Phosphorylation of Eukaryotic Translation Initiation Factor 2α Coordinates rRNA Transcription and Translation Inhibition during Endoplasmic Reticulum Stress

Jenny B. DuRose,1 Donalyn Scheuner,3 Randal J. Kaufman,3 Lawrence I. Rothblum,2 and Maho Niwa1*

Division of Biological Sciences, Section of Molecular Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0377;2 Department of Cell Biology, College of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104;3 and Department of Biological Chemistry, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Michigan 48109

Received 26 February 2009/Returned for modification 1 April 2009/Accepted 14 May 2009

All living organisms in their lifetimes are subject to fluctuations in their environments. The evolution of systems to sense and respond to stressful conditions allows the organism to quickly counter the action of the stress, minimizing potential damage or possible death. A common strategy among stress responses is modulation of gene expression programs at all stages, including protein synthesis, to facilitate a return to homeostasis. Currently, two major mechanisms have been described for regulation of protein production rates: one via regulation of the mammalian target of rapamycin (mTOR) pathway and the other by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α).

In response to growth signals and nutrients, mTOR signaling is responsible for coordinately regulating global translation initiation and ribosome biogenesis. mTOR modulates the rate of translation by phosphorylation of the eIF4E binding protein (4E-BP) (3, 8). In addition, mTOR activity modulates ribosome biogenesis by altering both synthesis of ribosomal proteins (7, 12, 47) and rRNA gene transcription by RNA polymerase I (Pol I) (26, 33, 41).

Regulation by mTOR can be thought of as a dial that can tune the efficiency of translation initiation and rRNA transcription up or down in order to balance the demand for resources to sustain cellular functions with the need for cell growth. While the mTOR pathway balances growth signals with nutrient availability, cells must also adjust the rate of protein synthesis in response to other challenges and stressful conditions.

The other major pathway that regulates translation is through phosphorylation of eIF2α and can be thought of as an emergency brake rather than a dial for modulating protein synthesis. The importance of this pathway is demonstrated by the evolution of at least four distinct eIF2α kinases in mammalian cells, each responding to a different set of stress conditions (4, 10, 28, 43, 50, 54). Under normal conditions eIF2α kinases are inactive, and upon stress stimulation they phosphorylate eIF2α, preventing recycling of the eIF2 complex and thus inhibiting the formation of the 43S translation initiation complex (11, 13, 19, 52). This allows cells to rapidly downregulate protein synthesis during stress even under conditions where growth signaling and nutrient availability are not immediately limiting, for example, during the unfolded protein response (UPR).

The UPR pathway monitors protein folding in the endoplasmic reticulum (ER). The synthesis, folding, and modification of proteins targeted to membranes or the secretory pathway takes place within the ER. When the protein-folding capacity of the ER is perturbed by environmental insult or when protein-folding demands are increased during developmental changes, unfolded proteins accumulate within the ER, resulting in activation of the UPR pathway. In mammals, there are three sensor molecules spanning the ER membrane that are

* Corresponding author. Mailing address: Division of Biological Sciences, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093. Phone: (858) 822-3451. Fax: (858) 822-0317. E-mail: niwa@ucsd.edu.
† Supplemental material for this article may be found at http://mbc.asm.org/.
‡ Published ahead of print on 26 May 2009.
responsible for initiating the UPR pathway: ATF6, IRE1, and PERK (PKR-like endoplasmic reticulum kinase). Each initiates ER stress through its luminal domain and transduces the signal across the ER membrane to the cytosol. All three UPR branches cooperate to increase production of ER chaperones and facilitate clearance of unfolded proteins from the organelle. However, PERK, the UPR-specific eIF2α kinase, has an additional function: to repress global translation initiation, thereby reducing the influx of nascent proteins into the stressed ER (27, 28, 50). In contrast to nutrient deprivation, where mTOR coordinately translates translation and ribosome biogenesis, translation repression through eIF2α phosphorylation during ER stress has not been reported to be associated with alteration in ribosomal concentration, although a recent study suggests that ribosomal components may alter their association with cellular proteins during ER stress (57a).

However, similar to nutrient deprivation, the continued investment in ribosome biogenesis during ER stress may also impose an unnecessary drain on cellular resources while translation is inhibited.

In this report, we have demonstrated that downregulation of rRNA gene transcription takes place immediately after the onset of ER stress. We have shown that RNA transcription repression occurs simultaneously with (rather than as a consequence of) translation repression. We have found that eIF2α phosphorylation by PERK is necessary for RNA transcription repression and that PERK activation results in dissociation of Pol I and RRN3/TIF-IA, a major Pol I transcription initiation factor, from the RNA promoter. Our data suggest that in addition to mTOR, there may be a second major pathway induced by eIF2α phosphorylation that coordinately regulates translation and ribosome biogenesis, ensuring proper adjustment of cellular resources during stress.

