Global Gene Expression Profiling Reveals Widespread yet Distinctive Translational Responses to Different Eukaryotic Translation Initiation Factor 2B-Targeting Stress Pathways†

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Global inhibition of protein synthesis is a hallmark of many cellular stress conditions. Even though specific mRNAs defy this (e.g., yeast *GCN4* and mammalian *ATF4*), the extent and variation of such resistance remain uncertain. In this study, we have identified yeast mRNAs that are translationally maintained following either amino acid depletion or fusel alcohol addition. Both stresses inhibit eukaryotic translation initiation factor 2B, but via different mechanisms. Using microarray analysis of polysome and monosome mRNA pools, we demonstrate that these stress conditions elicit widespread yet distinct translational reprogramming, identifying a fundamental role for translational control in the adaptation to environmental stress. These studies also highlight the complex interplay that exists between different stages in the gene expression pathway to allow specific preordained programs of proteome remodeling. For example, many ribosome biogenesis genes are coregulated at the transcriptional and translational levels following amino acid starvation. The transcriptional regulation of these genes has recently been connected to the regulation of cellular proliferation, and on the basis of our results, the translational control of these mRNAs should be factored into this equation.

The flow of genetic information into the proteome is regulated at many steps in a coordinated and highly organized manner. In response to stress, the gene expression program in a host of organisms is globally inhibited posttranscriptionally via effects on the rate of protein synthesis (3, 4, 9, 25, 41, 54, 55). This reduction in protein synthesis potentially serves to allow the turnover of existing pools of mRNA and protein while inducing translation of specific mRNAs (such as *ATF4* in mammalian cells and *GCN4* in yeast) via specialized control mechanisms (21, 23, 24, 38, 58).

Amino acid starvation of yeast activates a particularly well-defined pathway of translational regulation (Fig. 1A) (23). Depletion of amino acids leads to an accumulation of non-amino-acylated tRNA, which activates the Gcn2p protein kinase. As a result, Gcn2p phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2). eIF2 is a guanine nucleotide binding factor and in the GTP-bound form interacts with the initiator methionyl tRNA (Met-tRNA_i^{Met}) to form a ternary complex (eIF2-GTP-Met-tRNA_i^{Met}) that is competent for translational initiation. Following mRNA start codon recognition, the eIF2 bound GTP becomes hydrolyzed to GDP. A guanine nucleotide exchange factor, eIF2B, is responsible for

recycling eIF2-GDP to the translationally competent form eIF2-GTP. Starvation for amino acids and the subsequent phosphorylation of eIF2 α converts eIF2 from a substrate to an inhibitor of this guanine nucleotide exchange factor eIF2B (45). The resulting decrease in eIF2B activity generates reduced eIF2-GTP and ultimately leads to less ternary complex. Thus, the level of ternary complex within the cell is delicately poised, facilitating the rapid inhibition of global translation initiation. Paradoxically, translation of the *GCN4* mRNA is activated in response to low ternary complex in a mechanism involving short upstream open reading frames. Gcn4p is a transcription factor that activates many amino acid biosynthetic genes. Thus, activation of this transcription factor serves to overcome the imposed starvation, which initially led to the translational control (23).

Under conditions of nitrogen starvation, fusel alcohols accumulate as end product metabolites of amino acid breakdown pathways in yeast (12). The addition of fusel alcohols to yeast leads to a dramatic inhibition of translation initiation (Fig. 1A). This regulation targets eIF2B independently of eIF2 α phosphorylation; therefore, like amino acid starvation, it still leads to decreased ternary-complex levels and activation of Gcn4p (4). Therefore, both amino acid starvation and fusel alcohols target eIF2B, leading to a dramatic global inhibition of translation initiation and a concomitant activation of *GCN4* mRNA translation. However, it is unclear whether the translation of all mRNAs is targeted equally or whether individual mRNAs important for physiological adaptation to each stress are maintained in a translationally active state.

