic ribosome-related mRNAs. Traditionally, RP mRNAs have been considered relatively unstable [60]. However, mRNA stability has been measured by methods that stop transcription, such as thiolutin addition or resorting to an *rpb1-1* thermosensitive allele. Both methods cause stress to yeast cells [61]. Recently developed alternative methods for mRNA half-life determination [21–23], which do not provoke stress, found that RP mRNAs had much higher stabilities than previously believed. Our results demonstrate herein that all stresses in yeast cells provoke sudden destabilisation in RP mRNAs (Fig. S5) and, to a lesser extent, in RiBi (Fig. S5) and other down-regulated mRNAs (Fig. 6). This likely explains the apparent instability of RP mRNAs when stressful methods are used to measure mRNA stability.

Albeit apparently contradictory, mRNA destabilisation also occurred transiently in the ESR up-regulated genes (Figs. 6 and 7), which fits and generalises the mRNA destabilisation reported for some up-regulated genes (discussed in [20] and in the Introduction). The ESR-up-regulated genes showed complex patterns for mRNA stability.

For instance, osmotic stress, previously considered to provoke initial stabilisation at 5–6 min [26,27], in fact caused sudden destabilisation before 5 min (Fig. 6C), which also seemed to occur in other stresses (Fig. 6C). Then a stabilisation phase took place between 5 and 20 min in all stresses (Fig. 6C). To our knowledge, no sequence-specific RBP protein has been described to regulate mRNA stability in this set of genes. However, it is also possible that mRNA stability in these genes could be regulated in other ways during stress responses. For instance, it has been proposed that the transcription from certain promoters can imprint mRNAs under stress conditions [62] in such a way that its stability in the cytoplasm is determined in the nucleus during transcription [25]. An RBP complex without RNA sequence specificity can be recruited in a promoter-dependent manner and remains associated as an RNA–protein complex after RNA export from the nucleus, which regulates its decay in the cytoplasm [20]. In fact it has been demonstrated that a given promoter determines not only the stability of the mRNA it transcribes [63], but also promoter sequences influence the subcellular localisation of mRNAs and the efficiency with which they are translated [64]. So it is possible that the changes which occur in the promoter of the ESR-regulated genes during post-stress transcription induction also imprint their mRNAs, which provokes changes in their cytoplasmic stability, and perhaps in their translatability. More work is necessary to clarify how the cross-talk between mRNA stability and transcription regulatory mechanisms occurs during stress responses.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2015.04.001.

Funding sources

This work has been supported by the Spanish Ministry of Science and Innovation and the Fondo Europeo de Desarrollo Regional [BFU2011-30197-C3-01, EUI2009-04147 (SysMo2) to J.A. and BFU2010-21975-C03-01 to J.E.P-O]; the Generalitat Valenciana [PROMETEO 2011/088, ACOMP 2014/253 to J.E.P-O]; the Generalitat de Catalunya [2014SGR-4, ICREA Academia 2009 Award to J.A.] and the Spanish Ministry of Education (AP-2009-2824) [predoctoral fellowship to D.C.].

Acknowledgements

We thank Montserrat Robledo for her skilful technical support and E. Herrero (Universitat de Lleida) for strains.

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