MATERIALS AND METHODS

Cell culture and treatment. All cells were cultured in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum (Cellgro) and 1% penicillin/streptomycin (Invitrogen) except for HEK293 cells (kindly provided by Dr. S. Vanier) which were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated in 10% CO2 at 37°C. Culture medium was changed every 2–3 days.

To test if eIF2α phosphorylation prevents translation, we used the yeast two-hybrid system to overexpress PERK in yeast. Yeast cells were transformed with a vector containing PERK and a vector containing a DNA sequence coding for α-factor (control) or a DNA sequence coding for α-factor combined with PERK. The yeast cells were grown in liquid medium containing 2% raffinose to induce α-factor expression. Then, cells were centrifuged and resuspended in 2% galactose/2% raffinose medium. After 3 h, total RNA was extracted and analyzed on 4% polyacrylamide gels containing 7 M urea.

Northern blotting. Total RNA isolated from whole cells or nuclei was analyzed on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. Membranes were probed with antibodies against PERK, ERK, α-tubulin, or Histone H3. Membranes were washed and re-probed with antibodies against GAPDH, COXIV, or Histone H3. Membranes were exposed to phosphor screens.

Detection and quantification. Chemiluminescence of Western blots, ethidium bromide staining of agarose gels, and phosphor screens were visualized with a Typhoon 9400 imager (GE Healthcare). Bands were quantified with ImageQuant, version 5.2, software (GE Healthcare).

RESULTS

Activation of UPR inhibits Pol I transcription. To test if changes in rRNA levels accompany UPR-induced translation repression, we measured their levels in RNA isolated from nuclei of NIH 3T3 cells upon UPR induction. We reasoned that this would give us a better estimate of nascent rRNA as it...
eliminates the majority of stable steady-state rRNA in the cytoplasm. We isolated nuclear RNA from an equal number of cells upon treatment without or with Tg, an inhibitor of the ER calcium ATPase that perturbs ER protein folding and induces the UPR. We found a striking decrease in the amount of RNA isolated from Tg-treated nuclei compared to untreated nuclei (Fig. 1A). When the nuclear RNA was run on a Northern gel and stained with ethidium, the 18S and 28S rRNAs decreased within the first hour of Tg treatment and was barely visible after 3 h (Fig. 1B, lanes 5 to 7). When we probed Northern blots from the same gel for 7SL RNA, an RNA Pol III transcript that is unchanged during the UPR, we found that its level remained constant, suggesting that not all transcripts are decreased in nuclear RNA extracts. In addition, quantitative PCR analysis of BiP mRNA revealed that the level of this UPR target gene was increased, as expected, in nuclear RNA from Tg-treated cells compared to untreated cells while the level of β-Actin mRNA did not significantly change during either treatment (unpublished data). Together, these results indicate that activation of the UPR specifically decreased the level of rRNA in the nucleus, which likely occurred by either decreasing its synthesis or promoting its degradation.

To determine whether the UPR pathway regulates rRNA synthesis, we measured the level of newly synthesized rRNA in total RNA isolated from MEFs during a UPR time course. Here, we chose to follow the disappearance of the rRNA primary transcript (pre-rRNA) to further substantiate our findings. Pre-rRNA is synthesized as a long 47S RNA which undergoes a number of cleavages and modifications before assembly into ribosomes (Fig. 1C). The nascent rRNA undergoes processing which begins with an endonucleolytic cleavage in the 5′ external transcribed spacer at the +650-nucleotide (nt) position (44). This processing of the 5′ external transcribed spacer occurs so rapidly that the vast majority of precursor rRNA in the nucleolus has already been cleaved; thus, the level of uncleaved pre-rRNA closely approximates that of...
the most newly synthesized rRNA (44). We designed a probe for an RPA that encompasses this initial cleavage site such that the uncleaved pre-rRNA generates a protected fragment that is 232 nt, whereas the possible postcleavage protected fragments are half the size and migrate much faster during gel electrophoresis (see Fig. S1A in the supplemental material). We found that nascent pre-rRNA decreased rapidly within the first hour of Tg treatment and decreased by nearly threefold after a 2-h Tg treatment relative to 7SL RNA (Fig. 1D, lanes 3 to 5, and E). The result of this RPA was confirmed by Northern blotting for full-length pre-rRNA (see Fig. S2 in the supplemental material) and was linear over a fourfold range of input RNA (see Fig. S1B in the supplemental material). The extent of UPR induction was measured by an RPA probe detecting both the spliced and unspliced forms of Xbp1 mRNA (see Fig S1A in the supplemental material). The unconventional splicing of the UPR transcription factor Xbp1 is mediated by the ER-resident transmembrane UPR component Ire1 (14, 45, 51, 56, 57). Splicing of the UPR intron results in a frameshift that is crucial for the formation of a fully active XBP1 transcription factor and upregulation of UPR target genes (57). Xbp1 splicing is a hallmark of UPR activation and was rapidly induced by Tg treatment (Fig. 1D, lanes 3 to 5). The decrease of pre-rRNA occurred with similar kinetics to the appearance of the spliced form of Xbp1 mRNA, suggesting that downregulation of rRNA occurs at an early point upon UPR induction (Fig. 1D, lanes 3 to 5).