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FIG. 1. Two stresses that target eIF2B to control translation initiation. (A) Diagram representing the amino acid starvation and butanol pathways of translational control in yeast. (B) Overview of the experimental strategy used. (C) Polyribosome profiles (A_{254}) for fractionated control (C) and stressed (S) yeast cultures. 40S (small ribosomal subunit), 60S (large ribosomal subunit), 80S (monosome), and polysome peaks are labeled. The ratio of the area under the polysomal (P) to monosomal (M) peaks is shown (P:M), and it indicates that 10-min 1% (vol/vol) butanol treatment causes dramatic inhibition of translation. Removal of essential amino acids causes a similar, but slower, response (10-, 15-, 20-, and 27-min time points are shown). Pooled fractions containing monosomal (MC and MS) and polysomal (PC and PS) mRNAs used for microarray analysis are diagrammed beneath the control, +butanol (10-min), and -amino acids (20-min) traces.

The use of expression-profiling techniques, such as microarray analyses, have enabled detailed quantitative comparisons of the levels of all cellular mRNAs in many organisms (26). More recently, this technology has been extended to analyze protein synthesis (2, 33, 48). In this study, we have adapted this technology for use with Affymetrix microarrays to analyze the mRNAs that are translationally maintained in response to the eIF2B-targeting stresses, amino acid starvation and fusel alcohol addition. In addition to transcriptional control, our results implicate translational control as a key component of the adaptive responses to both stresses. Critically, however, the translationally regulated mRNAs for each stress are different yet functionally appropriate in terms of adaptation to each stress. Therefore, the regulation of protein synthesis complements and enhances transcriptional regulation to allow the widespread reorganization of the gene expression program.

MATERIALS AND METHODS

Yeast growth conditions. The yeast strain yMK36 (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 GCD-S180*) (4) was grown at 30°C in a synthetic minimal medium with 2% (wt/vol) glucose, lacking nonessential but containing essential amino acids. For all experiments, mid-log-phase yeast (optical density at 600 nm, 0.7) was used. For severe amino acid starvation, cells were pelleted and resuspended in prewarmed synthetic medium lacking all amino acids for various times, and the 20-min time point was selected for the microarray analysis, whereas butanol was added at 1% (vol/vol) in liquid culture for 10 min.

Polysomal analysis and RNA preparation. Cell extracts were prepared essentially as described previously (3). Briefly, after incubation with cycloheximide (100 μ g/ml at 4°C), cells were pelleted and washed twice in 50 ml of ice-cold lysis

buffer (3). The cell pellets were resuspended in 500 μ l of lysis buffer and rapidly frozen in liquid nitrogen. Lysates were prepared by grinding the cell pellets under liquid nitrogen. The ground yeast was thawed on ice and cleared by successive centrifugation steps (5,000 $\times$ g for 5 min at 4°C in a clinical centrifuge, and then 16,000 $\times$ g for 30 min at 4°C in an Eppendorf microcentrifuge). Sixty A_{260} units were layered onto 35-ml 15 to 50% sucrose gradients. The gradients were sedimented via centrifugation at 16,900 rpm for 13 h in a Beckman ultracentrifuge. The gradients were collected as described previously (3) and fractionated into 15 2.2-ml aliquots. These were collected directly into 2 volumes of Trizol (Invitrogen) and mixed, and 1 volume of chloroform was added with glycogen (50 to 150 mg/ml) and sodium acetate (0.1 M; pH 4.5). After centrifugation, the aqueous phase was collected and 0.7 volume of isopropanol was added. The RNA precipitate was pelleted, washed in 80% ethanol, and resuspended in water. Equal quantities of RNA were analyzed by Northern blot analysis. For microarrays, the RNA samples were diluted, precipitated again by the addition of an equal volume of LiCl buffer (4 M LiCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA). The RNA pellet was washed twice with 80% ethanol and resuspended in water. The RNA quality was assessed using 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) (50). For comparison of polysomal RNA levels, fractions 4 to 8 and 11 to 15 from the gradient were pooled for monosomal and polysomal RNA samples, respectively (Fig. 1C). As a control, all 15 fractions from a second gradient were pooled. Total RNA samples were prepared according to standard protocols (<http://www.cogeme.man.ac.uk/Facilities/TRF%20Protocols.htm>).