The observed decrease of pre-rRNA also occurred when MEFs were treated with agents that induce UPR by a different mechanism, such as Tm, which perturbs ER protein folding by inhibiting glycosylation rather than releasing ER calcium (Fig. 1D, lanes 6 to 8, and E). The extent of rRNA downregulation during UPR was significant as it was comparable to treatment of MEFs with Ans, a ribotoxic drug that is a well-characterized inhibitor of rRNA transcription (Fig. 1D, lanes 9 to 11, and E). Ans treatment does not induce UPR, as indicated by the lack of Xbp1 splicing compared to untreated cells (Fig. 1D, compare lanes 1 and 2 with 9 to 11). These data suggest that loss of 28S and 18S processed forms of rRNA in nuclear RNA pools (Fig. 1B) was likely the result of a decrease in pre-rRNA available for processing in the nucleus. Furthermore, after examining the levels of the most nascent rRNA, we reasoned that the decrease in pre-rRNA observed in Fig. 1D was likely caused by a reduction of transcription.

In order to further investigate the effect of UPR on rRNA gene transcription, we performed a nuclear run-on assay to measure the transcriptional activity of the rRNA genes. In this assay, engaged RNA PolS continue transcription of nascent RNAs in nuclei isolated from UPR-induced cells in the presence of radiolabeled UTP. The level of labeled RNA was measured by hybridization to DNA fragments complementary to rRNA, the UPR target gene BiP, and 7SL RNA (Fig. 1F). Nuclei isolated from untreated cells generated significant levels of rRNA, consistent with robust transcriptional activity of RNA Pol I. In contrast, the level of radiolabeled rRNA generated from nuclei isolated from cells treated with Tg for 1 h was significantly reduced, correlating with the reduction of pre-rRNA detected by RPA (Fig. 1E and G). Production of 7SL RNA by Pol III in the run-on assay was not reduced during the UPR. In addition, we did not detect a significant decrease in transcription of a number of additional housekeeping genes by Pol II and Pol III during Tg treatment, suggesting that Pol I is specifically regulated during the UPR (see Fig. S3 in the supplemental material).

During the UPR, the decrease in rRNA transcription was accompanied by an increase in BiP transcription. BiP is an ER-resident chaperone and is a well-characterized transcriptional target of the UPR. Typically, an increase in BiP mRNA becomes detectable by Northern gels at a minimum of 2 h after Tg treatment (unpublished data). Thus, the ability to detect an increase in BiP mRNA and a decrease in pre-rRNA within 30 min of Tg treatment demonstrated the sensitivity of our assay. Taken together, these results suggest that activation of the UPR pathway leads to a decrease in rRNA transcription. Since most transcriptional changes associated with UPR reported to date have been those which increase, our observation that rRNA transcription decreases would be one of the few exceptions. Furthermore, the abundance of rRNA relative to all other transcripts (80 to 90%) suggests that a reduction in rRNA transcription leading to a nearly threefold decrease in pre-rRNA may have a significant impact on the production of ribosomes and, hence, the protein synthesis capacity of the cell.

The PERK signaling branch regulates rRNA synthesis during UPR. To investigate how the ER transduces the signal to downregulate rRNA transcription in the nucleolus, we analyzed the level of pre-rRNA in Ire1 and Perk knockout MEFs upon UPR treatment. First we followed the level of pre-rRNA in wild-type and Ire1−/− MEFs upon Tg or Tm treatment and found that rRNA was downregulated equally well in both wild-type and knockout MEFs (Fig. 2A and B). While Ire1−/− MEFs were unable to splice Xbp1 mRNA, activation of UPR was evident from the increase in the level of unspliced Xbp1 transcript itself (Fig. 2A, lanes 7 to 12), presumably mediated by activation of the ATF6 branch of the UPR (57). These data suggest that the Ire1 signaling pathway is not required for rRNA downregulation during the UPR.

We then tested the involvement of the PERK pathway in regulating rRNA transcription. Upon treatment of wild-type MEFs (Perk−/−) with Tg, we found that the level of pre-rRNA decreased nearly threefold within 2 h and remained repressed over the following 12 h (Fig. 2C, lanes 4 to 8, and D). In contrast, the level of pre-rRNA remained unchanged in Perk−/− MEFs treated with Tg for the first 4 h (Fig. 2C, lanes 12 to 14, and E). Ultimately, after 12 h of Tg treatment, the level of pre-rRNA was reduced to the same extent as in wild-type MEFs treated for 1 to 2 h (Fig. 2C, compare lane 16 with lane 5). The decrease in pre-rRNA at later times was not due to a loss in viability, as >95% of all cells excluded the vital dye, trypan blue, over the entire 12-h time course (unpublished data). These results suggest that PERK is the major regulator of rRNA transcription during the first 4 h of UPR activation, resulting in a very rapid decrease in rRNA transcription that is sustained over time. Interestingly, the PERK-independent decrease of pre-rRNA in Perk−/− MEFs correlated with an increase in eIF2α phosphorylation between 5 and 12 h (see Fig. S4A in the supplemental material). The phosphorylation of eIF2α that occurs late in the UPR time course is presumably mediated by another eIF2α kinase, GCN2 (25).

Simultaneous downregulation of translation and pre-rRNA is independent of mTOR pathway. UPR activation resulted in
a rapid repression of translation through PERK-mediated eIF2α phosphorylation; thus, it is possible that reduced synthesis of components necessary for ribosome biogenesis are feeding back on rRNA transcription. We therefore measured the kinetics of pre-rRNA decrease and translation inhibition upon treatment with UPR inducers Tg and Tm. To measure global translation, we briefly pulsed cells with [35S] methionine-cysteine in order to label nascent proteins at the times indicated in the figures. In this assay, the level of 35S incorporation in whole-cell extracts is used as a measure of global protein synthesis. Based on our previous kinetic studies of UPR activation, we know that PERK is activated within 15 min of Tg treatment and 60 min of Tm treatment (18). We found that the level of cellular translation was decreased rapidly as incorporation of [35S]methionine-cysteine decreased by 20% within 15 min of Tg treatment and by 50% within 60 min of Tm treatment (Fig. 3). For each sample, we also examined pre-rRNA levels by RPA and found that the reduction in pre-rRNA occurred at a similar rate as translation inhibition (Fig. 3A, lanes 2 and 9). In some samples, the decrease in pre-rRNA was detected prior to the decrease in 35S incorporation. Thus, the similarity of the kinetic decrease of both pre-rRNA and translation is consistent with the idea that rRNA transcriptional repression is a primary response to UPR induction rather than the result of reduced translation of necessary components.

Coordinate regulation of rRNA transcription and cellular translation has been reported to take place via the mTOR signaling pathway. Inhibition of mTOR activity by Rap downregulates protein synthesis by inhibiting phosphorylation on ribosomal protein S6 kinase (7, 12, 47), and 4E-BP (3, 8). The mTOR signaling pathway also affects rRNA transcription initiation by a mechanism involving S6K (26, 41). To test for possible involvement of the mTOR pathway in UPR-induced rRNA transcriptional repression, we followed the phosphorylation of ribosomal protein S6 by S6K. Rap treatment resulted in a mild decrease in cellular translation (45%) while the levels of phospho-S6 and total S6 were significantly decreased, as expected (Fig. 3A, Rap). In contrast, we found that phosphorylation of S6 was unchanged upon Tg and Tm treatment. In addition, we found no change in the phosphorylation of 4E-BP upon Tg or Tm treatment (unpublished data), suggesting that activity of mTOR and its downstream substrates 4E-BP and S6K are unchanged during UPR. Together, these results suggest that the canonical substrates of the mTOR pathway are not involved in downregulating rRNA transcription during the
UPR and that there may be a novel signaling pathway from the ER to the nucleolus.