Affymetrix gene chip expression microarray analysis. Microarray experiments were performed using a yeast S98 chip oligonucleotide array (Affymetrix, Inc.) according to the manufacturer's instructions (<http://www.affymetrix.com/support/technical/manuals.affx>). Approximately 10 μ g of polysomal, monosomal, or total RNA was processed into biotinylated cRNA according to Affymetrix protocols; 15- μ g biotinylated cRNA targets were fragmented and hybridized to the arrays at 45°C for 16 h. The arrays were then processed using Affymetrix fluidics protocol EukGE-WS2 (V4 450) and stained with *R*-phycoerythrin conjugated to streptavidin (Molecular Probes, Inc). Microarray images were acquired using the 2500 GeneChip scanner (Affymetrix) and Microarray Suite version 5.0 software. Robust multichip average normalization (5, 27, 28) and further analysis were

carried out using the Affymetrix library of procedures (Affy version 1.5.8) in Bioconductor (version 1.5) (<http://www.bioconductor.org>) within R (version 2.0.1) (<http://www.r-project.com>; 16). The datasets are publicly available at ArrayExpress (accession numbers EMEX-323 [butanol data] and E-MEX-324 [amino acid data]).

The normalized datasets were processed by calculating the simple $\log_2$ intensity ratios comparing polysome to monosome values across control and stressed samples (or the translation states). A second ratio of these values gives the change in translation state for an individual mRNA following stress. To facilitate comparison to the Northern analysis, the linear-scale normalized intensity values were used to calculate the appropriate polysome-to-monomosome ratios (see Fig. 4).

Northern analysis and PCR. Northern blots were performed as described previously (3). Essentially, 4 μ g of RNA from each of the pooled monosome or polysome fractions was separated on a 1% agarose-formaldehyde gel and transferred onto Hybond-XL (Amersham) membrane in $10\times$ SSC (0.3 M sodium citrate, 3 M sodium chloride). RNA was cross-linked to the membrane using a UV cross-linker (Stratagene). Specific probes were PCR amplified from yeast genomic DNA (primer sequences are available upon request). The PCR products were radiolabeled using the Megaprime DNA-labeling system (Amersham). Hybridization and washing were performed according to standard protocols. Images were acquired using a Typhoon 8600 Scanner (Amersham).

RESULTS

Amino acid starvation and butanol stress both lead to a rapid redistribution of ribosomes in polysome gradients, so that an accumulation of inactive 80S monosomes is observed in response to stress (Fig. 1C). This redistribution of ribosomes from polysomes to monosomes is a well-characterized consequence of the inhibition of translation initiation (22) and precisely correlates with the effects of stress on the rate of amino acid incorporation into protein (4, 55). Both the translational inhibition and polysome runoff observed under these stress conditions are not seen in specific mutants that are incapable of regulating translation initiation via eIF2B (3, 4, 25). Therefore, the global effects of these two stresses on translation initiation are entirely dependent upon the regulation of protein synthesis via eIF2B-dependent pathways.

A key question is whether translation of specific mRNAs is maintained following such stress responses to facilitate cellular adaptation. To investigate this, we made use of a strategy whereby the ratio of every mRNA in polysome (P) and monosome (M) fractions was compared between stressed and control yeast cells. A key preliminary aspect of this analysis was the identification of precise stress conditions that fulfill a number of predetermined criteria. First, the stress period should be relatively short to limit indirect effects. Second, the stress periods used should have an identical impact upon translation initiation. Therefore, as depicted in Fig. 1C, using the response to 1% butanol for 10 min, we examined the kinetics of the amino acid starvation response and as a result selected the 20-min starvation time point as being most similar to the butanol stress condition. Therefore, the polysome-to-monomosome ratios for the selected stress conditions were 0.36 for butanol and 0.34 for amino acid starvation (Fig. 1C). As the stress conditions employed have identical impacts upon polysome runoff and the polysome runoff is entirely dependent upon eIF2B control mechanisms, the impact of each stress on the *in vivo* activity of eIF2B was therefore anticipated to be very similar.