**Phosphorylation of eIF2α is necessary for rRNA downregulation.** Since mTOR is likely not involved, what is the mechanism of rRNA transcription repression during ER stress? The most-well-characterized substrate of the PERK kinase is eIF2α, which functions as a regulatory subunit of the eIF2 complex. In order to address whether eIF2α phosphorylation is involved in rRNA regulation during the UPR, we measured pre-rRNA levels in MEFs carrying a homozygous serine-to-alanine mutation at the conserved eIF2α phosphorylation site, serine 51 (eIF2α<sup>S51</sup>). It has been shown that upon UPR induction, PERK is activated in eIF2α<sup>S51</sup> cells; however, translation attenuation does not occur (48). We found that both wild-type (eIF2α<sup>S51</sup>) and eIF2α<sup>S51</sup> MEFs induced a UPR, as indicated by Xbp1 splicing during Tg or Tm treatment (Fig. 4A). In eIF2α<sup>S51</sup> MEFs, Tg or Tm treatment resulted in a rapid downregulation of pre-rRNA in coordination with translation inhibition (Fig. 4A, lanes 1 to 3 and 7 to 9, and B). In contrast, we found that eIF2α<sup>A/A</sup> MEFs displayed impaired translation attenuation, as expected, and also failed to downregulate pre-rRNA in response to ER stress (Fig. 4A, lanes 4 to 6 and 10 to 12, and B). Upon treatment with the rRNA transcription inhibitor ANS, the level of pre-rRNA decreased regardless of eIF2α phosphorylation, indicating that the lack of rRNA regulation in eIF2α<sup>A/A</sup> MEFs during Tg and Tm treatment is UPR specific (Fig. 4A, lanes 13 to 18, and B). In contrast to Perk knockouts, eIF2α<sup>A/A</sup> MEFs failed to downregulate rRNA over the entire 12-h Tg time course (Fig. 2C, Perk<sup>−/−</sup>; see also Fig. S4B, eIF2α<sup>A/A</sup>, in the supplemental material). These results indicate that phosphorylation of eIF2α is necessary for both the early and late phases of pre-rRNA downregulation during ER stress. It has been shown that the PERK-independent phosphorylation of eIF2α that occurs late after UPR induction is mediated by GCN2 (25), suggesting that perhaps multiple eIF2α kinases can regulate rRNA transcription. We found that stresses that are known to regulate rRNA synthesis through the mTOR pathway do not require eIF2α phosphorylation, as...
Rap treatment was still capable of downregulating pre-rRNA in eIF2α/A/A MEFs (see Fig. S4C, eIF2α/A/A, in the supplemental material). Together, these results imply that eIF2α phosphorylation-induced translation repression and rRNA transcription inhibition are intimately coregulated during ER stress, suggesting a unique regulatory mechanism that is likely independent of mTOR.

**PERK is required for disruption of the rRNA preinitiation complex.** As a translation initiation factor, the vast majority of eIF2α is located in the cytoplasm while the rRNA transcriptional machinery is in the nucleolus. So what is the mechanism of eIF2α phosphorylation-dependent rRNA transcription inhibition? To explore how eIF2α phosphorylation downregulates rRNA transcription, we monitored the formation of the preinitiation complex at the rRNA promoter. The major proteins involved in rRNA transcription initiation identified to date are the DNA-binding protein UBF and selectivity factor (SL1) and the non-DNA binding protein RRN3/TIF-IA (Fig. 5A). In order to measure promoter occupancy of preinitiation complex components, we employed a ChIP assay. In this assay Perk⁺/⁺ or Perk⁻/⁻ MEFs were treated with Tg for the times indicated in the figure, and cross-linked chromatin was immunoprecipitated with antibodies against the large subunit of Pol I, RRN3/TIF-IA, and UBF. We found that association of promoter DNA with both Pol I and RRN3/TIF-IA was decreased upon treatment with ANS, indicating that our assay is functioning as expected (Fig. 5B and C). Upon Tg treatment of wild-type MEFs, we found a decrease in the association of promoter DNA with both Pol I and RRN3/TIF-IA, suggesting

---

**FIG. 4.** eIF2α phosphorylation is necessary for rRNA downregulation. (A) Time course of eIF2α⁺/⁺ and eIF2α⁺/⁻ MEFs treated with Tg (200 nM), Tm (10 μg/ml), or ANS (10 μM). Total cellular RNA and protein were isolated at the indicated times. The top three panels are RPs probing for Xbp1, pre-rRNA, and 7SL. U and S indicate unspliced and spliced Xbp1-protected fragments, respectively. Bottom panels are autoradiographs of 35S-labeled whole-cell extracts analyzed by SDS-PAGE. Ten micrograms of total protein was loaded into each lane. (B) Quantitation of pre-rRNA and 35S incorporation in eIF2α⁺/⁺ (S/S) and eIF2α⁺/⁻ (A/A) MEFs as shown in panel A. Top panels are graphs of pre-rRNA levels relative to 7SL, and bottom panels are graphs of relative 35S incorporation. Levels at 0 h were given the value of 1, and each point represents the mean ± standard deviation of a minimum of three independent experiments.
that the rRNA preinitiation complex is disrupted during UPR activation (Fig. 5B and C). The decrease in Pol I and RRN3/TIF-IA promoter occupancy was PERK dependent as there was no change in \( \text{Perk}^{-/-} \) MEFs (Fig. 5B and C). We did not detect any change in the promoter occupancy of UBF after Tg treatment in either cell line (see Fig. S5A in the supplemental material). We were unable to determine the promoter occupancy of SL1 because antibodies against subunits of SL1 were not suitable for ChIP assays. Our assay was specific to the targeted proteins, as precipitation with a control antibody against GAPDH did not pull down significant quantities of rRNA promoter DNA (Fig. 5B to D). In addition to the preinitiation complex, we monitored the level of elongating Pol I by its association with the ITS-1 of the rRNA gene and found that there is a PERK-dependent decrease in the association of elongating Pol I with ITS-1 DNA upon Tg treatment (Fig. 5D).