Polysome gradients from the selected stress condition were separated into fractions, and monosomal (MS or MC) or polysomal (PS or PC) fractions were pooled. In addition, total

FIG. 2. Representative MA plots from the amino acid data set. (A to I) MA plots, each comparing two different microarray datasets (array¹ versus array²) from this analysis. *M* is plotted on the *y* axis and is given by $\log_2[\text{array}^1 \text{ intensity} / \text{array}^2 \text{ intensity}]$. Therefore, *M* equals 0 when the intensities across two arrays are equal. *A* is plotted on the *x* axis and is given by $0.5(\log_2[\text{array}^1 \text{ intensity}] + \log_2[\text{array}^2 \text{ intensity}])$. This is the average of the intensities across the two arrays. For the biological replicates (e.g., PC1 versus PC2, MC1 versus MC2, MS1 versus MS2, PS1 versus PS2, or TC1 versus TC2), the *M* values cluster tightly around 0, whereas for the control-versus-stress comparisons (e.g., PC1 versus PS1, MC1 versus MS1, or TC1 versus TS1) or for the polysome-versus-monomosome sample comparisons (e.g., MS1 versus PS1), the *M* value is more dispersed. The σ^2 value for each data set is depicted in the top left corner of the plots and gives a standard statistical measure of the variance in *M*.

RNA samples (TS or TC) were prepared from stressed and control yeast to generate standard transcript level changes. The resulting RNA samples were processed into cRNA and hybridized to Affymetrix microarrays (Fig. 1B). The analysis was performed in duplicate, and the data were processed and compared using the bioinformatics analyses described in Materials and Methods.

Standard MA plots show that the biological replicate samples for the pooled polysome and monosome fractions generate very modest dispersal (Fig. 2A to D). However, when the data set from stressed samples was compared with the control or polysomal RNA was compared to monosomal RNA, a substantial spread of the datasets was observed (Fig. 2E to G). The spread across MA plots observed between polysome-monomosome replicates and nonreplicates (Fig. 2A to G) is similar to the difference observed using more standard total-RNA samples (Fig. 2H and I). These results are representative of a wider set of MA plots (data not shown) that demonstrate that the procedure we have developed is highly reproducible and fulfils

FIG. 3. Translational control is both widespread and divergent. (A and B) Graphical representations of the translational microarray data for butanol addition and amino acid starvation, respectively. The polysome-to-monomosome ratio for the stress conditions ($\log_2[\text{PS}/\text{MS}]$) has been plotted against the polysome-to-monomosome ratio for the control conditions ($\log_2[\text{PC}/\text{MC}]$) for every mRNA. Those mRNA data points falling above or below a 0.9 cutoff have been colored red and blue, respectively. (C and D) The same plots as in panels A and B, only the translationally regulated mRNAs (up-regulated in red and down-regulated in blue) for amino acid starvation have been highlighted on the butanol plot (C), and vice versa (D).

all of the standard criteria generally applicable to microarray data (6).

Intensity values from the polysomal and monosomal samples were used to generate $\log_2$ polysome-to-monomosome ratios for each probe set on the microarray. This ratio was designated the “translational state” of an individual mRNA. These values were then plotted on a scatter plot for stressed (y axis) and unstressed (x axis) cells (Fig. 3A and B), where the intersection generates the “change in translational state” for the mRNAs, or the second ratio (PS/MS to PC/MC). The Affymetrix microarray system requires that equal amounts of cRNA be used for each RNA hybridization. Even though as a consequence of this the polysomal-to-monomosomal ratios become similar for stressed and unstressed samples for most mRNAs when global translation has been inhibited by the stress, the relative values of the second ratio will not be affected. This facilitates the identification of those mRNAs where translation is greater or less than the global inhibited trend.

Surprisingly, the two stresses give very different translational profiles (Fig. 3A and B). For instance, where a 0.9 (exponential scale) or 1.9 (linear scale) cutoff value in the change in translation state was used, 615 mRNAs (342 up; 273 down) moved within the polysome gradient following amino acid starvation

(Fig. 3B), whereas after fusel alcohol addition, only 167 mRNAs (97 up; 70 down) changed their profiles across the gradient (Fig. 3A). The cutoff used generates a manageable number of regulated mRNAs; however, in reality, this value may be quite conservative, given that following amino acid starvation, the classically translationally regulated mRNA *GCN4* lies below this value (even though the position of this mRNA within the gradient is altered after both stresses) (Fig. 4A and B). In order to assess whether a similar set of mRNAs moved within the polysome gradients following each stress, we highlighted those mRNAs that changed following amino acid starvation on the butanol stress plot (Fig. 3C), and conversely, on the amino acid starvation plot, we highlighted the butanol stress-regulated mRNAs (Fig. 3D). These plots demonstrate that those mRNAs that remain translated following the two stresses are different; indeed, only 20 mRNAs are up-regulated and 13 mRNAs down-regulated across both stresses. Overall, it is clear that the translational response as a whole is much more widespread than previously anticipated, and individual stress conditions regulate the translation of distinct pools of mRNA.