The same reaction was performed with antibodies against RRN3/TIF-IA, which did not significantly pull down ITS-1 DNA as expected as RRN3/TIF-IA is not known to travel with the elongating Pol (see Fig. S5B in the supplemental material). The PERK dependence of pre-rRNA downregulation and disruption of the preinitiation complex was specific to the UPR. Treatment of \( \text{Perk}^{-/-} \) MEFs with ANS reduced pre-rRNA to the same extent as wild-type cells (see Fig. S5C and D in the supplemental material). Furthermore, a ChIP assay of \( \text{Perk}^{-/-} \) and \( \text{Perk}^{-/-} \) MEFs treated with hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), a known rRNA preinitiation complex disrupter, showed similar levels of Pol I and RRN3/TIF-IA promoter dissociation (see Fig. S5E and F in the supplemental material). Together, these results suggest that PERK activation decreases the promoter occupancy of both RRN3/TIF-IA and Pol I, leading to a decrease in the number of elongating
transcription complexes on the rRNA gene. This correlates with results shown in Fig. 1, where the overall level of pre-rRNA and the synthesis of rRNA from isolated nuclei are decreased upon UPR induction.

**RRN3/TIF-IA is inactivated during UPR to reduce rRNA transcription.** Since PERK is responsible for translation repression, a potential mechanism of the decrease in Pol I and RRN3/TIF-IA association with the rRNA promoter is a reduction in their cellular concentrations. Although antibodies to the TAF1 proteins of the SL1 complex were unsuitable for Western blotting, we found that the levels of the large Pol I subunit (RPA194), both isoforms of UBF, and RRN3/TIF-IA remain constant during UPR in both wild-type and Perk−/− MEFs, suggesting that translation inhibition is not leading to depletion of these essential rRNA transcription factors (Fig. 6A). Thus, the decrease in transcription factor association with the rRNA promoter may be a result of a decrease in their activity.

To identify the downstream effectors of PERK-mediated regulation of rRNA synthesis, we assayed Pol I transcription activity in nuclear extracts from untreated and Tg- and ANS-treated MEFs in vitro. In this assay, nuclear extracts were collected and analyzed by RPA against pre-rRNA and 7SL. The level of transcript produced by untreated extracts in the absence of exogenous RRN3/TIF-IA. Each bar represents the mean and standard deviation of three independent experiments.

**FIG. 6.** RRN3/TIF-IA is inactivated during the UPR. (A) Western blots from Perk+/+ and Perk−/− MEFs treated with Tg (200 nM) or dimethyl sulfoxide (NT; 0.1%) over a 12-h time course. Protein samples were run on denaturing polyacrylamide gels and probed with antibodies against RPA194 (Pol I large subunit), both isoforms of UBF, RRN3/TIF-IA, and β-actin. Note that the UBF antibody recognizes both isoforms of UBF protein. (B) Depiction of the rRNA transcription template (gray box) and the 632-nt transcript that results from correct initiation. The correct site of transcription initiation is indicated by an arrow. (C) Autoradiograph of 632-nt in vitro transcribed product resulting from transcription reactions using 25 μg of nuclear extracts and 10 ng of rDNA template in the presence of [α-32P]UTP. Purified RRN3/TIF-IA protein from untreated cells was added to reaction mixtures at the indicated concentrations (lanes 2 and 3). Nuclear extracts were isolated from MEFs subjected to 2 h of Tg (200 nM) or ANS (10 μM) treatment and compared to untreated (NT) cells. (D) The same experiment as in panel C except that partially purified UBF protein was added to the reaction mixture in a concentration of 2.5- and 5-fold excess (2.5× and 5×, respectively) of the endogenous level of UBF (lanes 2 and 3). (E) Quantitation of transcription products as shown in panel C relative to the level of transcript produced by untreated extracts in the absence of exogenous RRN3/TIF-IA. Each bar represents the mean and standard deviation of three independent experiments. (F) HeLa cells were transfected for 24 h with empty vector or FLAG-RRN3 before treatment with Tm (0.5 μg/ml). Total cellular RNA was collected and analyzed by RPA against pre-rRNA and 7SL. (G) Quantitation of transcription products as shown in panel D relative to the level of transcript produced by untreated extracts in the absence of exogenous UBF. Each bar represents the mean and standard deviation of three independent experiments.
DISCUSSION

All organisms depend on their ability to maintain cellular homeostasis in order to ensure their survival. As such, it is essential that organisms adjust their metabolic states according to external conditions. For example, when growing cells are deprived of nutrients, they downregulate their translational capacity by inhibiting translation initiation and ribosome biogenesis (24, 29, 39). While downregulation of these processes may come at the cost of cellular growth, given the amount of energy and resources they consume, it is likely to be highly beneficial when nutrients are limiting. Conversely, when ample nutrients are available, cells will increase their rates of ribosome biogenesis and protein synthesis, promoting growth. In either case, the mTOR pathway predominantly controls global changes of translational capacity in response to nutrients or growth factor signaling.