We confirmed these microarray results using standard Northern blotting techniques for a diverse set of mRNAs in terms of both the overall abundance of the mRNA and the

FIG. 4. The microarray analysis is confirmed by Northern blotting. (a) Northern blot data for the *GCN4*, *GLC7*, *PGK1*, *PUT4*, *CLG1*, and *DBP2* mRNAs across polysomal (P) and monosomal (M) fractions from butanol-stressed (S) and control (C) yeast. For comparison, the control and stressed polysome-to-monomosome ratios ([PC/MC] and [PS/MS]) as measured from the Northern blots and the microarrays are depicted on the right as histograms. (b) As for panel a, except the mRNAs *GCN4*, *GLC7*, *PGK1*, and *PUT4* were assessed following amino acid starvation.

change in abundance across polysomal gradients. Figure 4A and B show that there is a close correlation between the microarray data (using the linear-scale polysome-to-monomosome ratios for control and stress samples) and the quantitated Northern blots. In particular, the *CLG1*, *DBP2*, *GCN4*, and *GLC7* mRNAs for the butanol stress and the *PUT4*, *GCN4*, and *GLC7* mRNAs for amino acid starvation show increased abundance in polysomal fractions following stress. In contrast, for both stresses, the *PGK1* mRNA shows little change in polysomal distribution for either the microarray or Northern blot analysis.

In order to assess whether the response to these stresses is coordinated in terms of transcript level and translational activity, we plotted the change in translation state against the change in total transcript level following each stress for every mRNA (Fig. 5A and B). mRNAs that are translationally up-regulated and also change at the transcript level and those mRNAs that are translationally down-regulated and also change at the transcript level are depicted, along with other translationally regulated mRNAs (Fig. 5A and B, red, blue, and yellow points, respectively). Figure 5A shows that the overlap between changes in transcript level and the translational response to butanol stress is very minimal. Conversely, following amino acid starvation, a significant number of mRNAs are coregulated (Fig. 5B). For example, of the 598 genes for which the transcript level increases following amino acid starvation, 130 also increase translationally, whereas only

15 decrease translationally. Similarly, of the 672 genes for which the transcript level decreases, 128 also decrease translationally, whereas only 9 increase translationally. This coregulation of transcript level and translation has been noted previously following heat shock and rapamycin treatment of yeast and has been termed "potentiation" (48). The fact that we do not observe such coregulation for butanol stress suggests that this phenomenon is more specific than previously anticipated.

As well as allowing a comparison of the translational and transcriptional impacts of different stresses, these results were analyzed in terms of the precise function of the regulated genes and the likely impact the changes in translation state or mRNA level would have on cellular physiology. Using a combination of the MIPS (42), SGD (29), and YPD (7) databases, we defined 15 functional categories and placed those genes that are regulated at either the translation or transcript level relative to the global trend into these categories (Fig. 6; see Tables S1 to S8 in the supplemental material).

Fusel alcohols, such as butanol, affect protein synthesis (4), membrane transport (34), the cell wall, mitochondria (40), and cell morphology as a signal for nitrogen limitation (12, 32, 36). Figure 6A and Tables S1 and S2 in the supplemental material show that there is a remarkable correlation between the physiological impact of butanol on yeast cells and the individual mRNAs whose translation is regulated as a response to this stress. Genes involved in the cell cycle, protein synthesis, cellular transport, and nitrogen metabolism are clearly overrep-