In this report we have identified an alternate pathway for coordinately regulating ribosome biogenesis with protein synthesis (Fig. 7). The downregulation of rRNA that we see upon ER stress appears to occur independently of the mTOR pathway. We have demonstrated that during ER stress, PERK signaling is necessary for downregulating rRNA synthesis in addition to its well-documented effects on inhibiting global translation initiation. When PERK is activated by ER stress, it phosphorylates eIF2α, preventing the formation of active ternary complexes and thereby inhibiting initiation of translation (28, 50). Under these conditions, we found that the rRNA preinitiation complex is disrupted by a PERK-dependent dissociation of Pol I and the basal factor RRN3/TIF-IA from the rRNA promoter. In addition, we found that the decrease of pre-rRNA occurs with the same kinetics as translation inhibition and that phosphorylation of eIF2α is required for rRNA downregulation during ER stress.

Synthesis of rRNA comprises the majority of transcriptional activity in growing cells, with rRNA constituting 80 to 90% of all RNA within the cell. Thus, changes in rRNA transcription are likely to have significant consequences, particularly during stress. Mutation of the conserved eIF2α phosphorylation site rendered cells incapable of responding to ER stress, suggesting that phosphorylation of eIF2α is a key molecular event in regulating rRNA transcription. This is the first time that phosphorylation of eIF2α has been implicated in the regulation of rRNA synthesis.

To date, four eIF2α kinases have been identified in mammals (PKR, HRI, PERK, and GCN2), and each is activated by a distinct set of stresses. Our finding that rRNA downregulation during ER stress requires eIF2α phosphorylation raises the question of whether all eIF2α kinases utilize this pathway to regulate rRNA transcription. While an eIF2α-dependent rRNA transcription inhibition has never been reported, there is evidence in the literature that stresses leading to eIF2α phosphorylation also downregulate rRNA transcription. We found that for each stress where eIF2α phosphorylation is known to occur, there was at least one independent study that found that rRNA transcription was inhibited under the same conditions (Table 1). This is consistent with the idea that phosphorylation of eIF2α is associated with inhibition of rRNA transcription although none of the studies to date has reported the connection. If other eIF2α kinases signal through eIF2α phosphorylation to regulate rRNA transcription, this pathway...
may actually represent a major mode of regulating rRNA synthesis during stress in general.

Transcription of rDNA in the nucleolus requires at least three basal factors: RRN3/TIF-IA, UBF, and SL1. While all three of these transcription factors are highly regulated, RRN3/TIF-IA is the major target of environmental stresses such as nutrient deprivation, oxidative stress, and treatment with translation inhibitors (9, 30, 40, 41). Here, we found that during ER stress there is a PERK-dependent dissociation of RRN3/TIF-IA and Pol I from the rRNA promoter; however, the concentration of these proteins did not appear to change, suggesting that their activity was altered during the UPR. Additionally, we were able to restore transcriptional activity of UPR-treated nuclear extracts by the addition of active affinity-purified RRN3/TIF-IA but not with UBF, which is consistent with the idea that RRN3/TIF-IA is inactivated during UPR.

The major mechanism by which RRN3/TIF-IA activity is regulated is through phosphorylation. To date, at least eight phosphorylation sites on RRN3/TIF-IA have been identified, and we are now just beginning to understand how the pattern of RRN3/TIF-IA phosphorylation affects its activity (5, 16, 23, 40, 41, 46, 49). For example, inhibition of mTOR by Rap leads to inactivation of RRN3/TIF-IA through hypophosphorylation of serine 44 and hyperphosphorylation of serine 199 (41). Oxidative stress or treatment with the peptidyl transferase inhibitor ANS results in RRN3/TIF-IA inactivation through an inhibitory phosphorylation on threonine 200 catalyzed by JNK2 (40). It is not exactly clear why so many different phosphorylation sites regulate RRN3/TIF-IA activity, but perhaps it allows the rRNA transcription machinery to more tightly integrate signals coming from multiple pathways. Different patterns of phosphorylation may result in different levels of activity or may differentially alter cellular localization. In growing cells RRN3/TIF-IA localization is restricted to nucleoli; however, its localization has been reported to shift to the nucleoplasm during ANS treatment (40) or to the cytoplasm during Rap treatment (41). Perhaps this is an indication that RRN3/TIF-IA has additional functions during stress other than simply regulating Pol I transcription. Future work to determine the UPR-induced phosphorylation sites of RRN3/TIF-IA and identifying potential kinases or phosphatases that regulate RRN3/TIF-IA downstream of phosphorylated eIF2α will be one of the critical steps toward understanding the mechanistic details of how signals are transmitted from the ER to the nucleolus.

One of the major questions remaining to be answered is exactly how eIF2α phosphorylation in the cytoplasm is communicated with the nucleolus to regulate rRNA transcription. One interesting possibility is that the eIF2 complex itself directly communicates ER stress to the nucleolus. It is well established that the vast majority of eIF2 is localized in the

![Diagram](image_url)

**FIG. 7.** Model of PERK pathway controlling RNA transcription and translation in comparison to mTOR. (A) PERK pathway. Unfolded proteins in the ER lumen activate the PERK kinase. PERK phosphorylates eIF2α leading to inhibition of translation initiation and inhibition RRN3/TIF-IA activity, resulting in dissociation of RRN3/TIF-IA and Pol I from the preinitiation complex. Dashed lines indicate that direct interaction of components has not been demonstrated. (B) mTOR pathway. Under favorable conditions, signaling from growth factors and nutrients activates mTOR leading to phosphorylation of 4E-BP and S6K. Inhibition of 4E-BP and activation of S6K by mTOR phosphorylation cooperate to increase the efficiency of translation initiation. While the entire pathway has yet to be elucidated, mTOR and S6K activity lead to phosphorylation of rRNA transcription factors increasing transcription of rRNA.

<table>
<thead>
<tr>
<th>Stress source or type</th>
<th>eIF2α kinase</th>
<th>rRNA transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Reference</td>
<td>Increase or decrease</td>
</tr>
<tr>
<td>Oxidative</td>
<td>HRI</td>
<td>38</td>
</tr>
<tr>
<td>Amino acid deprivation</td>
<td>GCN2</td>
<td>58</td>
</tr>
<tr>
<td>Heat shock</td>
<td>HRI</td>
<td>38</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>PERK</td>
<td>35</td>
</tr>
<tr>
<td>UV irradiation</td>
<td>GCN2</td>
<td>34</td>
</tr>
<tr>
<td>Viral infection</td>
<td>PKR</td>
<td>30</td>
</tr>
<tr>
<td>Rap</td>
<td>GCN2</td>
<td>36</td>
</tr>
<tr>
<td>ANS</td>
<td>None</td>
<td>reported</td>
</tr>
<tr>
<td>UPR</td>
<td>PERK</td>
<td>28</td>
</tr>
</tbody>
</table>

* ↑, upregulation; ↓, downregulation.*

TABLE 1. Comparison of activation of eIF2α kinases and rRNA transcription during stress

VOL. 29, 2009 PHOSPHO-eIF2α REGULATES rRNA SYNTHESIS 4305

Downloaded from mcb.asm.org on Jul 14, 2009.
cytoplasm; however, an increasing amount of evidence suggests that eIF2 is present in the nucleus and may be directed to the nucleolus upon eIF2α phosphorylation. A number of studies have described that a small proportion of the eIF2 complex is localized to the nucleus or nucleoli (6, 15, 22, 37, 55). Specifically, two independent studies have indicated that during postischemic brain reperfusion, phosphorylated eIF2α accumulates in the nucleus and the nucleolus (15, 22). In another study, nearly all cytoplasmic eIF2α relocalized to discrete puncta within the nucleus that resemble nucleoli within 30 min of treatment with the CRM1 nuclear export inhibitor leptomycin B (6). While clear demonstration that these puncta are indeed nucleoli will be required, these results strongly suggest that the eIF2 complex rapidly shuttles between the cytoplasmic and nuclear compartments under normal conditions. In this regard, it would be interesting to examine if the shuffling of the eIF2 complex was altered in response to stresses that induce eIF2α phosphorylation and whether nuclear eIF2α plays a role in downregulating RNA transcription during stress.

The UPR has provided a unique opportunity to uncover the effects of eIF2α phosphorylation on RNA transcription as many of the stresses that activate eIF2α kinases also affect mTOR activity. While this may complicate study of the role of other eIF2α kinases in regulating RNA gene transcription, the UPR has allowed us to begin dissecting the molecular details of how PERK-mediated eIF2α phosphorylation can regulate rRNA transcription in the nucleolus during ER stress. Further understanding of the regulatory mechanisms and ability to uncouple eIF2α phosphorylation and rRNA synthesis will be necessary to provide answers to such questions.

ACKNOWLEDGMENTS

Perk+/- and Perk−/− cells were a gift from Douglas Cavener. We thank Peter Geiduschek and James Kadonaga for valuable discussion and Peter Geiduschek for critical reading of the manuscript. This work was supported by ACS RSG-05-01GMC, Searle 03-G107, and CRCC 6-447140-34384 to M.N.; R01GM069841 and R01HL057346 to R.J.K.

REFERENCES