FIG. 5. Functional comparison of alterations in transcript level and translation in response to stresses. (A and B) Graphical plots comparing transcript level ($\log_2[\text{TS}/\text{TC}]$) with the change in translation state ($\log_2[\text{PS}/\text{MS}] - \log_2[\text{PC}/\text{MC}]$) after (A) addition of butanol and (B) starvation for amino acids. Cutoff values of 1.0 and 0.9 for the change in transcript level and translation state, respectively, are depicted as dashed lines. Translationally regulated mRNAs that are not transcriptionally regulated are colored yellow. Translationally up-regulated mRNAs that are also regulated at the transcript level are colored red, whereas translationally down-regulated mRNAs that are also regulated at the transcript level are colored blue. (C) The same plot as in panel B, except that the coordinately down-regulated mRNAs at both the transcript and translation levels from the ribosome biogenesis/translation functional class have been colored green and the coordinately up-regulated mRNAs from the carbon metabolism/energy functional class have been colored purple.

resented. For example, there are a number of translationally up-regulated RNA helicases (*DED1*, *DBP2*, and *DBP9*), which could form part of an adaptational response to the global inhibition of protein synthesis brought about by butanol. Indeed *DED1* in particular has been shown to suppress eIF4E mutants, and *ded1* mutations cause defects in translation initiation (10). In addition, in the up-regulated cell cycle class of mRNAs, *CLN3* is particularly interesting, as it has been demonstrated that this mRNA is translationally regulated (47) and increased *CLN3* overcomes the cell cycle block in specific eIF4E mutants (8). Genes such as *TOK1*, *PMP2*, *PMP1*, and *PMA1* that are involved in the cellular transport of potassium ions and protons across the plasma membrane are also translationally up-regulated in response to butanol. It is particularly striking that both the *PMA1* plasma membrane ATPase gene and the *PMP1*-*PMP2* proteolipid genes, which are associated with and required for Pma1p activity (44), are coregulated at the translational level following butanol addition. Following butanol addition to yeast, there is a rapid increase in proton leakage across the plasma membrane (data not shown). Therefore, a translational up-regulation of the *PMA1* proton pump gene and the associated *PMP1* and *PMP2* genes could ultimately form part of an adaptive response to combat this butanol-dependent increase in proton leakage. Butanol also causes the translational regulation of genes involved in amino acid and alcohol metabolism. For instance, a number of amino acid, ammonia, or purine/pyrimidine permeases, as well as other enzymes and transcription factors involved in amino acid/alcohol metabolism, are translationally regulated in response to butanol (see Tables S1 and S2 in the supplemental material). The regulation of these genes seems likely to form part of a metabolic reconfiguration, based on the perception that amino acids are being catabolized to fusel alcohols. This highlights both the realignment of the metabolic profile of yeast as a response to alcohols and the central theme that these alcohols serve as signaling molecules communicating the metabolic consequences of amino acid catabolism to cells. Of particular note is the translationally down-regulated *YNL274c* mRNA, which encodes a potential α -ketoisocaproate reductase and therefore may be involved in one possible pathway of fusel alcohol production (13). Translational control of this enzyme could therefore serve as part of a negative feedback loop to ultimately decrease the level of fusel alcohols.

Amino acid starvation generates a significant translational state change for 615 mRNAs. It is clear that several processes are coregulated at the translational level in response to this stress. The most prominent example of this is the translational down-regulation of genes involved in ribosome biogenesis (Fig. 6C; see Table S4 in the supplemental material). This translational control of ribosomal-subunit biogenesis is coordinated with a decrease in transcript level for the ribosomal proteins, factors involved in ribosome biogenesis, and translation factors (Fig. 6D; see Table S8 in the supplemental material). Similar decreases in the mRNA abundance for this class of gene have been noted in response to many stresses and may be due to effects on transcription or mRNA stability (Fig. 6B and D) (18, 35, 46). The translational regulation of this same functional class of genes (Fig. 6C) demonstrates the coordinated manner in which gene expression is controlled in response to stress.

Intriguingly, over this very short period of amino acid star-

FIG. 6. A functional classification of the regulated mRNAs reveals rational yet distinct responses to stress. Shown is a summary of the functional classification in Tables S1 to S8 in the supplemental material.

vation, no significant increase in transcript level was observed for genes involved in amino acid biosynthesis (see Table S7 in the supplemental material). This reflects other studies, where Gcn4p has been shown to gradually accumulate over several hours of amino acid starvation (via either 3-AT or amino acid removal) (1, 25). Therefore it seems that the induction of the amino acid biosynthetic genes occurs gradually over a more prolonged amino acid starvation regime as a consequence of this translational induction of the Gcn4p transcription factor (39, 43). In contrast, several amino acid permease genes (*PUT4*, *HNM1*, *CAN1*, *BAP2*, *DIP5*, and *AVT6*), other nitrogenous compound permeases (*FCY2* and *MEP3*), and genes that regulate amino acid permeases (*NPR2*, *GLN3*, and *AS11*), as well as plasma membrane protease genes (*YPS1*, *YPS5*, *YPS7*, and *MKC7*) and other genes involved in protein degradation (*STE13*, *YBR139w*, *RPN14*, *UFD1*, *CDC34*, *UBC6*, *UBC8*, and *UBC9*), are translationally activated (see Table S3 in the supplemental material). This translational induction of permeases, proteases, and protein degradation pathways could form part of an early amino acid scavenging response to starvation. In addition, many genes involved in carbohydrate metabolism and mitochondrial function are translationally up-regulated following amino acid starvation (Fig. 6C; see Table S3 in the supplemental material). Overall, it seems that glucose uptake, metabolism to and from storage carbohydrates (glycogen/trehalose), diversion to the pentose phosphate pathway, and the tricarboxylic acid cycle are translationally up-regulated. This suggests that there is a reorganization of the carbohydrate storage capacity and a metabolic preparation for the subsequent increase in amino acid biosynthesis by the accumulation of appropriate carbon skeletons as starting material. Overall, these data point to an initial phase of the amino acid starvation response, where amino acid levels are maintained by enhanced protein salvaging and intermediary metabolite accumulation. At later times, based on previous transcriptional profiling experiments, it is clear that these early responses persist and are augmented by the well-characterized Gcn4p-dependent activation of amino acid biosynthesis (43).

An analysis of functional categories for the coregulated or

potentiated mRNAs following amino acid starvation (Fig. 5C; see Tables S3 and S4 in the supplemental material) shows that, surprisingly, there is a quite specific overlap in these responses. For example, 34 genes involved in carbohydrate metabolism are up-regulated (Fig. 5C), whereas 67 genes involved in ribosome biogenesis are down-regulated (Fig. 5C) in terms of both transcript level and translation. This coregulation of specific functional classes of genes explains a large proportion of the "potentiation" observed for amino acid starvation and suggests a higher level of organization for the control of gene expression than previously anticipated.

DISCUSSION

The results in this study show that following cellular exposure to stress, there is a rapid, concerted switch in both transcript level and translation to generate a coordinated change in the cellular proteome. This creates highly specific stress responses in which cellular energy is conserved, the metabolic profile of the cell is reorganized, and cellular adaptation is facilitated. The number of mRNAs that are translationally regulated in response to stress was previously considered minimal. However, this study suggests that as many mRNAs are differentially regulated at the level of translation as at the level of transcription. In addition, this translational regulation is a highly refined process, so that even though the stresses we have studied impact upon the same translation initiation factor, eIF2B, they have quite different outcomes in terms of the specific mRNAs that are translationally controlled.

In the yeast *Saccharomyces cerevisiae* there is just one eIF2 α kinase, Gcn2p, whereas in mammalian cells, four kinases perform the same function, i.e., regulate protein synthesis via inhibition of eIF2B. These four eIF2 α kinases—GCN2 (the amino acid control kinase), PKR (the double-stranded RNA-activated protein kinase), HRI (the heme-regulated inhibitor), and PERK/PEK (the PKR-like endoplasmic reticulum eIF2 α kinase)—are regulated independently in response to a host of different cellular stresses (49). In addition, eIF2B is subject to more direct regulation in mammalian cells in response to a

variety of signaling inputs. Specific growth factors, hormones, and nutrients (e.g., amino acids) have all been shown to impact upon the level of eIF2B activity in various cell types (49). Overall, therefore, eIF2B serves as a focus for mechanisms that relay information about the cellular environment and status to the protein synthetic machinery.

Studies using transgenic mice in which specific eIF2 α kinase genes are “knocked out” or in which the site of eIF2 α phosphorylation is mutated by a targeted “knock-in” strategy (all of which prevent the inhibition of eIF2B in response to stress) have identified a host of phenotypes associated with specific kinase and “knock-in” mutants (11, 19, 20, 37, 52, 59, 61). In addition, the inherited neurological disorder leukoencephalopathy with vanishing white matter, or childhood ataxia with diffuse central nervous system hypomyelination, is associated with mutations in the genes encoding the eIF2B subunits and hence is explained by reduced eIF2B function (53). This disease has highly variable clinical symptoms so that in addition to the neurological symptoms (of varying severity and age of onset), ovarian failure, pancreatitis, hyperplasia of the kidneys, cataracts, hepatosplenomegaly, and fetal abnormalities have also been identified (15, 57). This plethora of conditions and phenotypes associated with both unregulated and down-regulated eIF2B activity has been explained by the hypothesis that additional environmental factors and cell-specific factors contribute to the overall proteomic outcome (56, 60).

In this study, we showed that eIF2B can be down-regulated by two different mechanisms to generate highly variable translational outcomes. These data have important implications for the study of eIF2B regulation in mammalian cells. The differential regulation of specific mRNAs in different cells or in response to different stresses/stimuli may contribute to the pleiotropic symptoms and phenotypes associated with the eIF2B mutations or control mechanisms described above.

An obvious focus for future experimentation will be an investigation of how different stresses can target the same general translation factor to generate different proteomic outputs. Possible explanations for this are that there are distinct pools of eIF2B and regulation of one pool versus another could therefore influence the mRNAs that are translated. Alternatively, although these stresses both act via eIF2B, there could be other modulatory inputs on the translation initiation pathway that ultimately change which mRNAs are either translationally selected or disregarded following the global inhibition of translation by stress. For instance, one possibility is that mRNA binding proteins might be involved in this differential selection of mRNAs. Indeed, a recent study has shown that the Puf family of RNA binding proteins bind to distinct pools of mRNA and therefore are likely to impact upon their translation, transport, and stability (17). However, the specific Puf binding pools of mRNA show little overlap with the data sets generated here (data not shown). Therefore, it seems unlikely that the Puf RNA binding proteins contribute to the differential translational regulation that we observed, but the concept that specific RNA binding proteins are involved is an avenue for future research.

In this study, we also observed the previously described phenomenon of “potentiation” (48). This explains the coregulation of transcriptional and translational controls of gene expression in response to rapamycin and heat shock treatments.

The “potentiation” phenomenon seems likely to be more specific than previously anticipated, in that this effect is evident following amino acid starvation but not following butanol addition. We also find that potentiation can be largely explained by the coregulation of translation and transcription for specific functional classes of mRNA following amino acid starvation, i.e., the ribosome biogenesis/protein synthesis functional category is down-regulated, whereas carbohydrate metabolism is up-regulated, in terms of both translation and transcript level.

Transcriptional coregulation of genes involved in ribosome biogenesis has been highlighted recently and shown to require the Sfp1p transcription factor (14, 30, 31). It has also been suggested that this regulation is critically linked to cellular proliferation and that ribosome synthesis may serve as a measure for cell cycle progression (31, 51). On the basis of this, a set of 236 transcriptionally coregulated genes have been defined and termed the *Ribi* regulon (31). In our data set, 179 of these mRNAs are down-regulated at the transcript level following amino acid starvation (data not shown). Moreover, we have analyzed translational regulation, and approximately 20% of the *Ribi* regulon is also down-regulated at the translational level following amino acid starvation. Therefore, the transcriptional regulation of the *Ribi* regulon makes up a significant part of the transcriptional down-regulation we see in the ribosomal-subunit biogenesis functional category. In addition, there is significant overlap between our translationally down-regulated ribosomal-subunit biogenesis functional data set and the *Ribi* regulon defined previously. This has important implications for the control of cellular proliferation, as a correlation between transcriptional regulation of ribosome biogenesis genes and cell size control has recently been highlighted (31, 51). The addition of translational control for these mRNAs to this equation would potentially reduce the response time of cells to stress and allow them to rapidly coordinate it with the cell cycle.

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